Profound coordinated alterations of intratumoral NK cell phenotype
and function in lung carcinoma

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Non standard abbreviations: ADC, Adenocarcinoma; CD, Cluster of Differentiation; DC, Dendritic Cells; FACS, Fluorescence-Activated Cell Sorting; HLA, Human Leukocyte Antigen; IL, Interleukine; KIR, Killer-cell Immunoglobulin-like Receptor; MHC, Major Histocompatibility Complex; MICA/B, MHC I related Chain A/B; NCR, Natural Cytotoxicity Receptor; NK cell, Natural Killer cell; NSCLC, Non-Small-Cell Lung Cancer; SCC, Squamous Cell Carcinoma; LCC, Large Cell Carcinoma; PBMC, Peripheral Blood Mononuclear Cells; TGF, Transforming Growth Factor; TIL, Tumor Infiltrating Lymphocytes; TNM grade, Tumor Node Metastasis grade, ULBP, UL16-Binding Protein.

Precis: The tissue microenvironment in human lung carcinomas suppresses the tumoricidal activity of natural killer cells, thereby contributing to immune escape and progression.
ABSTRACT

Both the innate and adaptive immune systems contribute to tumor immunosurveillance in mice and humans, however, there is a paucity of direct evidence of a role for natural killer (NK) cells in this important process. In this study, we investigated the intratumoral phenotypic profile and functions of NK cells in primary human tumor specimens of non-small cell lung carcinoma (NSCLC). We used in situ methods to quantify and localize NK cells using the NKp46 marker and we characterized their phenotype in blood, tumoral and non-tumoral samples of NSCLC patients. Intratumoral NK cells displayed a profound and coordinated alteration of their phenotype, with a drastic reduction of NK cell receptor expression specifically detected in the tumoral region. According to their altered phenotype, intratumoral NK cells exhibited profound defects in the ability to activate degranulation and IFN-γ production. We found that the presence of NK cells did not impact the clinical outcome of patients with NSCLC. Finally, we showed that tumor cells heterogeneously express ligands for both activating and inhibitory NK receptors. Taken together, our results suggest that the NSCLC tumor microenvironment locally impairs NK cells, rendering them less tumorcidal and thereby supportive to cancer progression.
INTRODUCTION

Lung cancer is one of the leading causes of cancer death and its incidence continues to increase worldwide (1). Innate as well as adaptive immune components infiltrate human lung tumors. A strong CD3+ T cell infiltration (2, 3) and several DC subsets were found in lung tumors. However, despite the presence of an immune cell infiltrate, ineffective anti-tumor immunity is common in Non-Small-Cell Lung Cancer (NSCLC), and the correlation between tumor-infiltrating immune cells and the prognosis of patients with lung cancer remains controversial. In a recent study, we observed that the density of mature DC which home exclusively in the tertiary lymphoid structures present in the tumor stroma is highly associated with a favorable clinical outcome (4).

Direct evidences for a role of NK cells in tumor immune surveillance are limited. In murine models, animals lacking NK cells or NK cell receptors have a higher incidence of spontaneous tumors (5-7). In humans, a 11-year follow up correlative study of the general population showed a correlation between low NK cell cytotoxicity in peripheral blood and increased cancer risk (8). Furthermore, NK cells were associated with a good prognosis in colorectal (9), gastric (10) and lung (11) carcinomas, but these studies were based on the analysis of CD57 expression, which is not restricted to NK cells.

Tumor cells express molecules and release mediators (12) that allow their evasion from NK cells immunosurveillance (13). High levels of non classical MHC I molecules HLA-E and HLA-G that are inhibitory ligands for CD94/NKG2A and ILT2 respectively are present on tumor cells (14-16). Moreover, tumor cells also negatively regulate NK cell function by the release of immunosuppressive factors such as IL-10 (17) or TGF-β (18).
The tumor infiltrating NK cells have been characterized in few human studies. In renal cell carcinoma, intratumoral NK cells were found able to lyse target cells but only after in vitro IL-2 stimulation and had a distinct repertoire from blood NK cells from the same patients (19-21). In ovarian tumors, intratumoral NK cells display a reduced expression of DNAM-1, 2B4 and CD16, and impaired activation (22). The informations on NK cells infiltrating NSCLC are very scarce. In one study, CD3-CD56+ NK cells were found significantly reduced in tumor tissue as compared to adjacent non tumoral tissue (2, 3). However, another study describes a CD56brightCD16- NK cell subset highly enriched in tumor stroma, expressing NKp44, CD69 and HLA-DR activation markers but exhibiting reduced cytolytic potential (23). The identification of NK cells in situ has been hampered by the lack of specific reagents. Most if not all NK cells express NKp46 (24) and the recently developed NKp46 antibodies provide a new specific tool to detect NK cells in tumors. On the other hand, a large series of activating and inhibitory NK cell receptors and of their ligands have been described recently (25). Their identification is of prime importance to fully understand NK cell functions in tumors. We therefore focused our study on the quantification and localization of NK cells in NSCLC using the NKp46 marker, specific for NK cells, and characterized extensively the phenotype of CD3-CD56+ NK cells isolated from the tumoral and non tumoral distant regions of resected tumors, lung tissue from non-tumoral inflammatory pathologies and blood of NSCLC patients and healthy donors, by using a large panel of NK cell surface receptors and markers (n=17). The expression of a series of ligands for NKG2D, KIRs, DNAM1 and NCR (n=12) was also investigated on the tumor cells. Our results show that NKp46+ cells are mainly localized in the invasive margin of NSCLC. The intratumoral NK cells display a profound and coordinated alteration of their phenotype which is specifically
detected in the tumoral microenvironment. A similar phenomenon was observed in vitro by co-culture of blood NK cells with tumor cells, suggesting a tumor-induced local impairment of NK cells.
MATERIALS AND METHODS

Patients tumor samples and healthy controls

Human primary lung tumors, emphysema or bronchectasis tissues were obtained from Institut Mutualiste Montsouris (Paris) and Hotel Dieu hospital (Paris), on the day of surgery, with patient’s consent and agreement of the french ethic comittee (number 2008-133) in application with the article L. 1121-1 of french law. None of the patients received neo-adjuvant chemotherapy or radiotherapy. Patients with metastasis were ineligible. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood samples obtained from NSCLC patients or healthy volunteer donors, at the Centre National de la Transfusion Sanguine (Paris).

Cell lines

K562 cells derived from human leukemia cell line (American Type Culture Collection (ATCC) # CCL-243) were cultured in RPMI supplemented with 10%. A549 cells derived from human adenocarcinoma (ATCC # CCL-185) were cultured in DMEM supplemented with 10% FCS and 1% Ultroser G. The cumulative culture length of the cells was fewer than 6 months after resuscitation. Early passage cells were used for all experiments and they were not reauthenticated.

Immunohistochemistry

Paraffin-embedded tumors were retrieved retrospectively from patients diagnosed with early stage NSCLC at Institut Mutualiste Montsouris. The expression of NKp46, HLA-E and HLA-G was performed by immunohistochemistry as described (26) using monoclonal anti-NKp46 (R&D systems), -HLA-E (MEM-E/02, Exbio), or -G (5A6G7, Exbio).

Preparation of human single-cell suspension

Surgical samples were mechanically dilacerated, and single cell suspensions
obtained after non enzymatic disruption in the BD retrieval solution (BD Biosciences, Le Pont-De-Claix, France) for 1 h at 4°C were filtered through a 70 µm cell strainer (BD Biosciences). Non tumoral tumor tissue was obtained at >10 cm from the tumor. Cells were washed in 10% FCS/PBS medium and mononuclear cells were purified using Ficoll gradient. Tumor Infiltrating Lymphocytes (TILs) were obtained after CD45 positive selection using magnetic separation protocol.

Flow Cytometry

Single cell suspensions were analyzed by three-color flow cytometry and NK cells were defined as CD3-CD56+ cells within lymphocyte gate. NK receptor antibodies included in the analysis are listed in the Supplemental Table 1. Cells were incubated with conjugated antibodies or isotypic controls, for 20 min at 4°C and analyzed with FACScalibur cytometer (BD Biosciences). Flow cytometry data were analyzed using Cellquest Pro software (BD Biosciences). P values and Pearson correlation coefficients (r) were calculated between flow cytometry data of thirteen marker combinations among CD3-CD56+ intratumoral NK cells in thirty NSCLC patients. r values were plotted from r = min to r = max in matrix representation, followed by unsupervised hierarchical clustering using the GENESIS program (27).

Co-culture experiments

PBMC (1.5 x 10^6) from healthy donors or NSCLC patients, activated with 100U/ml IL-2 (Roussel-Uclaf) during 12 h, were cultured in the presence of lung cancer cell line A549 or autologous cancer cells (0.3 x 10^6), +/- transwell membrane (BD Falcon) and +/- 80ng/ml anti-TGFβ antibody (R&D systems). After 5 days of co-culture the phenotype of NK cells was analyzed by flow cytometry.

CD107a degranulation and IFNγ assays
PBMCs or TILs from NSCLC patients were cultured for 12 h in the absence or presence of 100 U/ml IL-2 and incubated with target cells at effector-target (E/T) ratios of 10:1 during 4 h, with monensin and PE Cy5-conjugated anti-CD107a (LAMP-1) mAb. Cells were then washed in PBS-FCS-EDTA and stained for 20 min at 4°C with FITC-conjugated anti-CD3 and APC-conjugated anti-CD56 or control conjugated isotypes. After fixation and permeabilization, the expression of IFN\(\gamma\) was detected by incubation with PE-conjugated anti-IFN\(\gamma\) for 30 min at 4°C.

**Method for NK cell quantification and statistical analysis**

After staining, whole tissue sections were scanned using NanoZoomer (Hamamatsu Photonics). Stained NKp46\(^+\) NK cells were then counted for 86 patients using NDP.View software in 10 fields of 1 mm\(^2\), in the center of the tumor (CT) and in the invasive margin (IM). Overall Survival (OS) and Disease-Specific Survival (DSS) curves were estimated by Kaplan-Meier method and differences between the groups of patients were evaluated using the log-rank test at minimal p value.
RESULTS

NK cell infiltration in lung tumors

NK cell infiltration was analyzed in tumoral and non-tumoral areas of lungs from 86 early stages NSCLC patients with adenocarcinoma (ADC) (n=69) or squamous cell carcinoma (SCC) (n=17), (Supplemental Table 2) by immunohistochemistry using the NK cell specific marker NKp46 (28). The NK cells were mainly localized in the invasive margin of the tumor (Figure 1A, B). However, they were rarely in contact with tumor cells and found outside the tertiary lymphoid structures (Figure 1C). Their appearance is large and granular. The NKp46 labeling in some intratumoral NK cells is localized into cytoplasmic granules (Figure 1D) and more diffuse in other NK cells. The numbers of NK cells per mm² ranged from 9 to 21 (median = 9, mean = 21) in the invasive margin (IM) and from 1 to 15 (median = 1, mean = 15) in the center of the tumor (CT) (Supplemental Figure 1). Twenty-two patients had more than 10 NK cells per mm² in the CT versus 42 patients in the IM. Altogether these results indicate that NK cells are recruited in the tumor microenvironment, where they localize mainly in the tumoral stroma rather than tumor nest.

Intratumoral NK cells exhibit a drastic reduction of a cluster of five receptors

TILs were isolated from fresh tumor tissues in a series of thirty NSCLC patients (Table 1) and the percentages of NK cells, defined as CD3⁻CD56⁺ cells were determined among lymphocytes. We confirmed the heterogeneity of NK distribution among patients, with values ranging from 1.7% to 34.4% with a mean of 8.6%, independently of the histological type, the TNM and the size of the tumor (Table 1). In most patients, the isolated intratumoral NK cells were mainly CD56dim. Their mean fluorescence intensities of CD56 expression were similar to that of blood NK cells from healthy donors (MFI 314 +/- 163 n= 26 and 309 +/- 111 respectively, n= 30).
However, in a minor proportion of patients (patients 11, 12, 25 and 28) the intratumoral NK cells were mainly CD56\textsuperscript{bright} (MFI of CD56 expression ranged between 579 to 1655) (Table 1).

We compared the expression of 17 NK receptors and markers on cells isolated from the tumor, the non tumoral distant tissue and peripheral blood (Figure 2A). An example of dot plots analysis is shown in supplemental Figure 2. The percentage of cells positive for the indicated receptors was analyzed, after gating on CD3\textsuperscript{−}CD56\textsuperscript{+} cells. These cells express NKp46, demonstrating that the CD3\textsuperscript{−}CD56\textsuperscript{+} gate includes NK cells. The expression of activating NK receptors NKp30, NKp80, CD16, NKG2D, and DNAM1 was reduced (from 30% to 60%) on intratumoral cells as compared to distant NK cells or blood NK cells from the same patients or healthy donors (Figure 2A). The expression of MHC class I receptors, including ILT-2, CD158a and h KIRs was also significantly reduced. On the opposite, the CD69 and NKp44 activation receptors, barely detectable on blood NK cells, were expressed on intratumoral NK cells with a median of 12% and 36% of NK cells positive for NKp44 and CD69, respectively (Figure 2A). The median percentages of NK cells positive for NKp46, CD94, NKG2C, NKG2D, and CD161 did not differ significantly between blood, non tumoral and tumoral lungs, contrasting to NKG2A expression which is slightly upregulated on intratumoral NK cells (Figure 2A). Finally, the phenotype of NK cells in two other lung pathologies, emphysema and bronchectasis (supplemental Figure 3) was analyzed and found similar to that of NK cells in normal lungs, except for NKp30 marker, which seems to be down-regulated in these non tumoral lung diseases. These results indicate that NK cells are present in the tumor microenvironment of NSCLC where they display a unique phenotype, which is not
detected at distance or in the periphery and may be induced by the tumor microenvironment.

**Co-modulation of the NK receptors in NSCLC**

To further investigate the modulation of NK cell receptors in tumors we searched for correlations between markers expression in the thirty patients. Pairwise comparisons of the markers were done by measuring Pearson correlation coefficient (r) and related P values. The relationships underlying these correlations were visualized by using unsupervised hierarchical clustering of r values (Figure 2B). Interestingly, this approach revealed three clusters. A cluster of five receptors that are co-regulated and contains CD16, ILT2, DNAM1, Nkp30 and Nkp80, the receptors found drastically reduced on intratumoral NK cells as compared to blood NK cells. These results suggest that similar mechanisms could be involved in their down regulation. We named this cluster “downR”. Two other clusters were detected. One including CD161, CD94 and NKG2A and a third one containing the activation markers Nkp44 and CD69 whose expression was upregulated on intratumoral NK cells.

To examine the distribution of the major phenotypic alterations among the thirty patients and their possible correlations with the histological type of the tumor and TNM, we performed an unsupervised hierarchical clustering of cell surface marker expression on intratumoral NK cells for each of patients (Figure 3). Two patterns of phenotypic alterations were observed. Whereas a low expression of the downR cluster correlated with high expression of CD69 in patients’ group A, the down regulation of only few members of the cluster correlated with low expression of CD69 in patients’ group B. These two groups did not differ in term of histological type or stage classification.
Reduced receptors expression of intratumoral NK cells is induced by the tumor

To investigate whether the tumor cells allow the selection of NK cells with an altered phenotype or induce phenotypic alterations of NK cells, NK cells isolated from blood of 6 healthy donors or one NSCLC patient were co-cultured with A549 NSCLC cell line or autologous tumor cells respectively. The phenotype of NK cells was determined after five days of co-culture in the presence or not of a transwell membrane, or in the presence of anti-TGF-β antibody. The expression of the following NK receptors was downregulated on NK cells from some patients after co-culture with tumor cells: NKp30 (3/7 co-cultures), NKp80 (5/7 co-cultures), ILT2 (2/7 co-cultures) and DNAM1 (6/7 co-cultures), whereas NKp46 expression was not affected, as observed in NK cells isolated from lung tumors (Supplemental Figure 4). The down-regulation of NKp30, NKp80 but not of DNAM1 was reversed in the presence of anti-TGF-β antibody. The expression of CD16 was not affected except for one donor, corresponding of NSCLC patient co-cultured in the presence of autologous cancer cells. Moreover, NKG2D was also downregulated after co-culture in 5/7 donors. Transwell experiments revealed that NKp30, NKp80, DNAM1 and NKG2D down-regulation seems be dependent on contacts between NK and tumor cells. On the contrary, ILT2 down-regulation was not dependent of cell contacts with tumor cells (Supplemental Figure 4).

Altogether these results indicate that tumor cells themselves can modulate the NK cell phenotype, by mechanism dependent on cell-to-cell contact and/or TGF-β.

Impaired CD107a degranulation and IFNγ secretion of intratumoral NK cells

The phenotype of intratumoral NK cells suggests an alteration of their functionality. We examined CD107a expression after a four hours incubation with various target cells. Degranulation of intratumoral NK cells as compared to blood NK cells was
significantly reduced after incubation with K562 cells, in the presence of IL2 (22% CD107$^+$ cells versus 41%) (Figure 4A and B). Moreover, intratumoral NK cells exhibited little if any capacity to degranulate when cultured with autologous tumor cells (16% CD107$^+$ cells) whereas blood NK cells from the same patient were fully effective (72% CD107$^+$ cells) (Figure 4A). No IFN$\gamma$ production was detected after IL-2 stimulation and contact with K562 cells, whereas it was detected in response to PMA and ionomycin (Figure 4C). These results indicate that intratumoral NK cells display impaired capacities to stimulate degranulation and IFN$\gamma$ production, via the activation receptors in contrast to blood NK cells from NSCLC patients, in accordance with their altered phenotype.

**Prognostic value of NKp46 cells**

To determine if NK cell infiltration in NSCLC has any impact on clinical outcome, we analyzed the prognostic value of NKp46$^+$ NK cells on patients survival. NK cells were quantified on paraffin embedded sections of 86 NSCLC in the center of the tumor, and in the invasive margin. Three years after surgery 73 patients were alive (85%), 13 of whom had relapsed, and 13 patients had died (15%) (Supplemental Table 2). Twelve deaths were NSCLC-related and one death was not. We investigated the prognostic significance of NK cell densities in each area of analysis for overall survival (OS) and disease specific survival (DSS) (Supplemental Figure 5). Analysis of Kaplan-Meier survival curves showed the lack of significant differences between the groups of patients with high or low NK cell densities. These results suggest that the presence of NK cells is not associated with clinical outcome at early stages of the disease.
Expression of NK ligands on tumor cells

We analyzed the expression of ligands of NK receptors on fresh tumor cells isolated from 12 NSCLC patients (Figure 5). A heterogeneous expression was observed in the different patients. NKG2D ligands expression was observed at high level (i.e. superior to 40% of tumor cells positive for the ligand) only in 3 patients out of 12. Classical MHC class I molecules were expressed in all patients, but on 18 to 95% of cells, depending the donor. The expression of HLA-E and -G was found on 35 to 90% of cells of 7 patients, DNAM1 ligands on 50 to 80% of cells of 6 patients and NCR ligands were rarely expressed, in only in 1 patient out of 5 tested for these ligands. Moreover, we did not observe any correlation between the expression levels of ligands on tumor cells and putative receptors on intratumoral NK cells from the same patients (10 patients). The expression of HLA-E ad G was also determined by immunohistochemistry on another series of 17 patients. We observed a strong expression of HLA-E on tumor cells in adenocarcinoma, squamous and large cell carcinomas, in 12/17 patients (Table 2 and Supplemental Figure 4A and C) and no expression in 5/17 patients (Supplemental Figure 4D). In contrast, HLA-E was not expressed by non tumoral epithelial cells (Supplemental Figure 4B). HLA-G was also expressed by tumor cells in adenocarcinoma (Supplemental Figure 4E, G), but not in SCC (Supplemental Figure 4H). However, it was also strongly expressed by epithelial cells in non tumoral proximal area in all histological types (Table 2).
DISCUSSION

We demonstrate that NK cells are enriched in NSCLC tumor microenvironment and localized in the stroma of the tumor. Phenotypic analysis of these intratumoral NK cells revealed an altered repertoire of NK cell receptors, with a coordinated decreased expression of a cluster of NKp30, NKp80, DNAM-1, CD16 and ILT2 receptors, when compared to the repertoire of NK cells from distal lung tissues or blood from the same patients or healthy donors. We observed that the capacities to stimulate degranulation and IFNγ secretion of these NK cells are abolished, which is not the case with circulating NK cells from the same patients. Finally, we found frequent high levels of HLA-E and HLA-G expression and undetectable or low levels of ligands for activating receptors or NCR on tumor cells. Interestingly, a down regulation of a similar set of activating receptors was observed by Mamessier et al. in intratumoral NK cells from human breast tumors (personal communication).

The analysis of NK cell infiltration in NSCLC microenvironment, based on the immunohistochemistry analysis of NKp46 marker, revealed that NK cells were mainly localized in the invasive margin of the tumor. Esendagli et al. described significantly lower amounts of NK cells in malignant tissues of NSCLC in comparison to non malignant area. However, the phenotype of these cells differ from that of classical NK cells since they were characterized by low expression of CD56, NKG2D and NKp46 (2). We observed that tumor infiltrating NK cells are mainly CD56dim, and CD56bright in some patients as described (23). A strong reduction of receptors including NKp30, NKp80, CD16, DNAM-1, ILT-2 and KIR was observed on intratumoral NK cells whereas blood NK cells from the same patients displayed no significant modification of their phenotype as compared to healthy controls. The expression of NKp80, CD16, DNAM-1, ILT-2 and KIR receptors were not modified on NK cells from lung specimen.
of patients with emphysema and bronchectasis. Altogether these results suggest that this phenotype is induced in the tumor microenvironment. Several mechanisms could explain the down-regulation of NK receptors, such as chronic ligands exposure or cytokine-induced down modulation. Consistent with the hypothesis that tumor cells can modulate NK phenotype, we showed that NK cells obtained from the blood displayed a similar receptor repertoire alteration (4 out of 5 receptors tested) when co-cultured during five days with lung tumor cells. An additional down-regulation of NKG2D was observed in vitro, which could be due to the high expression levels of NKG2D ligands on A549 cells (data not shown). Co-culture experiments show that the down-regulation of receptors was reversed in transwell assays, for NKp30, NKp80 and DNAM-1 suggesting that the mechanisms responsible for the downregulation of these receptors imply cell contacts between NK and tumor cells. Moreover, the addition of anti TGF-β antibodies reversed the downregulation of NKp30 and NKp80, but not that of DNAM1, suggesting that secretion of TGF-β could be one of the possible mechanisms that induces NKp30 and NKp80 down-regulation. The down-regulation of activation receptors on intratumoral NK cells could thus results from NK-tumor cells interactions. The subsequent down regulation of receptors could follow receptor-ligands interactions, as it has been described for DNAM-1 (22). The expression of DNAM1 ligand CD155 on NSCLC tumor cells could explain the down-regulation of DNAM1 in intratumoral NK cells.

Down-regulations of NKp30 and NKp46 have been described in cervical cancer (29), and downregulation of DNAM-1, 2B4 and CD16 in ovarian carcinoma (22). In addition, altered NKp30 and NKp46 NCR expression, and failure to lyse autologous MHC-I deficient tumor cells was observed on NK cell clones obtained from NSCLC tumors (30). Among KIR expressed by NK cells, we found that only CD158a,h was
significantly down-regulated in tumor environment. In respect with this intriguing result, it could be interesting to assess the HLA and KIR genotyping, in a large cohort of NSCLC patients to determine the impact of such parameters in the susceptibility to this disease.

We demonstrated that intratumoral NK cells fail to stimulate degranulation when cultured with autologous tumor cells or with K562, showing that they are deficient in their degranulation capacities. This impaired cytotoxic activity was not related to a defect in granzyme B or perforin expression (data not shown). In accordance with our results, Carrega et al. (23) have observed that intratumoral NK cells express activation markers NKp44 and CD69, and have a reduced potential to kill tumor cells (23). The intratumoral NK cells did not secrete IFN-γ even after stimulation with IL2. Indeed other stimuli like IL12 and IL-18 might be required for optimal cytokine production. The elevated concentration of TGF-β1 found in lung cancer patients (31) could be responsible for the low NK lytic activity (18, 31). Tumor cells release soluble form of MIC ligands, which can inhibit NKG2D function (32, 33). Finally, Myeloma-derived fibroblasts inhibit the IL-2 driven up-regulation of triggering receptors that are involved in the NK-mediated recognition and killing of tumor cells (34). Altogether these observations show that several mechanisms can be involved in the down-regulation of these receptors and reduced lytic activities of intratumoral NK cells.

The analysis of prognostic value of NK cell infiltration in NSCLC revealed that the presence of NK cells did not impact on clinical outcome. The overall survival and disease free survival were not significantly different in patients having high and low NK cell infiltrations. These results are in accordance with the fact that intratumoral NK cells display a strong down-regulation of activating receptors that are important for tumor cell recognition and killing and display impaired capacities to stimulate
degranulation. Indeed, the clinical outcome of patients would be more dependent on NK cell phenotype and functionality rather than on NK cell density.

The tumor cells in NSCLC specimen were characterized by high levels of non classical HLA-E and HLA-G, decreased expression of class I molecules, and undetectable or low levels of NKG2D and NCR ligands in most patients which may be involved in the tumor resistance to autologous NK cell-mediated lysis. We therefore suggest that the coordinated altered receptor repertoire and lack of lytic activity of intratumoral NK cells in NSCLC that mirrors an increased expression of inhibitory receptors ligands and low or undetectable activating receptors ligands on tumor cells is highly suggestive of a local impairment of NK cells activity towards tumors cells and that may contribute to cancer progression.
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REFERENCES


27. GENESIS software available at http://www.genome.tugraz.at.


### Table 1: Clinical characteristics of 30 patients with NSCLC studied for NK cell phenotype

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Pathological staging and histological types of lung cancer were determined according to the TNM staging system and to the histological classification of the World Health Organization.
Organization, respectively. The percentages of NK cells were determined by flow cytometry as CD3⁻CD56⁺ cells. PY: Packs per year. na: data not available.

**Table 2 : HLA-E and HLA-G expression by tumor cells**

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HLA-E and HLA-G expression was determined in ADC, in SCC and LCC in tumor area (TA), non tumoral proximal area (NTPA), non tumoral distal area (NTDA). The scores indicate the results of a semi-quantitative analysis of positivity. -: no detectable expression, +/-: less than 5%; +: 5-10%; ++: 10-50%; +++: 50-100%; ++++: 100% of cells express the ligand. na: data not available.
FIGURE LEGENDS

Figure 1

NK cells are present in lung tumor microenvironment. The presence of NK cells (in red) was analyzed in tumor sections of NSCLC patients (A-D) by using NKp46 immunohistochemical labeling on paraffin-embedded lung tumors. Original magnifications: x10 (A), X40 (B), X20 (C) and X40 (D) followed by computer magnification.

Figure 2

Altered phenotype of NK cells from lung tumors.

A. The expression of NK cell receptors was analyzed by flow cytometry on intratumoral NK cells (n = 30, grey box-plot), on NK cells from non tumoral distant lung (n=10, dashed box-plot), peripheral blood of patients (n=10, spotted box-plot), and healthy donors (n = 30, white box-plot). The percentages of CD3−CD56+ cells that expressed indicated NK receptors were determined relative to isotypic control staining. Plots show the range of data values obtained. Top and bottom whiskers, represent values of the top and bottom 25% of the cases, respectively; boxed area, interquartile range and the significant P values between groups; horizontal black line, median value. Receptor expression was compared between different groups using the PLSD Fisher test. P values are shown only for those comparisons that were statistically significant. P were: * p<0.05, ** p<0.01, *** p<0.001.
B. Correlation matrix of flow cytometry data. P values and Pearson correlation coefficients (r) were calculated between thirteen marker combinations among CD3⁻CD56⁺ intratumoral NK cells in thirty NSCLC patients, presented in Figure 2A. r values were plotted from $r = \text{min (green)}$ to $r = \text{max (red)}$ in matrix representation, followed by unsupervised hierarchical clustering using the GENESIS program.

**Figure 3**

**Hierarchical clustering of intratumoral NK cell markers**

Hierarchical clustering of thirteen marker combinations among CD3⁻CD56⁺ intratumoral NK cells in the thirty NSCLC patients. The percentage values obtained by FACS analysis in the figure 2A were clustered using the Genesis Software. Combinations of surface markers were plotted from the minimal (yellow) to the maximal (red) level of expression. Gray, not determined.

**Figure 4**

**Reduced functions of NK cells from lung tumors**

A. Direct cytotoxicity against K562 or primary autologous tumor cells determined by degranulation assay, using CD107a labeling and FACS analysis. Unstimulated or IL-2-stimulated PBMC from blood of NSCLC patients, or IL-2-stimulated tumor infiltrating lymphocytes (TIL) were used as effector cells, at a ratio E:T of 10:1. The percentages indicated give the proportion of CD107 positive PBMC after gating on CD3⁻CD56⁺ NK cells.

B. Mean percentages of CD107a expressing NK cells. IL-2-stimulated PBMC from blood (n = 4), or IL-2-stimulated TIL (n = 7) of NSCLC patients were used as effector cells, at a ratio E:T of 10:1, against K562 cells. The percentages indicated give the proportion of CD107 positive cells after gating on CD3⁻CD56⁺ NK cells. *p < 0.05 (Mann Whitney test).
C Impaired IFN-γ secretion by intratumoral NK cells of NSCLC patients. IL-2-stimulated TIL were used as effector cells, at a ratio E:T of 10:1. The percentages indicated give the proportion of CD107 and IFN-γ positive PBMC after gating on CD3-CD56+ NK cells.

**Figure 5**

**NK cells receptors ligands expression on NSCLC tumor cells.** Expression of MICA/B, ULBP1, ULBP2, ULBP3, HLA-ABC, HLA-E, HLA-G, PVR, Nectin, NKp30, NKp44 and NKp46 ligands on primary lung tumor cells of five to twelve NSCLC patients was analyzed by flow cytometry. Tumor cells were defined as CD45- large cells. The graphics represent the percentage of positive cells for ligands among tumor cells. Horizontal lines represent the mean percentage expression of each ligand.
Figure 1
Figure 2A

Figure 2B
Figure 3

![Heatmap diagram showing expression levels of various NK cell markers across different patient groups and tumor sizes.](image-url)
Figure 4

A

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<td>IL-2 stimulated PBMC</td>
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B

% of NK cells positive for CD107a

C

Blood

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Tumor

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01/06/2011
Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma

Sophia Platonova, Julien Cherfils-Vicini, Diane Damotte, et al.

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