Plasmacytoid dendritic cell subpopulations in head and neck squamous cell carcinoma

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Abstract. Human plasmacytoid dendritic cells (PDCs) are present in solid tumor tissue and metastatic cervical lymph nodes (CLN) in head and neck squamous cell carcinoma (HNSCC). We recently showed that classical PDC functions are heavily disturbed in the tumor microenvironment. In this study we present a new approach to the subject by introducing 3 PDC subsets in HNSCC, characterized by the surface markers CD25, CD56 and CD203c. The first subset, positive for CD25, is significantly induced by HNSCC *in vitro* and present in metastatic lymph nodes *in vivo*. This subset can be phenotypically subdivided into matured cells and into a group expressing early T cell markers. Functionally this subgroup is associated with the secretion of IL-8. The second subset, positive for CD56, constitutes 4-5% of all PDCs and is significantly down-regulated by HNSCC. Furthermore, this population sporadically expresses perforin/granzyme B and is absent in metastatic lymph nodes. The third subset, positive for the basophile marker CD203c, is inducible by crosslinking BDCA-2 in the presence of HNSCC and IL-4. Future studies will have to clarify the *in vivo* relevance of the different PDC subsets in HNSCC.

Introduction

Two main human dendritic cell subsets are to be distinguished: myeloid dendritic cells (MDCs) including langerhans cells, dermal dendritic cells and interstitial DCs versus plasmacytoid dendritic cells (PDCs) (1). Human PDCs are phenotypically defined by the expression of the surface markers CD123, BDCA-2 and HLA-DR. Furthermore, the classical PDC population is negative for the lineage markers CD3, CD14, CD16, CD19, CD20, and CD56. Functionally, PDCs are to a small extent capable of presenting antigens to T cells (2), however, their main known function is characterized by the ability to produce large amounts of IFN-α in viral infections *in vivo*. *In vitro*, IFN-α secretion is also triggered by CpG-oligonucleotides, which consist of unmethylated CpG-dinucleotides embedded in a certain sequence context within bacterial DNA (3,4). It has been demonstrated that human solid HNSCC tumor tissues are infiltrated by plasmacytoid dendritic cells and that the classical PDC functions are heavily disturbed in the tumor microenvironment (5). Degraded to paralysed bystanders, the ability of secreting IFN-α is impaired whilst the migratory activity is enhanced (6). Hence, we present a new approach to the subject by introducing new PDC subpopulations in HNSCC. Recently, the existence of a CD2 positive PDC subset with functional autonomy and presence in different tumor tissues was reported (7). Future studies will have to clarify the *in vivo* relevance of the different PDC subsets in the tumor microenvironment and metastatic CLN.

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Materials and methods

Isolation of PDCs. For *in vitro* studies plasmacytoid dendritic cells (PDC) were isolated from human peripheral blood provided by the blood bank of the University Hospital Lübeck, Germany. Blood donors were 18-65-year old healthy donors who were tested to be negative for allergies. PBMCs were obtained from buffy coats by Ficoll-Hypaque density gradient centrifugation as described elsewhere (8). PDCs were isolated by magnetic bead separation using magnetic labelled anti-BDCA-4 antibodies (Miltenyi, Bergisch Gladbach, Germany). Tumor tissue and patient's blood were obtained during operations of tumor patients based on approval from the ethics committee, Campus Lübeck. Informed consent was obtained from each patient.

Single-cell suspensions. Metastatic lymph nodes were cut into pieces and then covered with 31.5 mg collagenase/ml PBS and 3.99 mg/ml hyaloronidase/ml PBS in DMEM and incubated in a 37˚C water bath for 30-120 min. Digestion was checked every 30 min. In a further step 33.4 mg dispase/ml PBS was added for 30-60 min. Brefeldin A (eBioscience, San

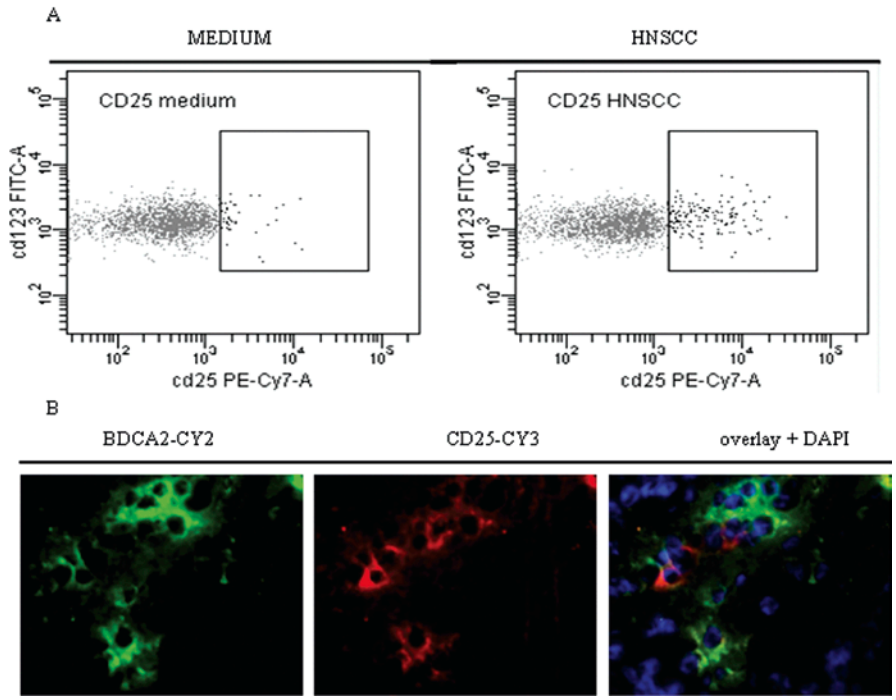

Figure 1. (A) CD25 positive subpopulation (5-20% of the main PDC population, p=0.009) was significantly induced after stimulation with the HNSCC cell lines PCI-1, PCI-13 and BHY *in vitro*. (B) Fluorescence staining of BDCA2 (green, CY2) and CD25 (red, CY3), in a metastatic lymph node.

Diego, CA, USA) was supplied during all steps. The digest was then passed through a 100-μm nylon mesh in order to remove clumps and washed several times.

Cell cultures. The permanent HNSCC cell lines BHY (DSMZ Germany), PCI-1 and PCI-13 (generously provided by Professor T. Whiteside, Pittsburgh Cancer Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, New York, NY, USA) supplemented with 10% FCS, 1 mM glutamine, and 0.1 mM sodium pyruvate. All compounds were purchased Endotoxin tested.

Fluorescence staining. Slices (6 μm) were cryo-sectioned and placed on an object holder. The object holders were then dried for 10 min, incubated in acetone for further 10 min and dried again for 10 min before washing them in PBS. In a further step the tissue samples were incubated with 30% goat serum (Vector) in PBS for at least 1 h. Then the primary antibodies (CD25, IgG1, mouse, Imgenex and BDCA-2, IgG1, mouse, Miltenyi) and the isotype control (IgG1, mouse, Dianova) were each transferred onto the slices in 2% goat serum at a concentration of 1:50 for 2 h (room temperature). Before adding the second of two primary antibodies, the first one was blocked with goat anti-mouse FAB-Fragments (Jackson immunoResearch) according to the Dianova protocol. Subsequent to washing with PBS for 15 min the object holders were incubated with secondary antibodies (Cy3, donkey anti-goat and Cy2, goat anti-mouse, Jackson immunoResearch) for 30 min in 10% goat serum. After another 15-min washing step the tissue was stained with DAPI for 1 min and then covered with Fluromount. The expression of BDCA2 and CD25 in lymph node-tissue was analyzed on a Zeiss Axiovert 200M.

Flow cytometry. PDCs were identified as CD123-PE positive, HLA-DR-PerCP positive, lineage cocktail-FITC (CD3, CD14, CD16, CD19, CD20, CD56; Becton-Dickinson, Heidelberg, Germany) negative and BDCA2-APC/PE (Miltenyi Biotec, Bergisch-Gladbach, Germany) positive cells. Surface antigen staining was performed using the antibodies CD1a-FITC, CD2-PE, CD3-PE, CD4-PERCP, CD4-APC-CY7, CD8-ARC-CY7, CD-25-APC/APC-CY7, CD34-PERCP, CD44-PE, CD56-PE, CD80-FITC, CD83-FITC, CD86-PE, CD117-APC, perforin-FITC, granzyme B-FITC, CD123-APC/FITC, BDCA4-APC, CD45RA-FITC, CD25-FITC/PE-CY7, CD127-FITC (eBioscience) and GITR-FITC, IL-2 FITC, IL-4-PE, IL-6-FITC, IL-8-FITC, IL-10-APC, TGF-β-PE, TNF-α-FITC (R&D Systems, Minneapolis, MN, USA). The isotype PE IgG1/IgG2a, the isotype APC IgG1/IgG2a and the isotype Fitc IgG1/IgG2b (Becton-Dickinson) were used as negative controls. Early apoptotic or dead cells were detected by staining the cells with Annexin-V-FITC and propidium-iodide-PE (Becton-Dickinson). Doublets were excluded by gating FSC-A against FSC-H. Only singlets were analysed. CD107a was up-regulated in the PDC population subsequent to stimulation with HNSCC, indicating cytokine secretion.

The amount of $2x10^5$ PDCs was incubated on ice in 50 μl 1% BSA (in PBS) with 1-2 μl of each antibody for 15 min. Then the cells were washed three times in 500 μl 1% BSA and subsequently analysed on a FACSCanto (Becton-Dickinson) equipped with FACSDIVA software. Intracellular staining was performed with the eBioscience staining buffer set according to the eBioscience protocol. Cell sorting was performed with a Dako moFlow.

Cytokine analysis. The Bio-Plex Cytokine Assay (Bio-Rad, München, Germany) was used for the determinations of

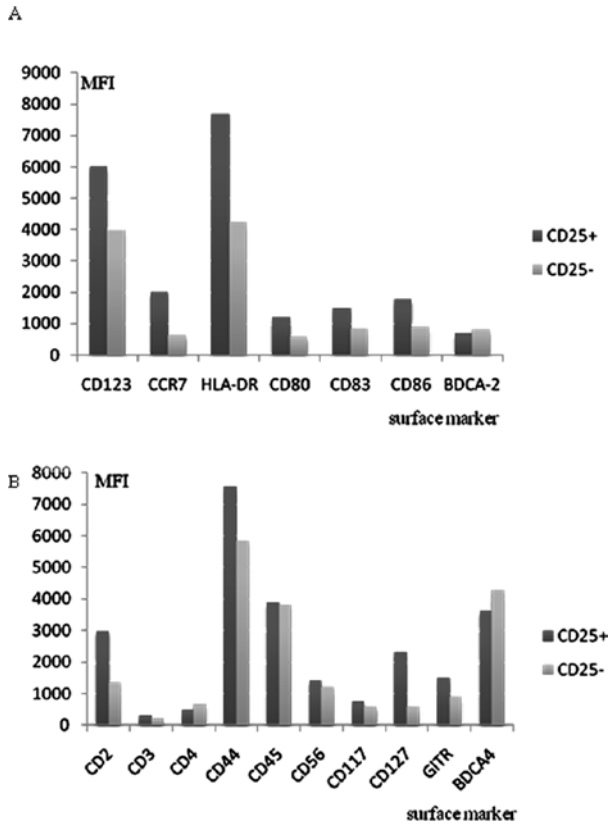

Figure 2. (A) CD25 positive subpopulation (40%) is matured on the contrary to the CD25 negative PDC population after stimulation with HNSCC cell lines *in vitro*. (B) The MFI and percentage of cells positive (data not shown) for T cell and thymocyte markers is elevated in the CD25 positive subpopulation. We detected higher levels of CD2, CD3, CD38, CD44, CD45RA, CD117 (c-kit), CD127 and GITR. CD4- and BDCA4-levels were decreased when compared to the main PDC population. CD56 positive cells were up-regulated in the CD25-subpopulation. *Two or three identical experiments were performed for each marker.

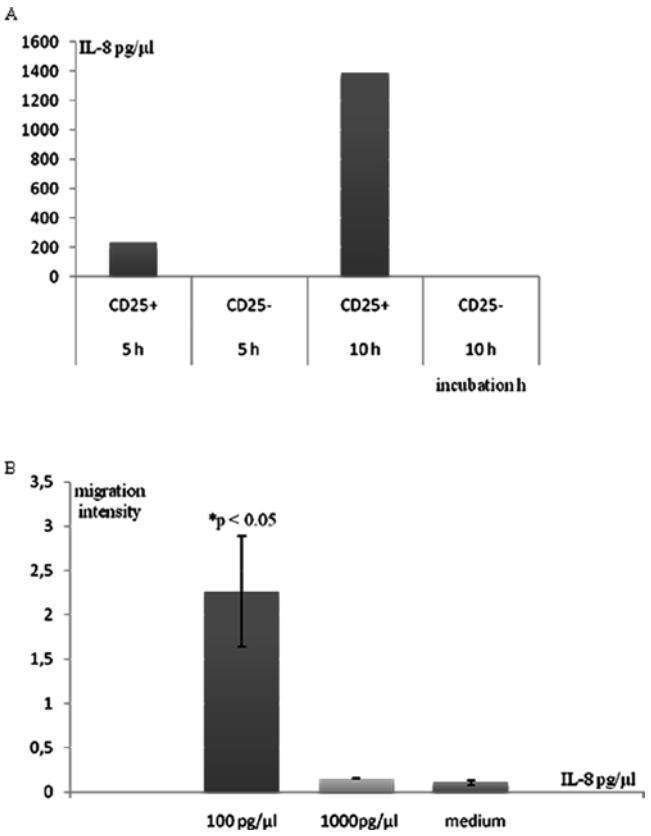

Figure 3. (A) Secretion of IL-8 above the levels of the HNSCC cell lines in the CD25 positive subpopulation after cell sorting. *Two identical experiments were performed. (B) The HNSCC cell line BHY migrated towards IL-8 (100 pg/µl) *in vitro*. *Three identical experiments were performed.

various cytokines such as interleukin-2 (IL-2), IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α. These cytokine assays allow the multiplexed quantitative measurement of multiple cytokines in a single small volume of cell culture supernatant. The protein array was analysed by a specialized microplate reader system (Bio-Plex Array Reader; Bio-Rad), and the data were calculated by the Bio-Plex Manager software.

Migration assay and MTT test. The cell line BHY ($4.8x10^5$ cells) was subjected to migration analysis using the ChemoTx system (NeuroProbe Inc., Gaithersburg, MD, USA). The microplate provided bottom wells for the chemoattractant IL-8. The cell suspensions were pipetted directly onto the sites on the top side of the filter sit and in hemispherical drops during incubation. Relative numbers of migrated cells were determined after 12 h using the MTT assay (In Vitro Toxicology Assay Kit, MTT-based; Sigma®-Aldrich Co., Steinheim, Germany).

Statistical analysis. The Kolmogorow-Smirnow test did not show a normal distribution. Therefore, we performed the Wilcoxon-Mann-Withney test for statistical analysis. $p<0.05$ was considered to be marginally significant.

Results

HNSCC induces a CD25 positive subpopulation exhibiting a mature phenotype, besides an early T-cell phenotype. The significant induction ($p=0.009$) of a CD25 positive PDC subpopulation was observed after stimulation with HNSCC (Fig. 1A). Furthermore, this subset was present in metastatic CLN (Fig. 1B).

Having been described as the early activation marker on PDCs (9), we analysed if tumor-induced CD25 positive PDCs would likewise evoke a mature phenotype. Subsequent to the stimulation with HNSCC cell lines for 12 h the expression of the maturation markers CD123, CCR7, BDCA2, CD80, CD83, CD86 and HLA-DR in the CD25-subpopulation was compared to the classical PDC population. A higher expression of the surface markers CD123, CCR7, CD80, CD83 and CD86 besides a distinct down-regulation of BDCA-2 was observed on 40% of the CD25-subpopulation when compared to the CD25 negative PDCs (Fig. 2A). The remaining CD25 positive cells showed higher expression levels for some of the following T cell markers when compared to the CD25 negative PDC population subsequent to incubation with HNSCC cell lines for 12 h. Following receptors were analysed: early T cell markers (CD1a, CD34, CD38, CD44, CD45, CD117 and CD127), common T cell markers (CD2, CD3, CD4) and Treg markers (GITR, CTLA-4) (Fig. 2B).

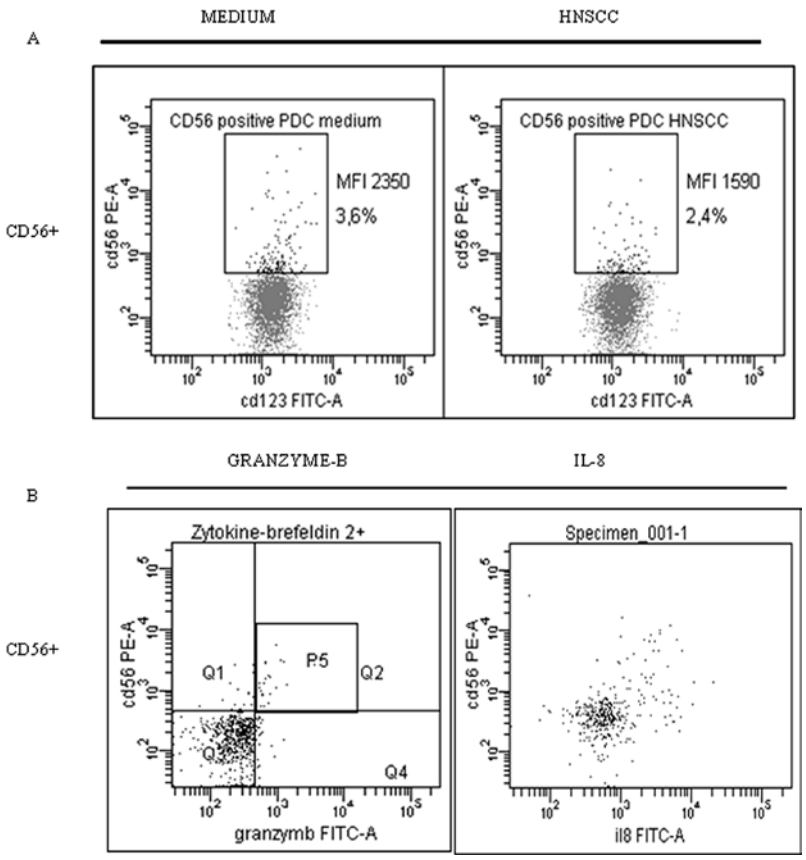

Figure 4. (A) We observed a significant (p=0.0007) down-regulation of the MFI and percentage of population in CD56 positive PDCs in healthy donors after stimulation with HNSCC. (B) Secretion of granzyme B and IL-8 in the CD56 positive subset.

The CD25 positive PDC subset reveals distinct functional properties. A Bio-Plex assay was performed after having sorted the CD25-subpopulation subsequent to incubation with HNSCC cell lines for 12 h. After 8 h of further incubation with HNSCC supernatants and medium alternatively, IL-6 and IL-8 were likewise detected in the supernatants of the CD25-subpopulation. The CD25-subpopulation secreted IL-8 to a greater extent than the control, whilst secretion of IL-6 could not be distinguished from the amounts produced by the tumor cell lines. We could not detect any cytokine production above the HNSCC controls in the CD25-negative PDC population after 8 h (Fig. 3A).

In vivo a significant IL-8 production could not be referred to a single subpopulation, because of being secreted alternatively by the CD25 positive, a CD56 positive and a phenotypically as yet unknown subpopulation which could not be classified. No significant differences could be found concerning intracellular cytokine production (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, TGF-β) in the CD25 positive subpopulation and the CD25 negative PDC population *in vivo*.

In addition, *in vitro* investigations showed that the HNSCC cell line BHY migrated actively towards IL-8 (100 pg/μl) in a period of 12 h (Fig. 3B).

HNSCC significantly down-regulates CD56 positive PDCs. The PDC population (4-5%) shows a CD56 positive phenotype (also partly positive for CD8) in healthy donors. In our study, we showed that this subpopulation was significantly (p=0.0007) down-regulated after incubation with the HNSCC cell line BHY (Fig. 4A). Although CD56 positive PDCs could be detected in the blood of tumor patients, we did not find a significant difference concerning the intracellular production of the cytokines IL-2, IL-4, IL-6, IL-8, IL-10 and TGF-β in comparison to the classical PDCs. *In vitro*, sporadic expression of perforin, granzyme-B, TNF-α and IL-8 was observed (Fig. 4B). CD56 positive PDCs were not present in metastatic lymph nodes.

BDCA-2 crosslinking in the presence of IL-4 and HNSCC induces a CD203c-positive PDC subpopulation. Besides the discovery of alterations concerning the CD25 and CD56 positive PDC subsets, we also observed the induction of a CD203c positive PDC subset in HNSCC. However, this population was not inducible by sole incubation with HNSCC. Crosslinking of the receptor BDCA-2 and addition of the cytokine IL-4 was mandatory for up-regulation of this surface marker. Furthermore, incubation with HNSCC and one of the mandatory components did not show any effect. BDCA-2 crosslinking in the presence of IL-4 in medium control induced a few CD203c positive PDCs, however, to a far lesser extent (Fig. 5).

Discussion

PDCs are to a small extent capable of presenting antigens to T cells (2), however, their main known function is characterized

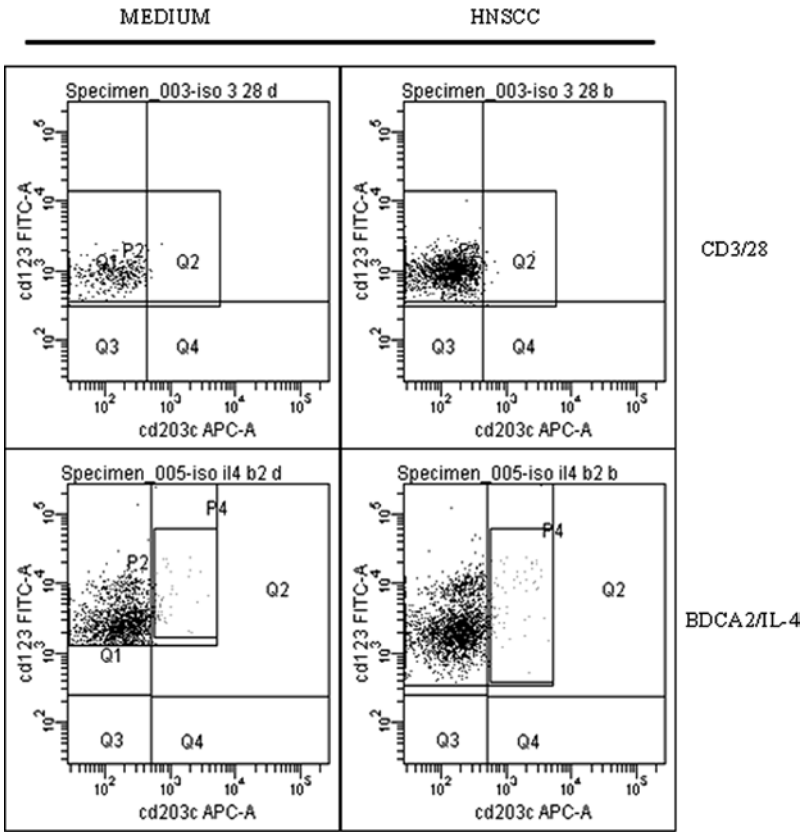

Figure 5. The new PDC subset positive for the Basophile marker CD203c was most effectively induced by crosslinking BDCA-2 in the presence of HNSCC and and IL-4 for 12 h. Sole incubation with HNSCC, or crosslinking CD3/CD28 did not induce the CD203c-positive subset. *Three identical experiments were performed.

by the ability to produce large amounts of IFN-α in viral infections *in vivo*. *In vitro*, IFN-α secretion is also triggered by CpG-oligonucleotides, which consist of unmethylated CpG-dinucleotides embedded in a certain sequence context within bacterial DNA (3,4). We have recently shown that crucial PDC functions are impaired in HNSCC (6). Here we provide a new approach to the subject by focusing on PDC subsets in HNSCC.

HNSCC has an impact on at least three PDC subsets, which are characterized by the surface markers CD25, CD56 and CD203c.

The CD25 positive subpopulation was significantly induced by HNSCC *in vitro* and present in metastatic lymph nodes. In contrast to the CD25 negative PDCs, which showed an impaired maturation subsequent to incubation with HNSCC (unpublished data) (10), the CD25 positive subset partly exhibited a classically maturated phenotype as described before (9). However, the colocation of the up-regulation of CD4, CD45RA in the PDC population subsequent to stimulation with HNSCC (unpublished data) besides the up-regulation of CD25 in this specific subset, led us to consider a totally different direction. Due to the fact that CD4 and CD45 are associated with the modulation of the T cell receptor (11), enhancement of positive selection (12) and pre-TCR signaling in thymocytes (13) besides CD123 being associated with early levels of differentiation (14) when highly expressed, we developed the assumption that these PDCs may be associated with peripheral early T cells. Indeed, those receptors which are elevated in the double negative stages 1-3 were partly up-regulated in the CD25 positive subpopulation, with exception of CD34 and CD1a. Only BDCA4 and CD4 were down-regulated when compared to the main PDC population. As down-regulation of CD25 is co-regulated with the up-regulation of BDCA-4 in murine thymocytes (15), the decrease of BDCA-4 may be linked to the up-regulation of CD25 in the HNSCC environment.

With exception of IL-8, which was secreted by the CD25 positive subset after exposure to HNSCC, we were confronted with major difficulties in distinguishing between cytokine-secretion by PDCs and HNSCC supernatants in the Bio-Plex assay, as HNSCC cytokine levels overlapped the production of cytokines by the PDC population. Furthermore, as supernatants containing few HNSCC cells were more effective in stimulating the PDCs than pure supernatants it was impossible to relate cytokine secretion to the different populations by Bio-Plex. Examining these results critically, the secretion of IL-8 may therefore be alternatively induced in the tumor cell population by the CD25 positive subset. Interestingly, secretion of IL-8 is not maintained by a single population *in vivo*. The relevance of IL-8 being alternatively secreted by mature CD25 positive PDCs, a CD56 positive subset, an unclassified subpopulation and even the tumor cells themselves subsequent to stimulation with the CD25- subpopulation remains to be resolved. In any case, IL-8 expression correlates with the angiogenesis, tumorigenicity, and metastasis of many tumors and is supposed to confer

chemotherapeutic resistance in cancer cells (16-18). These statements are underlined by the strong migratory activity towards IL-8 in the HNSCC cell line BHY *in vitro*.

The second PDC subset, which is characterized by expression of CD56 is significantly down-regulated subsequent to stimulation with HNSCC and is absent in metastatic lymph nodes. As this population sporadically displayed higher levels of perforin and granzyme-B when compared to the main PDC population, we suggest cytotoxic properties for this population. This subset may very well be associated with the murine IFN-producing killer dendritic cells (19) just as the monocyte derived $CD56^+$ dendritic cells (20) being down-regulated after stimulation with HNSCC cell lines and accordingly absent in metastatic lymph nodes *in vivo*.

The third subset is represented by CD203c positive PDCs and was induced by crosslinking BDCA-2 in the presence of IL-4 and HNSCC. Originally this marker was additionally used in order to exclude the basophile subset. CD203c is a glycosylated type II transmembrane molecule that belongs to the family of ecto-nucleotide pyrophosphatase/ phosphodiesterase (E-NPP3) enzymes. Among hematopoietic cells, expression of CD203c is restricted to basophiles, mast cells and their precursors, and has been described as specific for this lineage (21). Protein and/or mRNA expression of CD203c has also been found in solid tissues such as uterus or prostate (22). As CD203c has recently been demonstrated in human colon carcinoma such as neoplastic bile disease and is associated with malignant subversion and invasive properties (23,24), we propose a tumor benefitting role for the CD203c positive PDC subset in HNSCC. However, due to the size of the population, a functional analysis of this subset was not possible at present.

Future investigations and *in vivo* models will have to prove these theories and the clinical relevance for these PDC subsets *in vivo*.

Acknowledgments

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