Increased sMICA and TGFβ1 levels in HNSCC patients impair NKG2D-dependent functionality of activated NK cells

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Abbreviations: ADCC, antigen-dependent cellular cytotoxicity; HNSCC, head-and-neck squamous cell carcinoma; HSCT, haploidentical stem cell transplantation; KIR, killer cell immunoglobulin-like receptor; NCR, natural cytotoxicity receptor; NK, natural killer; NKG2D, natural-killer group 2, member D; vitsMICA/NKG2DL, soluble major histocompatibility complex Class I chain-related peptide A; TGFβ1, transforming growth factor beta 1; TIEM, tumor immune escape mechanism

Disseminated head-and-neck squamous cell carcinoma (HNSCC) escapes immune surveillance and thus frequently manifests as fatal disease. Here, we report on the distribution of distinct immune cell subpopulations, natural killer (NK) cell cytotoxicity and tumor immune escape mechanisms (TIEMs) in 55 HNSCC patients, either at initial diagnosis or present with tumor relapse. Compared to healthy controls, the regulatory NK cells and the ratio of pro/anti-inflammatory cytokines were decreased in HNSCC patients, while soluble major histocompatibility complex Class I chain-related peptide A (sMICA) and transforming growth factor β1 (TGFβ1) plasma levels were markedly elevated. Increased sMICA and TGFβ1 concentrations correlated with tumor progression and staging characteristics in 7 follow-up HNSCC patients, with significantly elevated levels of both soluble factors from the time of initial diagnosis to that of relapse. Patient plasma containing elevated sMICA and TGFβ1 markedly impaired NKG2D-dependent cytotoxicity against HNSCC cells upon incubation with patient-derived and IL-2 activated NK cells vs. those derived from healthy donors. Decreased antitumor recognition was accompanied by reduced NKG2D expression on the NK cell surface and an enhanced caspase-3 activity. In-vitro blocking and neutralization experiments demonstrated a synergistic negative impact of sMICA and TGFβ1 on NK cell functionality. Although we previously showed the feasibility and safety of transfer of allogeneic donor NK cells in a prior clinical study encompassing various leukemia and tumor patients, our present results suggest the need for caution regarding the sole use of adoptive NK cell transfer. The presence of soluble NKG2D ligands in the plasma of HNSCC patients and the decreased NK cell cytotoxicity due to several factors, especially TGFβ1, indicates timely depletion of these immunosuppressing molecules may promote NK cell-based immunotherapy.

Introduction

Head-and-neck squamous cell carcinoma (HNSCC) is a highly aggressive solid tumor originating from the epithelial lining of the upper aero-digestive tract, and regularly involves the nasal and oral cavity, lip, pharynx, larynx, and paranasal sinuses. The relative frequency of HNSCC correlates with tobacco and alcohol consumption as well as with human Papillomavirus infection and comprises approximately 4.5% of all malignancies.1,2 Classical therapeutic regimens for patients with HNSCC combine chemotherapy, radiotherapy and surgery. The 5-year survival curves over the last 30 years range from 30–65% and 5–58% for T1–T4 and N0–N3, respectively.3 The toxicity and morbidity proscribed by current therapies can severely impair quality of life, such that the prognosis for these patients remains poor.4,5 HNSCC patients have a high risk of impaired immune response due to (i) decreased leukocyte numbers, (ii) impaired leukocyte proliferative capacity, and (iii) increased numbers of CD4+CD25+Foxp3+ regulatory T cells (Treg), which are able to suppress all functions of T cells, natural killer (NK) cells and NK-T cells.6 Innate immune cells, such as NK cells, have stimulatory effects on the host defense by rapid activating the recognition of, migration towards and efficient elimination of tumor- or
otherwise “stressed” cells. NK cells are non-homogeneous, with CD56^bright/CD16^dim/neg NK cells possessing immunoregulatory functions (due to their high capacity for cytokine production), NK regulatory cells contrasting with CD56^dim/CD16^bright cytotoxic NK cells that express CD16 and killer cell immunoglobulin-like receptors (KIR) and are known to be highly cytolytic against virus-infected or malignant cells. Here, the activating receptors involved in the killing of transformed cells include the natural cytotoxicity receptors (NCRs) Nkp30, Nkp44 and Nkp46 that interact with poorly characterized ligands, and the NKG2D receptor [killer cell lectin like receptor K1 (KLRK1)], which recognizes a variety of well-defined ligands that are expressed by infected and transformed cells. Nevertheless, the soluble form of the NKG2D ligand (NKG2DL), also known as soluble major histocompatibility complex Class I chain-related peptide A (sMICA), is responsible for a systemic decrease in NKG2D surface expression on NK cells, CD8+ T cells and γδ+T cells, thereby significantly blocking the antitumor activities of these effector cells.

An association between plasma sMICA concentrations and disease Stage IV has been previously reported among Japanese HNSCC patients. In addition, decreased expression of NKG2D receptors has also been shown to impair NK cell function in HNSCC patients. Indeed, similar effects have been demonstrated in regards to other malignancies, such as colorectal cancer, prostate cancer, neuroblastoma, and several types of leukemia.

Interestingly, we demonstrated that sMICA and transforming growth factor β1 (TGFβ1) compromise the function of activated donor NK cells applied as an immunotherapeutic approach in children with neuroblastoma after haploidentical stem cell transplantation (HSCT). Thus sMICA/TGFβ1 play a critical role in the immune surveillance and effector cell-mediated killing of tumor cells with respect to both patient and donor NK cells.

In order to study whether similar pathological mechanisms play an important role in HNSCC patients, we set out to determine (i) the distribution of regulatory (CD56^bright/CD16^dim/neg) and cytotoxic (CD56^dim/CD16^bright) NK cells in HNSCC patients relative to age-matched healthy controls and (ii) the effect of plasma-released sMICA/TGFβ1 on the downregulation of the NKG2D-mediated tumor cell killing of both patient and donor-derived NK cells. Taken together, the present data indicate that individual inhibition patterns in HNSCC patients can be used to assess immune dysfunctions, which might help to design immunotherapeutic protocols to improve NK cell-mediated antitumor activity by scavenging soluble suppressive ligands.

**Results**

**Impaired distribution of immunocompetent cells in HNSCC patients**

Leukocytes were quantified in 55 HNSCC patients (not currently undergoing treatment) in order to assess whether the distribution of various cell immune cell subsets is altered in comparison to healthy controls. While median numbers of leukocytes and monocytes were significantly increased, CD3+ and CD19+ lymphocytes were reduced in HNSCC patients as compared to healthy controls (Fig. 1A). Although the absolute numbers of CD56+/CD3+ NKT cells and CD56+/CD5- NK cells remained constant, the proportion of immunoregulatory NK cells was decreased in HNSCC patients (median: 1.7% vs. 7.3% in controls), whereas the cytotoxic NK cell subpopulation was clearly increased (median: 94% vs. 87% in controls) as shown in Fig. 1B.

**Elevated sMICA and TGFβ1 plasma levels and altered cytokine profiles in HNSCC patients**

In 755 patients, we were able to quantify the plasma levels of sMICA and TGFβ1 both initially and at the time of relapse prior to treatment. We found a strong increase in sMICA and a moderate to strong rise in TGFβ1 in all patients (Fig. 2). This was confirmed in our complete patient cohort, with significantly lower sMICA levels (median: 83 vs. 475 pg/mL) and TGFβ1 levels (median: 24 vs. 45 x 10^4 pg/mL) for HNSCC patients at presentation as compared to relapsed patients (Fig. 3A, B). However, both markers showed values close to the detection limit with a mean of 22 pg/mL and 13 x 10^4 pg/mL measured in healthy controls.

With the exception of tumor necrosis factor α (TNFα), and the interleukins (IL)-6 and IL-8, the cytokine profile in the blood of our patient cohort was significantly altered relative to healthy controls (Mann-Whitney U-test). Patients displayed markedly decreased IFNγ, IL-2, and IL-12p70 and higher IL-10 concentrations, and significant differences in these particular cytokine levels were detected in HNSCC patients (in comparison to healthy controls) at presentation and relapse (Fig. 3C).

Grouping plasma sMICA levels from all HNSCC patients according to tumor characteristics revealed enhanced sMICA levels at progression and relapse as shown for tumor grading, disease stage and tumor size (Fig. 3D/F). Increased patient sMICA levels correlated with disease stage 4 (n = 28; median: 300 pg/mL), tumor size T4 (n = 23; median: 455 pg/mL) and tumor grading 3 (n = 12; median: 230 pg/mL), while patients with lower sMICA levels had disease stage 1 – 3 (n = 27; median: 47 pg/mL), tumor size T0 – T3 (n = 32; median: 47 pg/mL) and tumor grading 1 – 2 (n = 43; median: 66 pg/mL) (Fig. 3D/F). Moreover, TGFβ1 levels in HNSCC patients with disease stage 4 (n = 18; median: 30 x 10^4 pg/mL) and tumor size T4 (n = 18; median: 38 x 10^4 pg/mL) exhibited a 1.7 – 2.2-fold elevation in these protein levels in comparison to patients with disease stage 1 – 3 (n = 18; median: 18 x 10^4 pg/mL) and with tumor size T0 – T3 (n = 18; median: 17 x 10^4 pg/mL) (Fig. 3G, H). However, sMICA and TGFβ1 showed no significant correlations with lymph node status (N0 – N3) and, in case of TGFβ1, over the grading range of 1 to 3 (data not shown).

**High sMICA affects the cytotoxicity and viability of IL-2-activated NK cells ex-vivo derived from either HNSCC patients or healthy donors**

Functional experiments were conducted to investigate the impact of sMICA on the cytotoxic properties of NK cells. NK
Figure 1. Differences in blood leukocyte subpopulations between HNSCC patients and healthy controls. (A–C) Head-and-neck squamous cell carcinoma (HNSCC) patient (n = 55) vs. healthy control (n = 21) peripheral blood was collected and leukocytes were immunostained and analyzed via fluorescence cytometry. (A) Absolute numbers of immune cells were quantified [cells/μL] for CD14⁺, CD19⁺, CD3⁺, CD56⁺/CD16⁻ and CD56⁺/CD3⁺ cells. (B) Natural killer (NK) cells were subdivided (%) in circulating CD56⁺/CD16⁻ and CD56⁺/CD16⁺ subpopulations. Median for HNSCC patients (HNSCC) and healthy controls (C) are presented. Statistical analysis was performed by Mann-Whitney nonparametric U-test; P ≤ 0.05 was defined as statistically non-significant (n.s.).
cells were purified from HNSCC patients and healthy controls, stimulated with IL-2, and incubated with blood plasma (containing different sMICA concentrations) from either HNSCC patients or healthy individuals. After overnight incubation (16 h) with the respective plasma, the cytotoxicity of patient-derived NK cells (NK<sub>HNSCC</sub>) and NK cells from healthy donors (NK<sub>HD</sub>) against SCC-4 target cells (E:T-ratio of 10:1, 4 h) were individually tested and determined by fluorescence confocal microscopy.

The cytotoxicity of both NK<sub>HNSCC</sub> and NK<sub>HD</sub> were significantly inhibited when incubated with patient plasma containing high sMICA in comparison to NK cells pre-treated with the plasma of healthy controls (low sMICA) as summarized in Fig 4A/D (reciprocal proportional correlations) and exemplarily demonstrated in Fig. 4B, E. Interestingly, a greater decrease in effector cell viability was found for patient-derived NK<sub>HNSCC</sub> cells as compared to the more stable effector cell viability of healthy donor-derived NK<sub>HD</sub> cells in which both groups were pre-incubated with plasma of HNSCC patients containing high MICA levels. In contrast, both NK<sub>HNSCC</sub> and NK<sub>HD</sub> cells remained highly viable after incubation with plasma of healthy controls. Moreover, increased sMICA concentrations also correlated with decreased NK cell numbers and viability during the cytotoxic attack against SCC-4 cells (Fig. 4C).

In order to investigate an expected additive effect of sMICA and TGFβ<sub>1</sub> on NK<sub>HNSCC</sub> and NK<sub>HD</sub> cells, the patient plasma samples with high sMICA were stratified into two groups according to TGFβ<sub>1</sub> levels, i.e., high sMICA/high TGFβ<sub>1</sub> and high sMICA/low TGFβ<sub>1</sub> levels (relative to control plasma with low sMICA/low TGFβ<sub>1</sub>). NK cell-related cytotoxicity was more strongly inhibited in assays with patient plasma containing high levels of both sMICA and TGFβ<sub>1</sub> (HNSCC1) as compared to testing in patient plasma containing high sMICA and low TGFβ<sub>1</sub> levels (HNSCC2) (mean: 10 ± 10% vs. 24 ± 12%, respectively; Fig. 4F). Cytotoxicity in both cases was significantly blocked as compared to NK cells cultured with low sMICA/TGFβ<sub>1</sub> concentrations from healthy control plasma.

High levels of sMICA led to an inhibition of the NKG2D-dependent interactions between patient plasma-treated cells.
Figure 3. For figure legend, see next page.
NK\textsubscript{HNSCC} cells and adherent SCC-4 cells (Fig. 5D/2), as compared to the higher cytolytic activity of NK cells incubated with plasma derived from healthy controls (Fig. 5D/1). Moreover, patient plasma-incubated NK cells showed a significant decrease in viability as visualized by additional intracellular staining for activated caspase-3 (Casp-3) following methanol-fixation and subsequent permeabilization with Triton\textsuperscript{TM}X-100. Casp-3 staining activity was enhanced in a time-dependent manner (2–4 h) during co-incubation with target cells (Fig. 5D/2), as compared to the time-matched mono-cultured effector cell controls cultured in the absence of SCC-4 cells.

sMICA affects the tumor recognition and migration of NK cells

To describe the consequences that may be responsible for escape of HNSCC tumors from NKG2D-dependent immuno-surveillance, IL-2-activated NK\textsubscript{HNSCC} cells were incubated (16 h) with corresponding patient plasma containing high sMICA levels \textit{vs.} healthy plasma containing marginal sMICA concentrations as control. Early tumor cell recognition and infiltration of treated NK cells against adherent SCC-4 cells were detected by fluorescence microscopy after brief co-cultivation (15 min; Fig. 5A) and monitored via single cell experiments using time-lapse imaging (6 h; Fig. 5S). The experiments revealed impaired and disordered “effector-to-target” affinity and tumor cell infiltrations from NK cells cultured with plasma containing high sMICA levels as compared to the common antitumor activity of NK cells incubated with healthy control plasma (low sMICA). Supporting these observations, cytofluorimetric analysis revealed a marked decrease in NKG2D expression (mean fluorescence index (MFI): 21 \pm 6\% on IL-2-activated NK cells cultured (16 h) with patient plasma containing high sMICA concentrations, as compared to higher NKG2D levels (mean of 80 \pm 7\%) on activated NK cells cultured with control plasma (low sMICA) (Fig. 5B, left 2 columns). However, the viability in all treated NK cells was not affected after 16 h incubation with plasma from patients or controls (C \textit{vs.} HNSCC, Fig. 5C).

To verify the biological relevancy of these \textit{in-vitro} results, we next measured NKG2D expression on circulating NK cells \textit{in-vivo} using NK cells derived from the peripheral blood of various HNSCC patients (n = 12, HNSCC) and controls (n = 11, [C]). Patient NK cells showed a significantly lower NKG2D surface expression (mean MFI: 17 \pm 4\%) associated with higher relative levels of sMICA in plasma as compared to those from healthy individuals (mean MFI: 54 \pm 6\%) and corresponding to low sMICA concentrations (Fig. 5B, left 2 columns). Follow-up of 7 primary patients demonstrated increased sMICA/TGF\textsubscript{b1} levels and decreased NK cell cytotoxicity, stability of viability and reduced NKG2D expression on NK cells at relapse (Fig. 2).

Distinction between sMICA and TGF\textsubscript{b1}-mediated effects on NK cell-conditioned killing activity

To further verify the previous results and ascertain NKG2D-dependent anti-SCC-4 cytotoxicity of IL-2-activated NK cells isolated from HNSCC patients and healthy controls we next applied specific blocking agents and neutralization experiments with and without sMICA. In all E:T-ratios, the NKG2D-dependent cytotoxicity was strongly reduced when NK cell surface NKG2D was blocked with 20 \mu g/mL human anti-NKG2D polyclonal IgG antibody C-14, 750 pg/mL recombinant MICA (rMICA) or in the presence of patient plasma (HNSCC) containing high sMICA levels. An additive impact on the NK cell-mediated lysis was achieved by combining NKG2D receptor-blocking with anti-NKG2D antibodies and rMICA, resulting in strongly decreased NK cell cytotoxicity (Fig. S2). Moreover, healthy plasma (HP) controls containing low concentrations of TGF\textsubscript{b1} and sMICA displayed decreased NK cell cytotoxicity (relative to untreated controls) concomitantly with reduced NKG2D expression specifically in the presence of exogenous recombinant TGF\textsubscript{b1} and/or recombinant sMICA. However, both inhibitory effects could be reversed as introduction of TGF\textsubscript{b1} neutralization receptor antibodies and/or anti-sMICA antibodies increased the cytotoxic activity of NK cells pre-treated with patient plasma containing high plasma levels of TGF\textsubscript{b1} and/or sMICA (Fig. S4).

Discussion

Impaired immune surveillance is aggravated via tumor immune escape mechanisms in untreated HNSCC patients at primary diagnosis or relapse. Our present study identified impaired distributions in leukocytes, especially absolute higher leukocyte and monocyte numbers, but markedly decreased B and T cell numbers in untreated HNSCC patients as compared to healthy controls. This proportional decrease of immunocompetent cells might be due to marked upregulation of suppressor T\textsubscript{reg} throughout all disease stages in HNSCC patients, as previously described by Bose et al.\textsuperscript{6} Thus, increased numbers of suppressor T\textsubscript{reg} are not only involved in the observed decrease in various lymphocyte subpopulations but also in the inhibition of antigen-presenting cells, especially dendritic cells (DC\textsubscript{a}).\textsuperscript{21} In contrast to literature reports describing a strong decrease in the

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**Figure 3 (See previous page):** Tumor progression and relapse correlate with increased levels of soluble immunosuppressive factors. (A-B) sMICA and TGF\textsubscript{b1} concentrations in blood plasma of head-and-neck squamous cell carcinoma (HNSCC) patients with initial diagnosis (“HNSCC1”) and in patients at different time point of relapse with local tumor recurrence (“HNSCC2”) were compared with plasma samples from healthy controls (“C”). (C) Cytokine concentrations in collected blood plasma were investigated in initial (HNSCC1; n=9) and untreated relapsed (HNSCC2; n=12) patients (white bars) and compared with plasma samples from control individuals (C; n=11; [C]). (D-H) sMICA and TGF\textsubscript{b1} levels in HNSCC patients were grouped according to grading, disease stage and tumor size. Data show the median of the different columns. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor. Statistical analyses was performed by Mann-Whitney nonparametric U-test; P\leq 0.05 was defined as statistically non-significant (n.s.); \*statistically significant difference, P\leq 0.001.
Figure 4. For figure legend, see next page.
numbers of CD56<sup>+</sup>CD3<sup>–</sup> NK-T cells and especially in the levels of CD56<sup>–</sup>CD3<sup>–</sup> NK cells HNSCC patients, analysis of the immune status in our patients revealed no marked alterations in these lymphocyte subpopulations. However, we did find changes in different NK cell subsets. The regulatory NK cell subpopulation, which is able to trigger interactions between the innate and adaptive immune systems via both cytokine-release and “cross talk” with antigen-presenting DCs, was substantially decreased in our HNCC patients in comparison to age-matched healthy controls. This finding is consistent with that of Wulf et al. who observed decreased circulating regulatory NK cells in 70 HNCC patients throughout all tumor stages. We also detected higher numbers of cytotoxic NK cell subpopulations in HNCC patient peripheral blood than recorded in healthy controls. The observed changes in NK cell subpopulations among our HNCC patients taken together with the marked alterations detected in essential cytokine patterns suggests TIEM-mediated dysfunction of HNCC patient NK cells, an immunologic phenotype which was reflected by suppressed cytotoxicity against HNCC cells and reduced NK cell viability. Mukhopadhyaya et al. also observed decreased NK cell tumor or diseased lymph node infiltration as a percentage of total NK cells among different HNCC patients.

Moreover, Bose et al. showed that measurements of the cytokine secretory status of cultured MNCs from HNCC patients revealed decreased levels of Th1 cytokines (IFNγ, IL-12 and TNFα) and an increased release of Th2 cytokines (IL-4 and IL-10) opposite to the cytokine profile determined for cultured MNCs from healthy controls. In accordance with this report, we found strong alterations in the cytokine pattern of IFNγ, IL-2, IL-12p70 and IL-10 in our HNCC patients, where we could not detect any differences in TNFα concentration in cancer patients as compared to healthy controls. Nevertheless, the report of Bose et al., could not explain the engaging network of HNCC-dependent immunomodulation via soluble, tumor-derived molecules in relation to patient lymphocyte numbers and the specific cytotoxic functions of activated NK cells against HNCC cells.

An important question is whether malignant cells are recognized and killed by innate immune cells or they evade immune surveillance. Tumor-specific stress ligands that stimulate or inhibit effector cells are adequate molecular markers of solid tumor prognosis. For example, in a study by Wu et al., a significant correlation was found between high sMICA levels and prostate cancer disease progression. Moreover, a strong association between cancer stage and metastasis, and a marked rise in soluble MICA and MICB levels was recently demonstrated in a large study of 512 patients with various malignancies. SMICA-based TIEM in cancer patients are often mediated by matrix metalloproteinase (MMP)-dependent proteolytic cleavage (“shedding”) during tumor progression. Tumor shedding of the NKG2D ligands, which provide a means for tumor cell immune evasion, has been found to be regulated by ADAMs (a disintegrin and metalloproteases), 10 and 17, indicating that proteolytic cleavage of surface molecules is an undesirable characteristic of tumor cells.

The release of this NKG2D ligand is strongly associated with a systemically reduced NKG2D expression on the surface of circulating blood lymphocytes, especially cytotoxic CD56<sup>+</sup>CD3<sup>–</sup> NK cells, CD8<sup>+</sup> αβ and γδ T cells, resulting in attenuated recognition of transformed and malignant cancer cells and decreased tumor surveillance. The results of our current study confirm the impact of sMICA in the disease staging because untreated HNCC patients diagnosed with Stage IV disease had markedly elevated sMICA levels in their blood plasma as compared with those of lower disease stages. Additionally, a significant correlation between sMICA levels and both tumor size and tumor grading was detected in our patients. However, no associations between patients’ sMICA and increased frequency of regional lymph node (LN) metastasis could be proven. In contrast, a study of oral squamous cell carcinoma in Japanese patients demonstrated high sMICA levels in advanced cancer stages (Stage IV), sMICA concentrations correlating with increased frequencies of LN metastasis and poor clinical outcome. High sMICA levels coincided also with decreased survival rates in HNCC patients potentially synergizing with high sMICB levels.

Although sMICA appears to be a trend-setting for TIEMs of neuroblastoma and HNCC (CURRENT STUDY) the precise contribution of individual NKG2D ligands to TIEMs has not been evaluated yet. However, the plasma levels of the different NKG2D ligands vary considerably among patients and tumor entities. Therefore, in order to further elucidate the molecular conditions for these TIEMs and presumably associated thresholds for its clinical manifestation, it may be necessary to determine the plasma levels of other NKG2D ligands as well as important biomarkers.

We found elevated sMICA and TGFβ<sub>1</sub> levels in our untreated, relapsed HNCC patients as compared to both untreated primary HNCC patients and healthy individuals. Additionally the relapse of the disease seems to induce an increase of sMICA and TGFβ<sub>1</sub>, later manifesting in decreased NK cell viability and cytokotoxicity.
Figure 5. For figure legend, see next page.
viability and cytotoxicity as well as reduced NKG2D expression levels. However, after precise analysis via the TNM staging system, these relapsed HNSCC patients showed at least one advanced disease characteristic, such as tumor size, grading of tumor differentiation or elevated numbers of LN metastases as compared to the early staging after initial disease. In the relapsed patients this dramatic HNSCC tumor progression may have been caused by the reduction of an antitumor immune response by innate cells, such as regulatory NK cells, and this could be responsible for the upregulation of tumor-derived sMICA as compared to lower levels of this NKG2D ligand after primary diagnosis. This may partly reflect a time-dependent pattern in the appearance of different sMICA levels in HNSCC patient plasma, as we demonstrated during follow up in 7 patients. Similarly, multivariate analysis of multiple myeloma patients revealed strong correlations of this "independent predictive" factor sMICA (>305 pg/mL), associated with a poor overall and progression-free survival in those patients. Thus, the results are in line with our findings suggesting sMICA to be a potent prognostic marker in highly malignant diseases serving as identifier for high risk cancer patients. The latter has been confirmed by a report describing an association between increasing sMICA levels and oral squamous cell carcinoma disease Stage IV in Japanese patients. Moreover, expression of MMP-9 in samples from 43 HNSCC patients correlated with enhanced activity of inducible nitric oxide synthase (iNOS) and significantly higher cGMP levels, which together further contribute to increased tumor progression.

Beside the descriptive quantification of tumor markers and leukocyte subpopulations in the blood, our functional analyses applied showed impaired NK cell cytotoxicity in HNSCC patients. Our cytotoxicity experiments demonstrated that high sMICA/TGFβ levels identified in plasma from our HNSCC patients are jointly responsible for a drastic reduction of the NKG2D-mediated lytic activity against HNSCC cells and also for a decreased stability in IL-2-activated NK cells from both patients and healthy controls. Correspondingly to these results, Rossi et al. demonstrated a strong correlation between impaired NK cell viability and effector functions by inhibition of activation and also NKG2D and NKp46 expression. Furthermore, the visual monitoring of reduced cytotoxic properties from corresponding NK cells clearly represented a negative impact of patient-derived sMICA on these immune effector cells. The results of our current study revealed a relationship between high plasma sMICA levels and decreased target cell lyses by an inversely proportional coherence of $P<0.001$ and showed this for the first time in patients afflicted with HNSCC. Until now, only one group has described a direct effect of highly regulated and plasma-derived soluble tumor markers on NK cell functional inactivation. In an orthotopic murine model of HNSCC (SCC VII/5F) NK cell functions are strongly inactivated via downregulation of NKG2D and the antigen-dependent cellular cytotoxicity (ADCC) triggering CD16 receptors. Similarly, sMICA/B molecules are able to impair the NKG2D-mediated immunity against pancreatic carcinoma by directly diminishing the cytotoxicity of T and NK cells, which reflect the predominant sMICA-NKG2D interplay. In vitro studies further showed that tumor cell killing was completely inhibited by addition of a blocking anti-NKG2D antibody.

Our blocking experiments confirm these findings, since IL-2-activated patient-derived and donor NK cells, showed low killing activities by blocking of NKG2D with recombinant MICA (rMICA) and stronger effects in combination with TGFβ1 (rTGF) or NKG2D antibodies. As cytotoxicity was almost completely inhibited by the combinations of rMICA, rTGF and anti-NKG2D antibodies, this indicated not only a competitive NKG2D inhibition via specific antibodies to block the sMICA-NKG2D interaction but also a reduced NKG2D expression in the affected NK cells. Negative effects of high sMICA and TGFβ1 plasma levels detected in HNSCC patients could be extensively neutralized by blocking antibodies against sMICA and TGFβ1, indicating that both soluble factors are directly responsible for a diminished NK cell-mediated killing activity and reduced NKG2D expression. Accordingly to our results, the

**Figure 5 (See previous page).** Immunofluorescence microscopy and time-lapse imaging of NK cell killing activity. (A) Activated NKHNSCC Cells were cultured (16 h) with (i) patient plasma (HNSCC: 1:2 diluted with X-VIVO10) containing high sMICA (594 pg/mL) or (ii) heathy control (C) plasma with low sMICA levels (<20 pg/mL). NK cells were co-incubated with SCC-4 cells for 15 min at an effector-to-target (ET) cell ratio of 5:1. NKG2D (red) levels and CD45 (green) for both, controls (C) and HNSCC patients (HNSCC) plasma-incubated NK cells were analyzed by immunofluorescent staining with FITC- and PE-conjugated mAb, whereas co-incubated adherent SCC-4 cells were unlabelled. Double positive NK cells (CD45+/NKG2D+) showed positive signals by generation of overlay plots with exactly the same orientation. DAPI (4',6-Diamidino-2-Phenylindole, blue signals) was used to stain DNA from methanol-fixed effector and target cells for analyses of the nuclear morphology. (B) Flow cytometry was used to identify the NKG2D surface expression (mean fluorescence intensity, MFI) on NK cells pre-incubated with either patient plasma (MICA$_{high}$) or plasma from healthy controls (sMICA$_{low}$). NKG2D expression on NK cells in blood from healthy controls (C) and HNSCC patients (HNSCC) (C) Control of viability from all plasma-incubated NK cells was determined by flow cytometry with propidium iodide (PI). Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed by Student’s t-test, $^{*}_p<0.0001$ (IMFI): C vs. HNSCC. (D) Time-lapse monitoring (based on immunofluorescence microscopy) of cytotoxicity between NKHNSCC cells and adherent SCC-4 cells (ET ratio: 10:1). Activated NK cells isolated from patients or healthy donors were pre-incubated (16 h) with (2) corresponding patient plasma containing high sMICA (HNSCC: >400 pg/mL) or (1) low sMICA levels (<20 pg/mL) from healthy controls (controls). Time-dependent (15 min – 4 h) analysis of both cell types were accomplished by staining with FITC- and PE-conjugated mAbs against double-positive CD45+/NKG2D+ NK cells (green/red). Activated caspase-3 (Casp3-3, red) staining in apoptotic cells (after co-cultivations) by intracellular staining with PE-conjugated mAbs. Viable, non-apoptotic SCC-4 cells were unlabelled, whereas apoptotic SCC-4 cells showed enhanced Casp3 activity (apoptotic areas: white borders, see I/II). In overlay plots with exactly the same orientation, the double positive NK cells (CD45+/NKG2D+) showed a positive signal (yellow), whereas single positive, apoptotic SCC-4 cells (CD45+/NKG2D+/Casp3+) revealed no signals in the same plots (1: bottom slides, left). (2) Overlay plots of apoptotic NK cells (CD45+/Casp3+) generated double positive signals (yellow; apoptotic areas: red border) and showed a decreased NKG2D surface expression.
offending object of this specific immunosuppression could be TGFβ, potentiated by NKG2D-ligands subsequently resulting in decreased expression of NKG2D and NCRs on effector cells (i.e., NK cells, CD8+ T cells).\textsuperscript{39,40,41} Previously, our blocking experiments against highly expressed NKG2D receptors on activated NK cells pre-treated with rMICA or antibodies against NKG2D have shown a high degree of NKG2D down-modulation and attenuated killing functions of allogeneic NK cells against neuroblastoma cells.\textsuperscript{20} Most importantly, we showed in this clinical adaptive immunotherapy Phase I/II study with haploidentical, IL-2-activated NK cells that large numbers of activated donor NK cells can restore NK cell-mediated cytotoxicity by scavenging of sMICA in the plasma of patients.\textsuperscript{20}

In line with our findings regarding NKG2D downregulation is a recent report by Rossi \textit{et al.}, describing a minor NK cell apoptosis linked to enhanced downregulation of NKG2D, NKp44, NKp46, and CD25 on cytokine-activated NK cells,\textsuperscript{57} an event purportedly contributing to NK cell “burnout.” Accordingly, in a study by Bose \textit{et al.}, the expression of perforin, granzymeB and FasL are strongly downregulated in CD8+ T cells and NK cells from HNSCC patients underlying the inhibited cytotoxicity of HNSCC MNCs.\textsuperscript{6} Additionally, the lower viability of our analyzed NK cells in the presence of high sMICA levels observed during cytotoxic interactions could reflect the tumor cell capability to secrete apoptosis-promoting factors (such as programmed cell death ligand, PD-L1) that inhibit effector cells (T cells) functions in the tumor microenvironment,\textsuperscript{42} as observed with PD-1+ NK cells in multiple myeloma patients.\textsuperscript{43} Otherwise, the PD-1 expression on tumor-infiltrating effector cells could describe an early-initiating cytotoxicity against human papillomavirus (HPV)-linked HNSCC cells which may be re-stimulated by PD-1/PDL-1 inhibition and may correlate with a revised clinical prognosis in HPV-associated cancers.\textsuperscript{44}

The observed alterations in cytokine pattern (IFNγ, IL-2, IL-10, IL-12p70), decreased immune functions and impaired distributions of NK cell subpopulations among our HNSCC patients could directly disrupt different killing functions, including the maturation of mDCs via DC-NK cell “cross talk” and TNFα/IFNγ secretion, non-specific macrophage/NK cell-dependent cell lysis, and ADCC reactions.\textsuperscript{22,23}

In conclusion, our current findings unveil essential interrelations of soluble immunosuppressive NKG2D ligands via TIEMs in HNSCC patients at increased disease stages responsible for cytotoxic impairment of tumor immunosurveillance. In the last decade, the releasing of soluble NKG2D ligands has been extensively investigated with regard to their contribution to tumor pathologies and associated with a worse prognosis among cancer patients.\textsuperscript{45} This corollary could be responsible for a premature interaction of soluble tumor-derived ligands with various NCRs resulting in a receptor internalization and abrogated cytotoxicity mediated by various effector cell subsets.\textsuperscript{46}

Previously, a bi-national NK cell Phase I/II study (Clin-Gov-No-NCT01386619) demonstrated the safety, feasibility, and tolerability of using allogeneic un-stimulated and IL-2-activated NK cells in patients with multiple relapsed leukemia and highly malignant solid tumors after receiving HSCT.\textsuperscript{9,20,47} Taken together, the results of both our prior clinical study and our present findings in HNSCC patients revealed that allogeneic NK cells may be desirable to eliminate TIEM-derived limitations against patient NK cells. Therefore, it is rational to scavenge time dependent immunosuppressive ligands via acute depletion, such as by plasmapheresis, to utilize the tumoricidal properties of NKG2D and NCRs on activated NK cells for therapeutic benefit.

### Patients and Methods

**Patients**

Our study included 55 HNSCC patients (32 male and 23 female, from 18 to 99 years old). Forty of the patients were studied at primary diagnosis (Primary) and during the treatment follow-up as follows: 21 in complete remission (CR), 4 with disease progression (DP), and 15 with relapse (Rel) (Table 1). Fifteen patients were studied in a palliative situation at the time of diagnosis. No chemotherapy, radiotherapy and/or surgery were carried out at the time points of blood sample collection. Up to 21 age-matched healthy individuals (37±11yrs.) served as controls. Informed consent was obtained from patients, caretakers, and healthy controls. Procedures were approved according to guidelines of the medical ethics committee of the University Hospital Frankfurt (Ref. No. 143/09) and in accordance with national guidelines regarding human subjects.

**Cytokine analysis**

The BD CBA Kit is a tool for scavenging soluble cytokines, or various groups of cytokines, with beads of known size and fluorescence as a reference standard, allowing identification and quantitation of soluble molecules in blood via fluorescence cytometry. This system was applied to detect changes in cytokine levels in HNSCC patient sera (in comparison to healthy controls), as described previously.\textsuperscript{9}

**Quantification of sMICA and TGFβ1 in HNSCC patients**

The BAMOMAB MICA-Sandwich ELISA kit for sMICA (AXXORA GmbH, Germany), designed for quantitative analysis of soluble MICA in blood plasma was used as described previously.\textsuperscript{22,23} The MTPL ELISA for the quantitative measurement of TGFβ1 in human blood plasma was purchased from Milenia Biotec (Version 3.0, Germany). The majority of plasma from healthy controls revealed sMICA and TGFβ1 concentrations close to detection limits of engaged ELISAs (median [sMICA]: 22 pg/mL; median [TGFβ1]: 13 × 10^4 pg/mL, Fig. 3a/b, controls), whereas sMICA [TGFβ1] in the plasma from HNSCC patients ranged between 11-752 pg/mL [12-68 × 10^4 pg/mL] (Figure 3a/b, HNSCC1/2).
Immunomagnetic depletion and activation of CD56⁺CD3⁻ NK cells

Up to 100 mL heparinized blood from HNSCC patients was utilized to separate mononuclear cell (MNC) fractions using Ficoll. Native NK cells were further isolated from purified MNCs via “non-touched” depletion using the EasySep/C210 Human NK Cell Enrichment Kit (STEMCELL Technologies SARL, Germany). Unwanted cells are targeted for removal with tetrameric antibody complexes recognizing CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, glycophorin A and dextran-coated magnetic particles. The non-labeled cells were collected using an EasySep/C210 hand magnet according to the manufacturer’s recommendations. Freshly purified NK cells were ex-vivo expanded and activated with 1000 IU/mL IL-2 for 8–10 days as shown elsewhere. The purity of the separated CD56⁺CD3⁻ NK cells was 94.4 ± 3.3%.

Target cell line

The human adherent HNSCC cell line SCC-4 (ATCC: CRL-1624) was cultured in DMEM and GlutaMAX™ medium (GIBCO, Invitrogen, Germany). The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine (PAA Laboratories GmbH, Austria).

Cytotoxicity of activated NK cells from HNSCC patients

To determine the impact of tumor-derived sMICA on the NKG2D-mediated cytotoxicity, activated NK cells were incubated overnight (37°C, 5% CO₂, 250 rpm) with HNSCC patient plasma containing high sMICA levels or control plasma with low sMICA levels. All samples were diluted 1:2 in X-VIVO™ medium (Biowhittaker TMCambex Bioscience, Belgium). The cytotoxicity of these pre-treated NK cells against the MHC Class I neg HNSCC cell line SCC-4 (tested at an effector to target [E:T-ratio] ratio of 10:1) was determined within 4 h (37°C, 5% CO₂, 250 rpm) in a cell suspension co-culture. An adjusted gating strategy (Fig. S1) of a no-wash, single platform cytofluorimetric staining and analysis procedure was performed using a FC500 flow cytometer (Beckman Coulter, Germany). The effector cells were labeled with up to 4 different monoclonal antibodies (mAbs): CD45 FITC (fluorescein isothiocyanate), CD56 PE (phycoerythrin) or NKG2D PE, CD16 PC-7 (phycoerythrin-cyanin-7) in order to separate them from the target cell line SCC-4 labeled with CD9 FITC, CD9 PE or CD81 PE. The labeling of effector and target cells with mAbs was performed as described previously. The lytic activity of effector cells was calculated as the total loss of viable target cells as follows.

\[
\text{Cytotoxicity} = \frac{(1 - \text{concentration}_{\text{co-cultured target cells/µL}}} \text{concentration}_{\text{control cells/µL}}) \times 100\%
\]

Fluorescence microscopy/Immunofluorescence

Activated NK cells, pre-incubated with patient plasma (high sMICA) or control plasma, were co-incubated with SCC-4 cells on chamber slides (15 min–4 h, 37°C, 5% CO₂, E:T-ratio 5:1). Target cell recognition, HNSCC/NK cell interactions and the cytotoxicity of NK cells, were monitored by confocal fluorescence microscopy. Immunofluorescence, using FITC- and PE-conjugated mAbs, was used for discrimination of labeled NK cells (CD45⁺/NKG2D⁺) and unlabeled SCC-4 cells, as previously described.

Time-lapse microscopy

As a visual control for each corresponding NK cell-mediated cytotoxicity assay based on fluorescence cytometry, the killing activity of plasma-incubated NK effector cells against SCC-4 target cells was time-dependently monitored via confocal fluorescence microscopy. Therefore, very low numbers of single SCC-4

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**Table 1** The clinical parameters and frequencies of 55 HNSCC patients (initial diagnosis and relapse) summarized before undergoing any acute clinical therapeutic regimens. The 3 T0 patients presented with lymph node metastasis.

| Parameter                      | Frequency (%) |
|-------------------------------|--------------|
| **Age (mean: 59 years, range)** |              |
| 20–40                         | 4 (7.3)      |
| 40–60                         | 23 (41.8)    |
| 60–80                         | 25 (45.5)    |
| >80                           | 3 (5.4)      |
| *Sex*                         |              |
| male                          | 32 (58.2)    |
| female                        | 23 (41.8)    |
| **Tumor size**                |              |
| T0                            | 3 (5.4)      |
| T1                            | 15 (27.3)    |
| T2                            | 14 (25.5)    |
| T3                            | 1 (1.8)      |
| T4                            | 22 (40.0)    |
| **Lymph node status**         |              |
| N0                            | 29 (52.7)    |
| N1                            | 9 (16.4)     |
| N2                            | 16 (29.1)    |
| N3                            | 1 (1.8)      |
| **Metastasis**                |              |
| M0                            | 98 (96.4)    |
| M1                            | 2 (2.6)      |
| **Disease stadium**           |              |
| I                             | 13 (23.6)    |
| II                            | 5 (9.1)      |
| III                           | 9 (16.4)     |
| IV                            | 28 (50.9)    |
| **Tumor differentiation**     |              |
| Well                          | 4 (7.3)      |
| Moderate                      | 39 (70.9)    |
| Poor                          | 12 (21.8)    |
| **Tumor location**            |              |
| Floor of mouth                | 21 (38.2)    |
| Tongue                        | 15 (27.3)    |
| Upper gingiva                 | 6 (10.9)     |
| Lower gingiva                 | 7 (12.7)     |
| Buccal mucosa                 | 6 (10.9)     |
| **History of disease**        |              |
| Primary diagnosis             | 40 (72.7)    |
| Remission                     | 21 (38.2)    |
| Recurrence                    | 22 (40.0)    |
| Progress                      | 4 (7.3)      |
cells were grown scattered on 20 mm chamber slides (Nunc, USA) for 16 h (37°C, 5% CO₂). Subsequently, these adherent HNSCC cells were co-incubated for different periods with activated NK cells, pre-treated with patient (high sMICA) or healthy controls’ (low sMICA) plasma, at an E:T-ratio of approximately 10:1. Cytotoxicity of the effector cells was assessed by time-lapse microscopy and imaging (Fig. S3) as described previously.8,20,26

**Blocking and neutralisation experiments**

To demonstrate the sMICA triggered inhibition of NK cell-mediated cytotoxicity against SCC-4 cells, blocking assays against NKG2D receptors were carried out using NK cells collected from 4 relapsed HNSCC patients and 4 healthy controls. These effector cells were isolated and activated as described above. Subsequently, the cells were pre-incubated overnight with (i) recombinant MICA (rMICA, 750 pg/mL, RayBiotech, IP-03-367P, USA), (ii) human, anti-NKG2D polyclonal IgG antibodies (20 μg/mL, C-14, sc-5455, Santa Cruz Biotechnology, Germany), (iii) mixed solutions of both, 20 μg/mL anti-NKG2D antibodies and 750 pg/mL rMICA, (iv), patient plasma containing high sMICA levels, and (v) with low sMICA control plasma (Fig. S2). To analyze direct roles of TGFβ1 and sMICA on NK cell cytotoxicity and NKG2D expression, we incubated IL-2-activated NK cells with plasma of 3 patients containing high levels of TGFβ1/sMICA in the presence of TGFβ1 receptor (ab10518, ABCAM, Germany) and/or MICA antibodies (MAB13001, R&D systems, Germany). Additionally, control plasma of 3 healthy persons (low TGFβ1/sMICA) were incubated with NK cells in the presence of recombinant TGFβ1 (240-B-010, R&D systems, Germany) and/or recombinant MICA (Fig. S4). The altered NKG2D-dependent, NK cell killing rates against SCC-4 cells and NKG2D expression on those NK cells were measured at different E:T-ratios after 4 h (37°C, 5% CO₂), as described above.

**Statistical analyses**

The Mann-Whitney non-parametric U-test was used to compare leukocyte subpopulations and clinical pathological parameters of patients’ plasma sMICA/TGFβ1 from HNSCC patients compared to healthy controls. The Student’s t-test was used to assess the significance of the cytotoxicity from different, pre-incubated NK cells. A P-value ≥ 0.05 was considered statistically non-significant. Data of cytotoxicity and blocking assays are shown as the mean ± SD and represent 3 to 6 independent experiments.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

**References**

1. Shibuya K, Mathers CD, Boschi-Pinto C, Lopez AD, Murray CJ. Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. BMC Cancer 2002; 2:37; PMID:12502432
2. Ragin CC, Modugno F, Gollin SM. The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. J Dent Res 2007; 86:104-14; PMID:17251508; http://dx.doi.org/10.1177/154405910708600202
3. Howaldt HP, Kainz M, Euler B, Vorast H. Proposal for a new staging classification for cancer of the oral cavity: D35AK. J Craniomaxillofac Surg 2004; 32:285-90; PMID:154405910708600202
4. Mclearn C, Muldoon F, Collin SM. The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. J Dent Res 2007; 86:104-14; PMID:17251508; http://dx.doi.org/10.1177/154405910708600202
5. Howaldt HP, Kainz M, Euler B, Vorast H. Proposal for a new staging classification for cancer of the oral cavity: D35AK. J Craniomaxillofac Surg 2004; 32:285-90; PMID:154405910708600202
6. Bose A, Chakraborty T, Chakraborty K, Pal S, Baral R. Dysregulation in immune functions is reflected in tumor cell cytotoxicity by peripheral blood mononuclear cells from head and neck squamous cell carcinoma patients. Cancer Immun 2008; 8:10; PMID:18547033
7. Farag SS, Caliguri MA. Human natural killer cell development and biology. Blood Rev 2006; 20:123-37; PMID:16364519; http://dx.doi.org/10.1016/j.bler.2005.10.001
8. Kohls S, Bochennek K, Huenecke S, Zimmermann SY, Kauci S, Muller T, Wels WX, Klingebiel T, Esser R, Koehl U. A novel five-colour flow cytometric assay to determine NK cell cytotoxicity against neuroblastoma and other adherent tumour cells. J Immunol Methods 2007; 325:140-7; PMID:17663991; http://dx.doi.org/10.1016/j.jim.2007.06.013
9. Huenecke S, Zimmermann SY, Kauci S, Esser R, Brinkmann A, Tramsen L, Koenig M, Erben S, Seidl C, Tonn T, et al. IL-2-driven regulation of NK cell receptors with regard to the distribution of CD16+ and CD16- subpopulations and in vivo influence after haploidentical NK cell infusion. J Immunother 2010; 33:101E00802)
10. Coudert JD, Held W. The role of the NK cell activating receptor NKG2D in leukemia. Blood 2003; 102:1389-96; PMID:12714493; http://dx.doi.org/10.1182/blood-2003-01-0019
11. Bryceson YT, Ljunggren HG. Tumor cell recognition by the NK cell activating receptor NKG2D. Eur J Immunol 2008; 38(11):2957-61; PMID:18979516
12. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002; 419:734-8; PMID:12384702; http://dx.doi.org/10.1038/nature01112
13. Tamaki S, Sanefuji N, Kawakami M, Aoki K, Imai Y, Yamazaki Y, Yamamoto K, Ishihara A, Hatake K, Kiritaka T. Association between soluble MICA levels and disease stage IV oral squamous cell carcinoma in Japanese patients. Hum Immunol 2008; 69:88-93; PMID:18361952; http://dx.doi.org/10.1016/j.humimm.2008.01.010
14. Duray A, Demoulin S, Hubert P, Delvenne P, Saussez S. Immune suppression in head and neck cancers: a review. Clin Dev Immunol 2010; 2010:701657; PMID:21437225; http://dx.doi.org/10.1155/2010/701657
15. Daugupta S, Bhattacharya-Chatterjee M, O’Malley BW, Jr., Chatterjee SK. Inhibition of NK cell activity through TGF-beta 1 by down-regulation of NKG2D in a murine model of head and neck cancer. J Immunol 2005; 175:5541-51; PMID:16210663; http://dx.doi.org/10.4049/jimmunol.175.8.5541
17. Raffaello L, Prigione A, Iaoreth I, Camorano M, Leve-
reni I, Gambini C, Pende D, Steine A, Ferrone S, Piu-
toia V. Downregulation and/or release of NGK2D ligands as immune evasion strategy of human neuro-
blasto
toma. Neuro-Oncology 2004; 6:558-64; PMID:15458365;
http://dx.doi.org/10.1093/neo.04.0316
18. Wu JD, Higgins LM, Steine A, Cosman D, Haugk K,
Plymarte SR. Prevalent expression of the immunosup-
litary MHC class I chain-related molecule is counter-
acted by shedding in prostate cancer. J Clin Investig-
2004; 114:560-8; PMID:15314693; http://dx.doi.
10.1172/JCI200422206
19. Dourovina ES, Dourovina MM, Vider E, Suson RB,
O'Reilly RJ, Digong R, Vyas YM. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. J Immunol 2003;
171:6891-9; PMID:14662866; http://dx.doi.org/10.
1042/jimmunol.171.12.6891
20. Klues S, Herrencke S, Pechukl D, Eser R, Koch J,.
Brehm G, Sorensen J, Gardholw T, Brinkmann A, Bader P, et al. IL-2-activated haploidipotent NK cells restore NGK2D-mediated NK cell cytotoxicity in neuro-
blastoma by scavenging of plasma patients. Eur J Immunol 2010; 40:3255-67; PMID:20164445; http://
dx.doi.org/10.1002/eji.201040568
21. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006; 6:295-307; PMID:
16716866; http://dx.doi.org/10.1038/nri1806
22. Zwirner NW, Fuertes MB, Girart MV, Damaica CI,.
Rossi LE. Cytokine-driven regulation of NK cell func-
tions in tumor immunity: role of the MICA-NKG2D system. Cytokine Growth Factor Rev 2007; 18:159-70;
PMID:17324607; http://dx.doi.org/10.1016/j.
cytogfr.2007.01.013
23. Walter T, Dalod M, Robbins SH, Zitovgol V, Livier.
E. Natural-killer cells and dendritic cells: "Tumori funet la.
face". Blood 2005; 106:2252-8; PMID:15933055;
http://dx.doi.org/10.1182/blood-2005-03-1154
24. Wulf S, Prks R, Borngk J, Trenk K, Tellenberg W.
Decreased levels of circulating regulatory NK cells in patients with head and neck cancer throughout all tumor stages. Anticancer Res 2009; 29:3053-7;
PMID:19613153
25. Mukhopadhyaya R, Tatak K, Krishnan N, Rao RS,
Fakh AR, Naik SL, Gangal SG. Immunoreactivity of lymphocytes from draining lymph nodes, peripheral blood and tumor infiltrates from oral cancer patients. J Clin Lab Immunol 1989; 30:21-5; PMID:2641790
26. Watson MF, Spendlove I, Acre M, Bird B, Schut.
PMI. Reduced expression of plasma cell by shedding in prostate cancer. J Clin Investig 2006; 118:1445-52; PMID:
16184547; http://dx.doi.org/10.1182/jci200629832;
PMID:17218152; http://dx.doi.org/10.1016/j.
clinimm.2006.01.007
27. Tamaki S, Kawakami M, Yamanaka Y, Shimomura H,
Kamper M, Sattler A, et al. Age-matched lymphocyte subpopulation reference values in childhood and ado-
lescence. J Human Lymphocytes 2010; 10:497-101; PMID:20167255
28. Rehmann B, Schurt P, Brandhorst T, Mandia CI, Zwir-
er et al. Role of NGK2D in tumor cell lysis medi-
ated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of neoplastic origin. Eur J Immunol 2001; 31:1076-86; PMID:11306477; http://
dx.doi.org/10.1002/1097-0238(EIS).321076-0002.CO;2-Y
29. Ontberg JR, Dayrenc BE, Yuan M, Oflalogu E, Repa-
sky EA. Enhancement of natural killer cell (NK cyto-
toxic) activity by shedding in prostate cancer. J Clin Investig 2004; 114:560-8; PMID:15314693; http://dx.
doi.org/10.1182/blood-2004-11-22206
30. Cappel RA, Beckett LR, Sbisichi RA, Zhang J, Yu J,
Smith MK, et al. The PD-1/PD-1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT011, a novel monoclonal antibody. Blood 2010; 116:2286-94; PMID:20460501; http://dx.doi.org/10.1182/blood.
2010-02-271874
31. Bredoual C, Hans S, Merillon V, Van Rysswick C, Ravel B, Benhamouda N, Levionnois E, Nizard M, Si-
moneau P, Millet A, Brinser N, et al. Soluble MICA expressing tumor infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer. Cancer Res 2013; 73:128-38; PMID:23135914; http://dx.
doi.org/10.1158/0008-5472.CAN-12-2606
32. Baraganera A, Saquet C, Lopez-Perez F, Salvador F.
Secretory pathways generating immunosuppres-
sive NGK2D ligands: New targets for therapeutic intervention. Oncoimmunology 2014; 3:28497; PMID:25050215;
http://dx.doi.org/10.4161/onci.28497
33. Ulrich E, Koch J, Gerwenka A, Steine A. New pros-
spects on the NGK2D/NGK2D system for oncology. Oncoimmunology 2013; 2:e26097; PMID:23453908; http://dx.doi.org/10.4161/onci.26097
34. Huernecke S, Behl M, Falder C, Zimmermann SY, Kloe-S, Bremm M, Ulrich E, Sorensen J, Quaiser A, Erben S, et al. Clinical grade purifica-
tion and expansion of NK cell products for an opti-
mized manufacturing protocol. Front Oncol 2013; 3:118; PMID:23736825; http://dx.doi.org/10.3389/
fonc.2013.00118
35. Huernecke S, Behl M, Falder C, Zimmermann SY,.
Bochhennek K, Tramsh E, Reib E, Klaarnen D, Kamer M, Sattler A, et al. Age-matched lymphocyte subpopulation reference values in childhood and ado-
lescence: application of exponential regression analysis. Eur J Haematol 2008; 80:532-9; PMID:18284628
36. Rheinwald JG, Becket MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cul-
tures from human squamous cell carcinomas. Cancer research 1981; 41:1657-63; PMID:7214336
37. Rheinwald JG, Becket MA. Defective terminal differ-
centiation in culture as a consitent and selectable char-
acter of malignant human keratinocytcs. Cell 1980; 20:629-32; PMID:6491961; http://dx.doi.org/10.1002/eji.200939728
38. Wilson EB, El-Jawhari J, Neilson AL, Hall GD, Melcher AA, Meade JC, Cook GP. Human tumour immunity eva-
uation via NGK2D-beta blocks NK cell activa-
tion but not survival. Oncotherapeutic reestoration of anti-tumour activity. PloS one 2011; 6:e22842
39. Gyio M, Conrini P, Grisini S, Boero S, Musso A, Poggi.
A. Soluble HLA-I-mediated secretion of TGFBeta1 by human NK cells and consequent down-regula-
tion of anti-tumor cytolytic activity. Eur J Immunol 2009; 39:3459-68; PMID:19830740; http://dx.
doi.org/10.1002/eji.200939728
40. Ghio M, Contini P, Negrini S, Boero S, Musso A, Poggi.
A. Soluble HLA-I-mediated secretion of TGFBeta1 by human NK cells and consequent down-regulation of anti-tumor cytolytic activity. Eur J Immunol 2009; 39:3459-68; PMID:19830740; http://dx.doi.org/10.1002/eji.200939728