

Decreased Levels of Circulating Regulatory NK Cells in Patients with Head and Neck Cancer throughout all Tumor Stages

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Abstract. *Background:* Natural killer (NK) cells play a crucial role in innate immunity as effectors against tumor cells and pathogen-infected cells. Human NK cells can be subdivided into two functional subsets, the immunoregulatory CD56^{bright} NK cells and the cytotoxic CD56^{dim} NK cells. NK-mediated host defence against tumor cells is strongly impaired in patients with head and neck squamous cell carcinoma (HNSCC). *Materials and Methods:* NK cells were isolated from peripheral blood of 70 HNSCC patients and 22 healthy donors by magnetic bead separation and subsequently analyzed using flow cytometric as well as immunohistochemical methods. *Results:* In this work we demonstrate that the population of circulating immunoregulatory CD56^{bright} NK cells is lower in the peripheral blood of patients with HNSCC as compared with that in healthy donors, regardless of the individual tumor stage or tumor type. *Conclusion:* These data underline the complex network of HNSCC-mediated immunomodulation as part of the immune escape mechanisms of malignant head and neck tumors.

As effector members of the innate immunity, natural killer (NK) cells play a major role in anti-infection activity and tumor surveillance. NK cells can directly kill target cells to which they are capable of adhering within 1-4 hours without prior activation, priming or assistance by cytokines (1-3). NK cells can be triggered through various receptors depending on

specific ligands presented by target cells in a given encounter (4-8). They are defined by the expression of CD56, an isoform of the neural-cell adhesion molecule with unknown function and the presence of Fc γ receptor III (CD16) (3). Two subsets of NK cells can be distinguished by the surface density of CD56, namely the CD56^{dim} and CD56^{bright} subpopulations. The majority of about 90% of peripheral blood NK cells belong to the CD56^{dim} subset, which has been shown to be responsible for the natural cytotoxicity against tumor targets (9). Compared to the CD56^{dim} subset, CD56^{bright} NK cells are known to secrete higher levels of different cytokines such as interferon (IFN)- γ , tumor necrosis factor (TNF)- β , granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-10 and IL-13 in response to monokine stimulation. As a counterpart, CD56^{bright} NK cells represent the immunoregulatory subset (10).

The CD56^{dim} NK cells express a low density of CD56 but high levels of Fc γ RIII (CD16), whereas CD56^{bright} NK cells show no or only little CD16 expression (11). Moreover, the two NK cell subsets can be distinguished concerning their receptor expression profiles. While the CD56^{bright} NK cell subset expresses high amounts of CC-chemokine receptor 7 (CCR7) and L-selectin (CD62L), the CD56^{dim} NK cells show no expression of these proteins but high levels of leukocyte function-associated antigen 1 (LFA1) instead (11, 12).

However, NK cells isolated from cancer patients exhibit strongly impaired antitumor functions, also in head and neck squamous cell carcinoma (HNSCC) (13, 14). Cells of head and neck cancer are known to develop molecular strategies to escape from efficient anti tumor immune responses. It is supposed that tumor production of various immune suppressive mediators contributes to massively compromised immune functions within the malignant transformation process (11, 15-18). In this work, we investigated circulating immunoregulatory NK cells in HNSCC patients and healthy donors, considering tumor stage and type.

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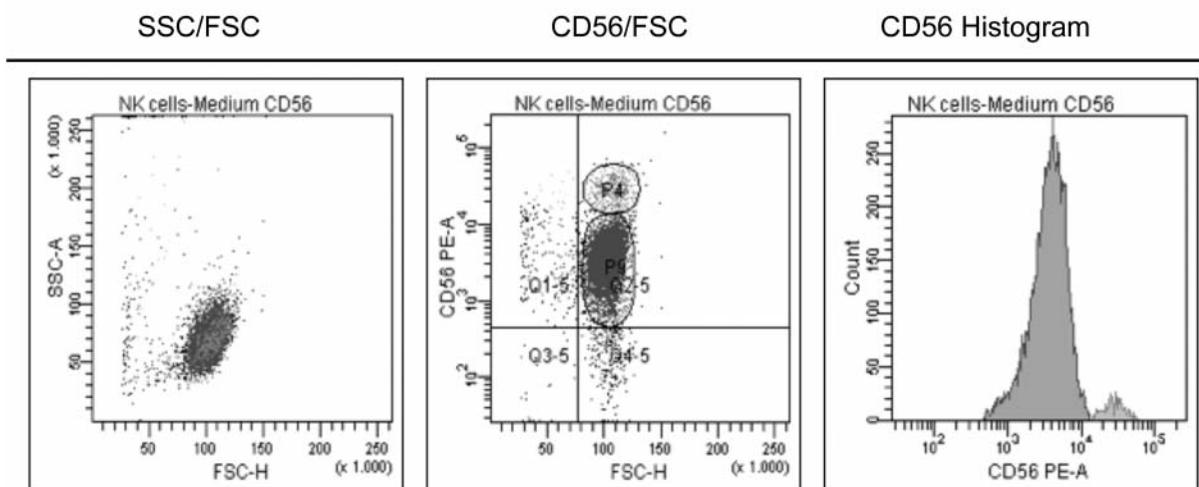


Figure 1. Flow cytometric analysis of isolated NK cells. Isolated NK cells were characterized by SSC/FSC properties and identified by the expression of the characteristic surface antigen CD56 using phycoerythrin (PE)-labeled anti-CD56 antibodies. NK cell isolation resulted in average purities of about 95%. CD56^{dim} (P4) and CD56^{bright} (P9) subsets could clearly be distinguished with respect to their CD56 expression levels.

Materials and Methods

Peripheral blood samples. Blood samples were obtained from patients with head and neck cancer (n=70) and from healthy blood donors (n=22). The buffy coats were provided by the blood bank of the University of Lübeck, Germany. Blood donors were 18 to 65 years old healthy men or women who were tested as being negative for HIV and hepatitis B and C viruses.

Isolation of NK cells. Peripheral blood of patients with HNSCC was directly obtained in CPT vacutainers. After centrifugation, the interphase containing the PBMCs was extracted and NK cells were isolated. Human peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque density gradient centrifugation. Depletion of non-NK cells (T-cells, B-cells, dendritic cells, monocytes, granulocytes and erythroid cells) in order to isolate intact NK cells was carried out using a cocktail of biotin-conjugated antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123, and Glycophorin A, and Anti-Biotin Micro Beads, according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow cytometry. Surface antigen staining was performed as described elsewhere (19). Cells were stained with phycoerythrin (PE)-conjugated anti-CD56 antibodies (BD Biosciences, Heidelberg, Germany). Propidium iodide was used to identify dead cells. Samples were analyzed on a FACSCanto (BD Biosciences) and data acquisition was performed using the FACS DIVA software (BD Biosciences).

Fluorescence microscopy. Fluorescence microscopy was used to analyze the NK cell abundance in solid HNSCC. Therefore, tumor tissues and metastatic lymph nodes were preserved at a temperature of -80°C in freezing medium and 6 µm slices were cut in a Leica cryotome and placed on an object holder. The object holders were

then dried for 10 min, incubated in methanol for a further 20 min and dried again for 5 min before washing them in phosphate-buffered saline (PBS) three times for 5 min. Primary antibodies were diluted with antibody dilution buffer (DCS Labline, Hamburg, Germany) and incubated at 4°C overnight. The CD56 mouse antibody (US Biological, Swampscott, Massachusetts, USA) was diluted 1:400, the CD16 rabbit antibody (Santa Cruz, Heidelberg, Germany) was diluted 1:50. In a further step, the tissue samples were washed three times in PBS for 5 min. The secondary antibodies were also diluted with antibody dilution buffer (DCS Labline, Hamburg, Germany) and incubated for 45 min at room temperature. The secondary antibody goat anti-mouse-Cy2 was diluted 1:100 and the secondary antibody goat anti-rabbit (Dianova, Hamburg, Germany) was diluted 1:200. The tissue samples were washed again three times in PBS. The nuclei were stained with DAPI (1 µg/ml) for 1 min. The samples were washed again three times for 5 min in PBS and were embedded afterwards in Fluoromount (Southern Biotechnologies Birmingham, Alabama, USA).

Samples were viewed on a Zeiss Axiovert 200M microscope by either differential interference contrast (DIC) microscopy or fluorescence microscopy using different fluorescent filter sets according to the fluorescent staining (AHF Analysentechnik AG, Tübingen, Germany). Cells were photographed using a Zeiss AxioCam MRm Rev.3 FireWire (D) and the Zeiss AxioVision Rel. 4.5 software. Numerous solid HNSCC were analyzed to indicate the infiltration of both cytotoxic CD56^{dim} and immunoregulatory CD56^{bright} NK cell subsets.

Results

Identification of NK cell subsets in peripheral blood and tumor tissue. Human natural killer (NK) cells were isolated from peripheral blood and identified by flow cytometry using phycoerythrin (PE)-labeled anti-CD56 antibodies. Our data

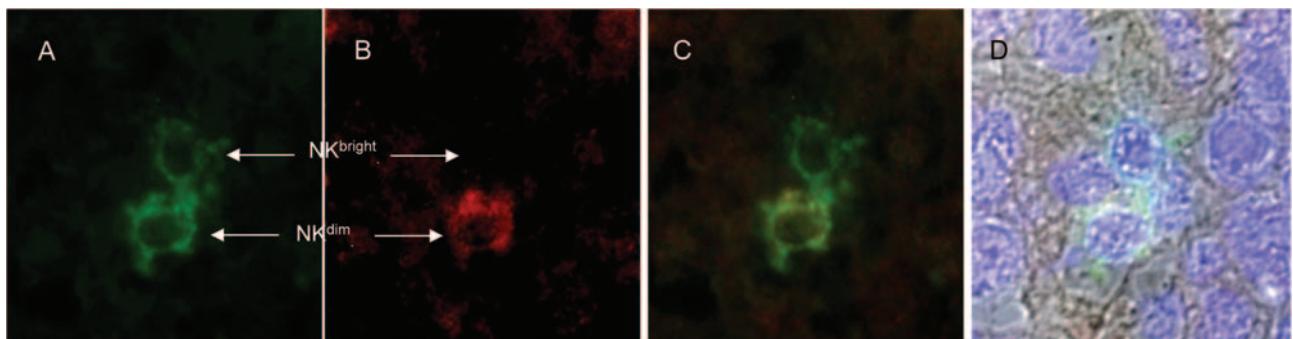


Figure 2. Localization of NK cells in tumor tissue by use of a rabbit anti-CD56 antibody and a secondary Cy2-conjugated anti-rabbit antibody, as well as a mouse anti-CD16 antibody and a secondary Cy3-conjugated anti-mouse antibody. DAPI was used to visualize the nuclei of the cells. A, Two NK cells expressing CD56. B, CD56^{dim} and CD56^{bright} NK cells identified by their expression of CD56 and CD16. C, Overlay of CD16-Cy3 and CD56-Cy2. D, Overlay of DAPI, bright field, CD56-Cy2 and CD16-Cy3.

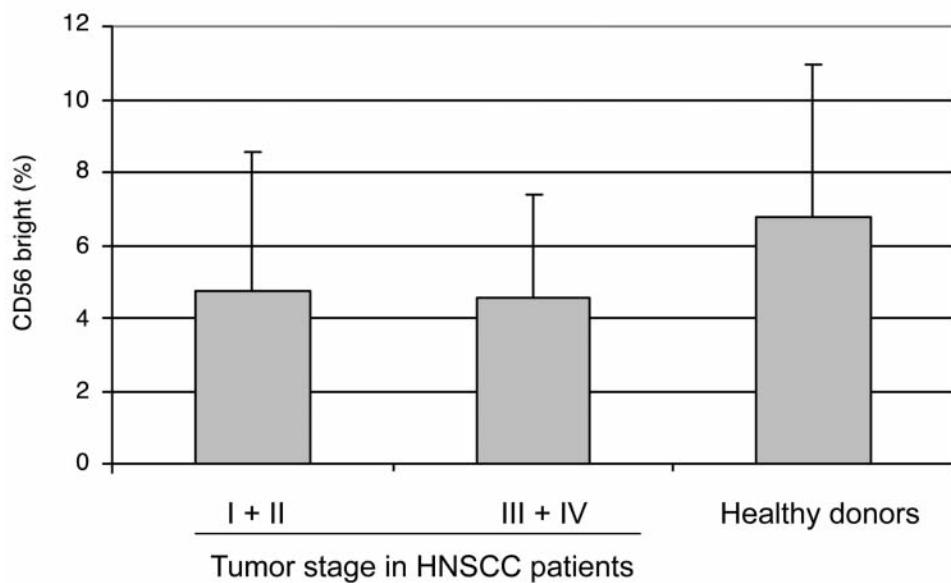


Figure 3. Abundance of circulating CD56^{bright} NK cells of HNSCC patients (UICC stages) and healthy blood donors. Data are shown as means \pm SEM.

revealed average purities of isolated NK cells of about 95%. NK cell subpopulations CD56^{dim} and CD56^{bright} were clearly distinguished with respect to their CD56 expression levels (Figure 1). Although these two NK cell subpopulations are often distributed into 'regulatory' and 'cytotoxic' NK cells, the precise role of surface protein CD56 for NK cell function is still mostly unclear.

Both NK cell subsets were also identified in solid HNSCC using immunofluorescence microscopy. It was possible to distinguish cells using co-staining of CD56 and the specific surface marker CD16, which allows the identification of the CD56^{dim} subset. Our data clearly indicate that solid HNSCC

are infiltrated by both cytotoxic CD56^{dim} and immuno-regulatory CD56^{bright} NK cell subsets (Figure 2).

Lower levels of circulating CD56^{bright} cells in patients with HNSCC. We were interested as to whether head and neck cancer leads to a modulated ratio of these NK cell populations in the peripheral blood. The abundance of CD56^{bright} and CD56^{dim} NK cell subsets in the peripheral blood of patients with HNSCC was analyzed with respect to different tumor stages using flow cytometry. Peripheral blood of healthy donors was used as an internal control. Our data show lower levels of circulating CD56^{bright} NK cells in the

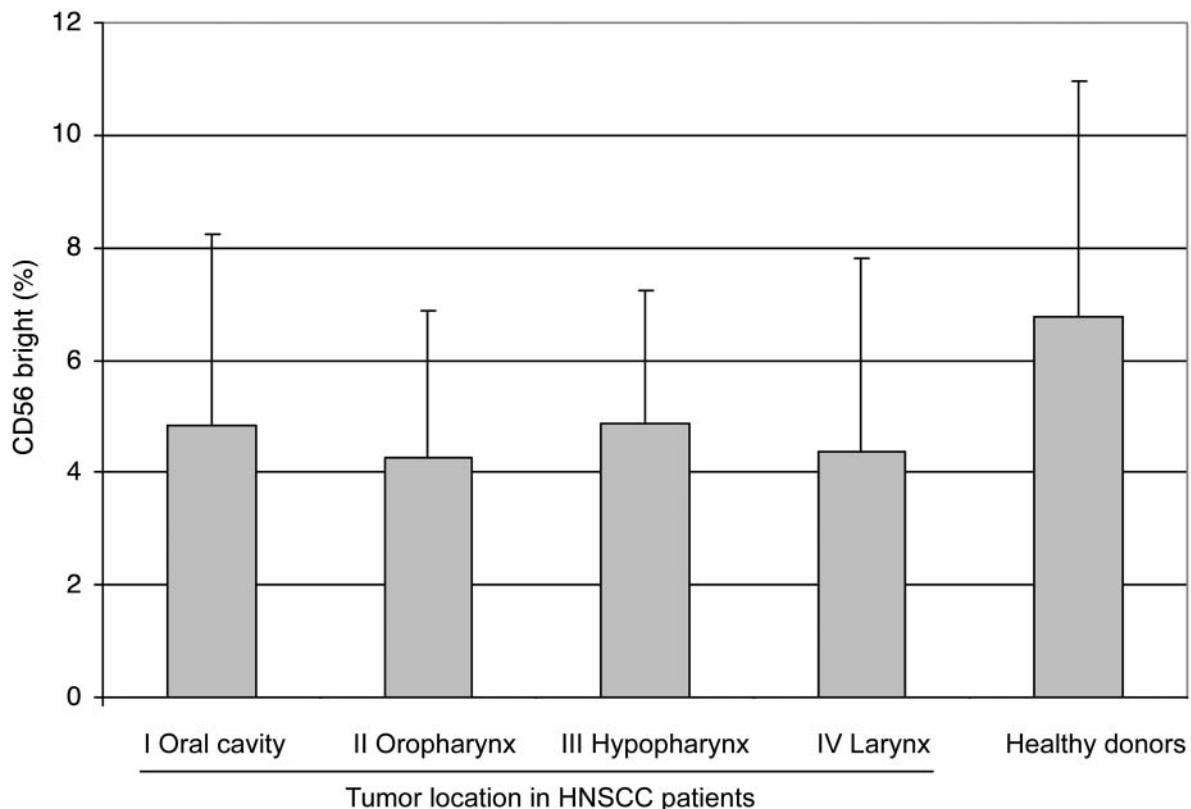


Figure 4. Abundance of circulating CD56^{bright} NK cells of HNSCC patients and healthy blood donors. Data are shown as means \pm SEM.

peripheral blood of patients with HNSCC as compared to those in the healthy control, whereas no differences could be detected between tumor stages I+II and III+IV (Figure 3).

Furthermore, the abundance of circulating CD56^{bright} regulatory NK cells was evaluated with respect to different origins of HNSCC. Therefore, tumors were subdivided into oral cavity, oropharynx, hypopharynx and larynx, where no correlation between CD56^{bright} NK cell abundance and tumor type was found (Figure 4).

In summary, our data demonstrate reduced CD56 bright levels as an early immunomodulatory event in patients with HNSCC regardless of tumor stage and tumor type.

Discussion

In recent years, a variety of immunoregulatory processes concerning different subsets of immune cells have been described and the concept has emerged that peripheral tolerance to tumors is maintained and enhanced by regulatory immune cells such as Tregs (20).

CD56⁺ natural killer (NK) cells, which comprise approximately 10% of human PBMCs and up to 30% of

intrahepatic mononuclear cells, are critical for innate anti-viral and antitumor host defence. They can be divided into two subsets, dim and bright, according to their CD56 surface density expression. Dim NK cells are cytolytic and comprise about 90% of NK cells, whereas bright NK cells are immunoregulatory, principally through cytokine production. Bright NK cells, which lack perforin granules, display homing receptors required for migration to secondary lymph nodes (1, 3, 10).

In this study, we investigated whether HNSCC modulates the abundance of circulating NK cell subsets in the peripheral blood with respect to different tumor stages and tumor types. We found that patients with HNSCC reveal a lower abundance of circulating CD56^{bright} NK cells compared to healthy blood donors, with strong individual deviations. The observed decrease was shown to be completely independent of different tumor types and tumor stages.

These observations are in accordance with other studies, which suggest that malignant tumors, independently of the disease stage, site or nodal involvement, have a strong impact on the homeostasis of lymphocytes long after the tumor has

been removed, even in the absence of any chemo- or radiotherapeutic treatments (21). In addition, previously published data demonstrate increased levels of CD56^{dim} NK cells and reduced levels of CD56^{bright} NK cells in patients with breast cancer (22). Previously, CD56^{bright} NK cells were shown to be able to differentiate into CD56^{dim} NK cells in the presence of synovial fibroblasts, and the resultant new population of CD56^{dim} NK cells revealed a lower cytotoxic capacity than the original CD56^{dim} subset. Differentiation of the majority of CD56^{bright} into CD56^{dim} NK cells was observed about 10 days after their transfer into NOD-SCID mice (23). Thus, whether the observed shift of circulating NK cell subsets in patients with head and neck cancer occurs due to stress factors of the HNSCC microenvironment has to be further elucidated.

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