Cytotoxic Markers and Frequency Predict Functional Capacity of Natural Killer Cells Infiltrating Renal Cell Carcinoma

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Abstract

Purpose: Renal cell carcinoma harbors high numbers of infiltrating lymphocytes with apparent limited efficacy in tumor control. This study focused on the natural killer (NK) cells infiltrating renal cell carcinoma.

Experimental Design: Tumor-infiltrating lymphocytes (TIL) were isolated from renal cell carcinoma and analyzed for NK cell frequency and phenotype (n = 34). NK cells were enriched and tested for effector function.

Results: Two renal cell carcinoma subtypes were identified, one containing high (>20%) of the lymphocyte population, n = 14), the other low (20%, n = 20), NK cell numbers. NK cells of both groups were noncytolytic ex vivo but differed in CD16 and cytotoxic effector molecule expression as well as in their capacity to acquire cytotoxic activity: The majority of NK cells from tumors with high NK cell content (high NK-TIL) were CD16bright, whereas few CD16bright NK cells were found in tumors with low NK cell frequencies (low NK-TIL). The CD16 dichotomy correlated with different capacities to develop cytotoxicity after short-term activation with interleukin-2 ex vivo: Low NK-TIL remained noncytolytic against K562 and unresponsive to signals via the activating receptor NKp46 despite expression of receptor and adaptor molecules. In contrast, high NK-TIL acquired cytotoxic function. As described for peripheral CD16bright NK cells, NK cells from high-NK tumors showed high per cell expression of granzyme A, granzyme B, and perforin. NK cells from low NK-TIL resembled CD16neg/dim peripheral NK cells with few cytotoxin+ cells and lower expression of perforin.

Conclusion: The extent of NK cell infiltration and the expression of markers (CD16 and cytotoxins) predict the functional capacity of NK cells infiltrating renal cell carcinoma and can be used to characterize subgroups of renal cell carcinoma.

Renal cell carcinoma (RCC) is a progressive tumor that accounts for 80% to 85% of malignant kidney tumors and 3% of all adult malignancies in the Western world (1, 2). About 30% of patients exhibit metastases at the time of diagnosis (2).

Few therapeutic options exist for renal cell carcinoma because it does not respond to chemotherapy or irradiation. Renal cell carcinoma seems to be immunogenic and some patients respond to systemic immunotherapeutic agents, including IFN-α and/or interleukin-2 (IL-2; refs. 3, 4). However, it is unclear why only some patients show remarkable regression of metastatic lesions whereas others exhibit rapid tumor progression under identical cytokine therapies. Because systemic cytokine therapies often inflict serious adverse effects, it is desirable to spare nonresponding patients from treatment. Multiple studies have attempted to elucidate variables that distinguish responders from nonresponders, thereby showing prognostic significance for successful immunotherapies (5–10). Conflicting results have been reported regarding the importance of peripheral lymphocyte subpopulations (6–8) and the presence of higher numbers of T cells in renal cell carcinoma tissues seems to correlate with a poor prognosis (9, 11, 12).

For some solid tumors, such as squamous cell lung cancer (13), gastric (14), colorectal cancer (15), and head and neck squamous cell carcinoma (16), the presence of intratumoral natural killer (NK) cells was correlated with improved survival rates. NK cells are part of the first defense against neoplastic growth (17), because, unlike T cells, they are not tolerized against autologous cells and can attack cells with aberrant

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expression of MHC class I molecules (18, 19). Whereas down-regulation of MHC class I molecules allows tumor cells to escape recognition by tumor-specific CTL, this should render them susceptible to NK cell lysis (20). Indeed, it has been shown that NK cells can control tumor growth in vivo and prevent dissemination of malignant cells (21, 22).

For renal cell carcinoma, little is known about the characteristics and significance of tumor-infiltrating NK cells (NK-TIL). We previously showed that NK-TIL differ from autologous peripheral NK cells regarding their expression of inhibitory receptors, which may contribute to functional deficits within tumor tissues (23). Furthermore, we showed that some renal cell carcinoma tissues had a higher percentage of NK cells, which could be activated against tumor cells after stimulation with low-dose IL-2, whereas other NK-TIL populations were refractory to a similar activation procedure. In the present study, we have further characterized the NK-TIL of renal cell carcinoma and defined markers that predict their functional capacity.

**Materials and Methods**

**Patients and healthy blood donors.** After informed consent, samples of histologically diagnosed clear cell carcinoma were obtained immediately after surgical resection \( (n = 33) \). Urological Department of Ludwig-Maximilians-University Munich, Urological Clinic Planegg, Munich, Germany; Department of Urology, University of Heidelberg, Heidelberg, Germany; Peripheral blood mononuclear cells (PBMC) were obtained from renal cell carcinoma patients \( (n = 22) \) and healthy donors \( (n = 10) \). Detailed patient characteristics \( (24–26) \) are shown in Table 1.

**Isolation of TIL and PBMC.** TIL were isolated from tissues immediately following surgical resection by mechanical disruption or enzymatic digestion (23, 27). Differences in lymphocyte composition, expression of surface markers, or intracellular cytotoxic effector molecules were not observed using these methods (not shown). Only mechanical disruption was used to isolate TIL for functional studies. PBMCs were purified by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were used directly for functional and phenotypic investigations or cryopreserved for further analyses.

**Effector and target cells.** Effector cells were cultured in TIL medium \( (RPMI 1640, 10\% \text{ pooled human serum, } 2 \text{ mmol/L L-glutamine, } 100 \text{ units/mL penicillin, } 100 \mu\text{g/mL streptomycin, and } 1 \text{ mmol/L sodium pyruvate (all from Invitrogen, Life Technologies, Eggenstein, Germany); supplemented with } 20 \text{ units/mL recombinant human IL-2 (Cetus, Emeryville, CA)} \). CD56^+ cells were positively selected using magnetic bead separation and the AutoMACS System (Miltenyi, Auburn, CA).

Primary activated human NK cells \( (2DL2^*\text{NK}) \) were generated by allogeneic stimulation of CD3-depleted PBMC from a healthy donor and maintained in TIL medium with 5% heat-inactivated fetal bovine serum and 300 units/mL recombinant human IL-2. 2DL2^*\text{NK} cells, the human leukemia NK cell lines NKL (kindly provided by M. Lopez-Botet, Department of Health and Experimental Sciences, University Pompeu Fabra, Madrid, Spain), YT \( (A. \text{ Krenskey, Department of Pediatrics-Immunology and Transplant Biology, Stanford University, Stanford, CA)} \), and NK-92 \( (E. \text{ Vivier, Centre d'Immunologie de Marseille-Luminy, CNRS-INSERM-Université de la Méditerranée, Marseille, France)} \) were used as positive controls.

Cytotoxic activity was evaluated using the MHC class I−negative cell line K562. The murine mastocytoma cell line P815 was used for redirected lysis experiments.

**Cell-mediated lympholysis.** Chromium release assays were done as described (28). Briefly, Na^2(51Cr)O_4 (NEN, Boston, MA)–labeled target cells were incubated with effector cells for 4 hours at indicated effector-to-target cell ratios. For redirected lysis experiments, P815 cells were preincubated with antibodies against NKp46 (hybridoma supernatant, clone 45G8, 1:400 final concentration, IgG1, kindly provided by M. Colonna, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Washington, DC) or isotype control antibody MOPC21 (IgG1, 200 ng/mL final concentration, Sigma-Aldrich, GmbH, Taufkirchen/Munich, Germany)

### Table 1. Clinicopathologic variables of renal cell carcinoma patients

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<th>Tumor stage</th>
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<td>IV</td>
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<td>T_{2}N_{0}M_{0} G_{2}</td>
<td>IV</td>
<td>A-SD (44)</td>
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</table>

NOTE: Only clear cell carcinomas were selected. Patients did not receive any treatment in addition to surgery.

Abbreviations: M, male; F, female; A, alive; A-DF, alive-disease free; A-PR, alive-progression; A-SD, alive-stable disease; DOD, dead-of-disease; ND, not determined.

*Age, in years, at operation; mean = 61.3y for high-NK group and 61 years for low-NK group.

1. Tumor classification according to (24, 25).

2. Follow-up was completed until August 2005 with a median follow-up of 34 months (range: 12–45). Numbers in brackets are months of survival after operation.
for 20 minutes at room temperature before the release assay. Spontaneous release was determined by incubating target cells alone; total release was determined by directly counting labeled cells. Percentage cytotoxicity was calculated as follows: percentage specific lysis = [experimental counts per minute (cpm) – spontaneous cpm] / [total cpm – spontaneous cpm] × 100. Duplicate measurements were done in all experiments.

**Immunophenotyping of lymphocytes.** Lymphocytes were analyzed without cultivation using monoclonal antibodies (mAb): CD3-PerCP (SK7), CD16-FITC (3G8), perforin-FITC (6c9), granzyme A-FITC (CB9), and isotype controls (IgG2b and IgG1; all from Pharmingen, San Diego, CA); CD56-APC (N901) and NKp46-PE (BAB281; Beckmann/Coulter, Westwood, MA); CD3ε-FITC (G3), granzyme B-PE (GB11), and isotype controls (IgG2a, IgG1; Serotec, Oxford, United Kingdom); and NKG2D-PE (FABI139D; R&D Systems, Minneapolis, MN). After incubation with each antibody for 45 minutes on ice, cells were washed, fixed with 1% paraformaldehyde (30 minutes; ice), permeabilized with 1% saponin (Sigma-Aldrich), and then incubated with antibodies for intracellular proteins. Cells were analyzed in parallel to functional assays using four-color flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, CA).

**Semiquantitative reverse transcription-PCR.** Total RNA of 0.5 × 10⁶ lymphocytes was extracted using the RNAqueous-4PCR kit, including a DNase digestion step (Ambion, Austin, TX) and reverse transcribed into cDNA using oligo-d(T)₁₂₋₁₈ primer and Superscript II (Invitrogen, Life Technologies) with the following conditions: 10 minutes at 70°C, 70 minutes at 42°C, and 5 minutes at 95°C. Reverse transcription-PCR (RT-PCR) of adaptor molecules DAP10 and DAP12/KARAP (killer cell – activating receptor-associated protein) and housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was done from cDNA using Taq polymerase (Invitrogen, Life Technologies) and primer pairs as follows: 1 minute at 95°C, 30 seconds at 62°C, 1 minute at 72°C, and final extension for 7 minutes at 72°C. Primer sequences (Genzentrum, Munich, Germany) were as follows: 5′-ATGATC-CATCTGGTCACTAC-3′/5′-TGACGCCCTGGCTGGAATATG-3′ (DAP10), 5′-CTAGGGGGCGACATACGAC-3′/5′-GATTCCGGGTCTAATG-3′ (DAP12), and 5′-GATTCCGGGTCTGAGTAC-3′/5′-GCCTACGT- CAGGATGACC-3′ (GAPDH). RT-PCR products were analyzed on 2% agarose gels.

**Real-time quantitative RT-PCR.** Total RNA was isolated from cryopreserved tissue using the silica gel-based RNA isolation protocol (RNeasy-Mini; Qiagen, Hilden, Germany) and heat-activated Taqman DNA polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA). Amplification conditions and validation were done as described (29, 30). Predeveloped Taqman reagents with optimized primer and probe concentrations for human 18S rRNA, β-actin, and CXCL12/SDF-1 were obtained from Applied Biosystems.

**Statistical analysis.** Due to the limited number of patients analyzed, nonparametric statistical models were used to determine the significance of differences in sample groups. P values were calculated according to Wilcoxon rank-sum (S-Plus 6.2 Professional, Seattle, WA).

**Results**

**High frequency of CD16bright NK cells correlates with high NK cell content in renal cell carcinoma tumors.** We previously showed that some renal cell carcinoma tissues were infiltrated by significant numbers of NK cells (23). In the present study, we analyzed the expression of FcγRIII (CD16) and compared NK-TIL from tumor tissue with their corresponding autologous peripheral NK cell populations. Peripheral NK cells (NK-PBMC) of renal cell carcinoma patients contained an average of 89.5% CD16bright NK cells, with a very narrow

### Table 2. CD16 surface expression NK cells correlates with the frequency of NK cells in renal cell carcinoma tumors

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<tr>
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<th>%NK (range)</th>
<th>%CD16+ (range)</th>
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<td>NK-PBMC</td>
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<td>12.7 (2.0-26.0)</td>
</tr>
<tr>
<td>NK-TIL</td>
<td>22</td>
<td>59.3 (24.8-96.9)</td>
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<tr>
<td>High NK-TIL</td>
<td>7</td>
<td>35.4 (22.0-62.1)</td>
</tr>
<tr>
<td>Low NK-TIL</td>
<td>14</td>
<td>12.2 (5.9-17.9)</td>
</tr>
</tbody>
</table>

*Number of lymphocyte populations investigated.
†Mean percentage (range) of CD3+CD56+ NK cells.
‡Mean percentage (range) of CD16+ cells within gated CD3+CD56+ NK cells.
§PBMC from renal cell carcinoma patients.
∥TIL from renal cell carcinoma tissues, grouped according to their NK cell content (high-NK-TIL, >20% NK; low-NK-TIL, ≤20% NK).
¶P value between both groups of NK-TIL was <0.0001 (other P values are given in the text).
range of 72.4% to 98.7% (Table 2). Thereby, they did not differ from NK-PBMC of healthy donors that contain an average of 90% CD16bright NK cells (31, 32). In contrast, NK-TIL had an average of only 59.3% CD16bright NK cells with a broad range of 24.8% to 96.9% among different patients.

The percentage of NK cells plotted against the frequency of CD16bright NK cells separated the TIL samples into two groups (Fig. 1A): TIL with high NK content (high-NK-TIL, >20% of NK cells) showed very high percentages of CD16bright NK cells (average of 89.9%) within a narrow range (82.0-96.6%). TIL with a low frequency of NK cells (low NK-TIL, <20% of NK cells) displayed a lower percentage of CD16bright NK cells (average of 43.5%). NK-PBMC from all patients (Fig. 1A; Table 2) or healthy donors (not shown) showed high percentages of CD16bright cells independent of the NK content of the tumor (Fig. 1A).

The high NK-TIL group resembled NK-PBMC (P = 0.9), whereas low NK-TIL showed significantly fewer CD16bright NK cells, compared with NK-PBMC or high NK-TIL (P < 0.0001 for both groups). The CD16neg/dim NK population present in TIL was either CD56bright or CD56neg/dim. A correlation to the source of the NK cells from TIL with high or low NK content was not obvious. Figure 1B illustrates the similarity of high NK-TIL to NK-PBMC and the difference to low NK-TIL. All TIL samples differed from PBMC in terms of lymphocyte composition, such as CD4-to-CD8 T-cell ratios and the percentage of NK cells, excluding the possibility that TIL represented contaminating peripheral rather than tissue-derived lymphocytes (not shown).

**NK cells from high-NK tumors have higher cytolytic activity than NK cells from low-NK tumors.** In peripheral NK cells, the expression of CD16 (CD16bright versus CD16neg/dim) correlates with cytotoxic activity (32, 33). To investigate the cytotoxic capacity of NK-TIL, we used the MHC class I–negative cell line K562 that lacks ligands for all known NK cell inhibitory receptors and, therefore, is recognized by all NK cells. Hereby,
we could show that the lack of cytolytic activity was the result of a deficiency in the lytic capacity of the NK cell population and not caused by NK cell inhibition through inhibitory receptor-ligand interactions with MHC. Renal cell carcinoma generally expresses MHC class Ia and Ib molecules that serve as inhibitory ligands for NK cells and thus were unsuitable for our analysis. We also enriched NK cells from different sources to comparable amounts (60-95%) to exclude that low NK cell numbers or detection limits of the assay explained a lack of cytotoxicity.

In contrast to NK-PBMC, NK cells directly isolated from tumors did not lyse K562 even after enrichment (23). Therefore, NK cells were cultured in low-dose IL-2 (20 units/mL) for 24 or 48 hours before assessing their cytolytic activity. Enriched NK-PBMC of all renal cell carcinoma patients or healthy donors lysed K562 target cells after short-term activation by IL-2 (Fig. 2A). NK cell–depleted lymphocytes (T cell enriched, <5% NK cells) had lytic activities below 5% against K562 (negative controls; not shown). NK cells enriched from high NK-TIL similarly lysed K562 after short-term activation and even nonenriched TIL populations, containing 35% to 42% NK cells, exhibited significant cytolytic activity (Fig. 2A). In contrast, enriched low NK-TIL had significantly lower cytolytic capacities than NK-PBMC and high NK-TIL \( (P = 0.014 \text{ and } P = 0.046, \text{ respectively}) \). Specific lysis values were below 10% lysis for all low-NK-TIL samples, except for one with >20% lytic activity that was tested at an elevated effector-to-target ratio.

We next investigated whether NK cells responded differently to triggering of the activating receptor NKp46. NKp46 is exclusively and constitutively expressed by NK cells (34). It represents a major activating receptor for NK cells and does not require other coactivating stimuli (35). Redirected lysis experiments using an antibody to the NKp46 receptor were done with selected samples tested in parallel in the K562 assay. NK-PBMC of healthy donors (192, 131) and renal cell carcinoma patients (2989, 2936), and high NK-TIL clearly showed cytotoxicity against P815 target cells loaded with mAb against NKp46 (Fig. 2B). Low NK-TIL (shown are low NK-TIL 2936 and NK-TIL 3088) exhibited only background lysis of NKp46-loaded P815 target cells, even at an effector-to-target ratio of 40:1. The observed differences in high NK-TIL and low NK-TIL to NKp46 triggering corresponded to the cytotoxicity directed against K562, consistent with the observation that lysis of K562 is mediated, in part, by NKp46 triggering (35).
The percentage of specific lysis of P815 was dependent on the concentration of anti-NKp46 antibody, confirming that lysis was due to the strength of triggering and not to unspecific recognition of target cells (not shown). P815 cells loaded with isotype antibody always showed <10% lysis (not shown).

NK-TIL from different tumors varied in their cytotoxic capacity after ex vivo culture and NK cells originating from tumors with a high NK cell content had higher cytotoxic capacity. This observation correlates with our observation that NK cells from tumors with high NK cell infiltration differentially expressed CD16. Thus, the association between CD16 expression and cytototoxicity, previously defined for peripheral NK cells (32, 33), also extends to renal cell carcinoma–infiltrating NK cells. Of importance is that these two populations coexist in PBMC, whereas they are segregated in renal cell carcinoma tissues either because certain tumor tissues specifically recruit or differentially retain the CD16\textsuperscript{bright} or CD16\textsuperscript{dim} NK subgroup.

High NK-TIL and low NK-TIL express NKp46 receptors and corresponding adaptor molecules. We examined whether activating receptors other than CD16 or corresponding adaptor molecules might also discriminate the two NK-TIL groups. NKp46 molecules were expressed at the cell surface of the noncytolytic low NK-TIL as seen for peripheral NK cells. NK-PBMC and NK-TIL of patient 3088 are shown as examples in Fig. 3A. Comparable staining patterns were observed for all cells of the high and low NK-TIL that were studied (n = 31).

Others reported that surface density of NKp46 correlated with the cytotoxic activity (35). In our analyses, differences in fluorescence intensity observed in individual samples (Fig. 3A) could not be extrapolated to variations in density of NKp46 expression because each sample was immunophenotyped separately at the time it became available, thereby excluding a direct quantitative comparison of samples.

The CD3\textgreek{z} chain represents the crucial adaptor molecule for NKp46 and is responsible for the intracellular transmission of the activating signal. Because a loss of the CD3\textgreek{z} chain has been observed in T cells infiltrating renal cell carcinoma (36) and other types of cancer (37), we analyzed CD3\textgreek{z} expression of NK-TIL by intracellular immunofluorescent staining. As shown for patient 2936, low NK-TIL showed expression of the CD3\textgreek{z} chain comparable with autologous NK-PBMC despite their impaired cytototoxicity (Fig. 3B).

NKG2D, a receptor of the lectin-like family, represents another crucial activating receptor for NK cells (reviewed in ref. 23). Low NK-TIL of patient 2928 displayed surface expression of NKG2D on all NK cells comparable with autologous NK-PBMC. This was consistent for all low NK-TIL samples. The expression of DAP10 and DAP12/KARAP, which are adaptor molecules for various activating receptors (reviewed in ref. 18), was examined by semiquantitative RT-PCR. Figure 3D shows that low NK-TIL and PBMC of healthy donors and patients expressed both adaptor molecules at the RNA level. This was confirmed for five different TIL populations. Expression of these molecules could not be analyzed biochemically due to limited material. All these analyses showed that the absence of critical adaptor molecules was not likely to be responsible for the reduced cytotoxic capacity of low NK-TIL.

Low NK-TIL and high NK-TIL differ in the expression of perforin, granzyme A, and granzyme B. The cytolytic effector function of NK cells is directly mediated by the secretion of cytotoxic molecules, such as perforin, granzyme A, and granzyme B. Flow cytometry revealed that all NK-PBMC of renal cell carcinoma patients expressed these effector molecules (Fig. 4A, left column) similar to healthy donors (not shown). NK cells of the high-NK-TIL group (middle column) displayed comparable percentages of cytotoxic\textsuperscript{high} NK cells and, therefore, clearly resembled NK-PBMC. In contrast, NK cells of the low-NK-TIL group (right column) exhibited significantly reduced percentages of cytotoxic\textsuperscript{high} NK cells in every sample of the low-NK-TIL group compared with NK-PBMC or high NK-TIL (P < 0.02 for all three molecules) with a broader range (20-78% positive cells). There was no low-NK-TIL sample that had a high percentage of NK cells positive for all three molecules. For example, NK-TIL 3088 had 62% perforin\textsuperscript{high}, 54% granzyme A\textsuperscript{high}, and only 20% granzyme B\textsuperscript{high} NK cells; NK-TIL 2916 had 66% granzyme A\textsuperscript{high} and 70% granzyme B\textsuperscript{high}, but very few (27%) perforin\textsuperscript{high} NK cells. Finally, NK-TIL 2990 exhibited the highest number of positive cells in the low-NK-TIL category, but percentages of 78% for perforin, 64% for granzyme A, and 71% for granzyme B were all below those observed for NK cells of PBMC or high NK-TIL.

In addition to reduced percentages of cytotoxic\textsuperscript{high} cells, the expression level of cytotoxins, in particular that of perforin, was reduced in low NK-TIL compared with NK-PBMC and high NK-TIL. As illustrated in Fig. 4B, 80% to 90% of NK cells of PBMC showed high perforin expression levels. A similar pattern was seen for high NK-TIL (middle row), whereas the low-NK-TIL group consisted predominantly of NK cells with low perforin expression and fewer perforin\textsuperscript{high} NK cells (bottom row). This was most apparent for NK-TIL 2890 that had as few as 22% perforin\textsuperscript{high} NK cells of which only 11% were perforin\textsuperscript{high}. NK-TIL 2990 had the highest percentage of perforin\textsuperscript{high} NK cells within the low-NK-TIL group and still showed perforin\textsuperscript{high} expression in only 55% of NK cells (Fig. 4A).

Taken together, NK cells of the high-NK-TIL group had consistently higher percentages and also higher expression levels of cytotoxins than NK cells of the low-NK-TIL group. These results are consistent with the characteristics described for peripheral CD16\textsuperscript{bright} NK cells, which express high amounts of cytotoxins (31). On a functional level, the reduced cytotoxin expression of low NK-TIL may explain their reduced cytotoxic potential.

The Fas/FasL pathway, known as another important mechanism of NK cell–mediated cytotoxicity, could not be analyzed in our studies because our universal target cell K562 does not express Fas\textsuperscript{2} and is resistant to Fas-mediated killing (38).

Concordant cytotoxin expression determines cytotoxic strength of NK cell lines. Within the low-NK-TIL group, none of the samples had concordant expression of all three effector molecules at high frequency as was the case for all NK-PBMC and high-NK-TIL samples. Therefore, we speculated that to exert detectable cytotoxicity against K562, an NK cell population must contain a critical percentage of NK cells that express all three effector molecules above a certain threshold. To test this hypothesis, we examined the cytotoxic activity of four established NK cell lines against K562 and simultaneously analyzed their expression patterns of cytotoxic effector molecules.

\textsuperscript{7}C.S. Falk, unpublished data.
Phenotypic analysis of cytotoxic effector molecules revealed that NK-L, NK-92, and 2DL2’NK expressed all three effector molecules whereas YT completely lacked granzyme A (Fig. 4C). All NK cell lines, except YT, showed clear cytotoxic activity (Fig. 4D). Expression levels of effector molecules positively correlated with the percentage of K562 lysis (Fig. 4C and D). These results indicated that NK cell–mediated lysis of K562 cells is dependent on a coordinated expression of all three effector molecules and the expression levels of each cytotoxicinfluenced the extent of lysis.

**Discussion**

Renal cell carcinoma tissues harbor high numbers of infiltrating leukocytes that include almost every cell type of the immune system. However, the factors responsible for the inability of these cells to eliminate surrounding tumor cells are poorly understood. Many reports have described the functional inactivity of TIL present within renal cell carcinoma. This may be due to the direct inactivation of effector cells by tumor cells or by a lack of activating signals. Some of these mechanisms are known as cancer immunoediting or tumor escape mechanisms (reviewed in ref. 39).

Little is known about renal cell carcinoma–infiltrating NK cells. This is partly due to the fact that specific antibodies for NK cell receptors were defined only recently and mechanisms of NK cell function are just beginning to be resolved. We showed that some renal cell carcinoma tissues show infiltration by high numbers of NK cells that were unable to lyse otherwise NK cell–sensitive target cell lines if they were freshly isolated from tumors (23). This inactivity may contribute to the apparent unhindered growth of tumors. In the present study, we separated tumors into two groups based on the frequency of infiltrating NK cells: One group included tissues with infiltrates consisting of <20% NK cells (low NK-TIL), whereas the second group included tissues with >20% NK cells (high NK-TIL). We found that NK cells of the two groups differed in the frequency of FcγRIII (CD16)–expressing cells, in their cytotoxic ability, and in the expression of intracellular cytotoxic effector molecules. Low NK-TIL had reduced percentages of CD16 bright NK cells and were nonlytic against K562 target cells after short-term activation by low-dose IL-2, whereas high NK-TIL were mainly CD16 bright and acquired cytolytic activity, thereby resembling peripheral NK cells both phenotypically and functionally.

The importance and influence of CD16 expression in NK cell–mediated elimination of tumor cells remains to be resolved. Even for peripheral NK cells, where the distinction of CD16 neg/dim and CD16 bright was established (33), it is still discussed controversially whether the CD16 neg/dim population is a distinct subpopulation with unique function or a terminally differentiated NK cell (32, 33). Despite this controversy, it is accepted that CD16 neg/dim NK cells of peripheral blood, secondary lymphoid tissues, or other tissues, like the decidua, are less cytotoxic than CD16 bright NK cells, probably due to their reduced expression of cytotoxic effector molecules (32, 33, 40). The low expression of cytotoxins could be a reflection of their different functional role or could be due to an exhaustion of these molecules caused by recently executed cytotoxic effector function (32). Whatever the correct interpretation, the functional correlation of CD16 expression and cytotoxic capacity is in line with our finding that CD16 neg/dim low-NK-TIL showed concomitant reduced expression of cytotoxins that may be the primary reason for their diminished cytotoxicity. In support of this contention, we characterized NK cell lines and established that the expression levels of effector molecules correlated with the extent of K562 target cell lysis. Additional support for a correlation between perforin and cytolytic capacity is given by our observation that long-term stimulation (7 days) of low NK-TIL significantly increased perforin expression and restored lytic capacity (not shown).

The detailed characterization of TIL revealed that the frequency of NK cells within tumor tissues was predictive of their phenotype and effector function, such that a higher extent of NK cell infiltration was associated with the presence of an NK cell population with better cytolytic potential.

Migration and tissue homeostasis of NK cells are complex processes, which are regulated in part by the presence of chemokines and the expression of their corresponding receptors (41). It has been suggested that CXCL12/SDF-1 may play a role in the recruitment of CD16 bright NK cells (42). In our study, CXCL12/SDF-1 mRNA did not distinguish tumors of high or low NK content (P = 0.34 normalized to 18S rRNA, not shown). Recently, a correlation between the absolute number of NK cells in peripheral blood and the percentage of NK cells in tumors has been suggested (43). Absolute numbers of NK cells in blood are not available for our patient collective. However, the ratio of CD16 bright to CD16 neg/dim NK cells in peripheral blood lymphocytes did not correlate to that of TIL (not shown). The absolute number of infiltrating cells was very heterogeneous among tumors and TILs with low NK content were found for tumors with a low or high infiltration (not shown). A comparison of tumors according to their tumor-node-metastasis characteristics (Table 1) revealed that patients with distant metastasis were found only in the low-NK group. This important observation, however, requires a larger sample size to validate a correlation between NK infiltrates and tumor stage.

Although it is currently unknown why some renal cell carcinoma harbor more NK cells, the observation that the level of NK cell infiltration is associated with a distinct ex vivo functionality may serve as a predictive marker for clinical outcome in the future. Support for this contention can be drawn from other solid tumors (13–15). Moreover, it has been shown by others that infiltration levels and also NK cell activity correlated with survival rates (44–46) and with the response to immunotherapeutic treatment (47).

It is beyond the scope of this analysis to draw conclusions about the clinical outcome or response to immunotherapy with regard to NK cell infiltration. It is apparent that infiltrating NK cells, like T cells, were not able to control outgrowth of primary tumors. It remains to be determined whether patients with higher percentages of CD16 bright NK cells in their tumors have a better capacity to cope with remaining tumor cells after resection of the primary tumors. Relevant follow-up data are not yet available for our cohort of patients and ongoing investigations are required to assess this hypothesis.

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Cytotoxic Markers and Frequency Predict Functional Capacity of Natural Killer Cells Infiltrating Renal Cell Carcinoma

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