Natural Killer Cells Infiltrating Human Nonsmall-Cell Lung Cancer Are Enriched in CD56^{Bright}CD16^{-} Cells and Display an Impaired Capability to Kill Tumor Cells

Paolo Carrega, PhD[1]
Barbara Morandi, PhD[2]
Roberta Costa, MD[3]
Guido Frumento, MD[4]
Giuseppe Forte, MD[5]
Giuseppe Altavilla, MD[2]
Giovanni Battista Ratto, MD[4]
Maria Cristina Mingari, PhD[4]
Lorenzo Moretta, MD[6,7]
Guido Ferlazzo, MD[2]

[1] Department of Experimental Medicine, University of Genoa, Italy.

[2] Department of Human Pathology, School of Medicine, University of Messina, Italy.

[3] Santi A.B.C.A. Hospital, Alessandria, Italy.

[4] National Institute of Cancer Research, Genoa, Italy.

[5] Santa Croce e Carle Hospital, Cuneo, Italy.

[6] Giannina Gaslini Institute, Genoa, Italy.

[7] Center of Excellence in Biomedical Research, University of Genoa, Italy.

Funded by grants from Associazione Italiana Ricerca sul Cancro (AIRC), CIPE (02/07/2004, CBA project), Fondazione Banco di Sicilia (to G.F.), and a fellowship (to B.M.) from Fondazione Italiana Ricerca sul Cancro (FIRC).

The authors thank Drs. Rita Rotondo and Rosa Maria Lantermo for expert assistance in immunohistochemistry and Antonella D'Agostino for technical support and suggestions.

Address for reprints: Guido Ferlazzo, MD, Department of Human Pathology, Pad.H-Policlinico Universitario, Via Consolare Valeria, 98125, Messina, Italy; Fax: (011) 39 090 221-2043; E-mail: guido.ferlazzo@unime.it

Received March 23, 2007; revision received August 10, 2007; accepted September 4, 2007.

BACKGROUND. Despite natural killer (NK) cells being originally identified and named because of their ability to kill tumor cells in vitro, only limited information is available on NK cells infiltrating malignant tumors, especially in humans.
METHODS. NK cells infiltrating human nonsmall cell lung cancers (NSCLC) were analyzed with the aim of identifying their potential protective role in an antitumor immune response. Both relevant molecule expression and functions of NK cells infiltrating NSCLC were analyzed in comparison with autologous NK cells isolated from either peritumoral normal lung tissues or peripheral blood.
RESULTS. The CD56^{bright}CD16^{-} NK cell subset was consistently observed as being highly enriched in tumor infiltrate and displayed activation markers, including NKp44, CD69, and HLA-DR. Remarkably, the cytolytic potential of NK cells isolated from cancer tissues was lower than that of NK cells from peripheral blood or normal lung tissue, whereas no difference was observed regarding their capability of producing cytokines. With regard to their localization within tumor, NK cells were found in tumor stroma, whereas they were not in direct contact with cancer cells.
CONCLUSIONS. For the first time NK cells infiltrating NSCLC have been characterized and it is shown that they are mainly capable of producing relevant cytokines rather than exerting direct cancer cell killing. ***Cancer*** **2008;112:863–75.** © *2008 American Cancer Society.*

KEYWORDS: natural killer cells, nonsmall cell lung cancer, killer immunoglobulin-like receptor, membrane proteins, human.

Natural killer (NK) cells were originally identified on a functional basis as this designation was assigned to lymphoid cells capable of lysing tumor cell lines in the absence of prior stimulation in vivo or in vitro.[1] The molecular mechanisms underlying the ability of NK cells to discriminate between normal and aberrant cells have been elucidated only during the past decade. Nowadays there is evidence that NK cell recognition of target cells is guided by the balance of activating and inhibitory signals given by different groups of surface receptors. The main activating receptors constitutively found on all NK cells in peripheral blood are NKG2D and the natural cytotoxic receptors NKp30 and NKp46,[2] whereas NKp44 is inducible upon activation. All of these receptors are likely to recognize molecules that are up-regulated upon cellular stress.[3,4] With respect to inhibitory signals, human NK cells have been shown to express different human leukocyte antigen (HLA)-class I-specific inhibitory receptors. A family of these receptors, termed killer Ig-like receptors (KIR), recognizes shared allelic determinants of HLA class I molecules,

DOI 10.1002/cncr.23239
Published online 17 January 2008 in Wiley InterScience (www.interscience.wiley.com).

whereas other receptors display a looser pattern of recognition and are characterized by a broad specificity for different HLA class I molecules (LIR1/ILT2) or recognize the HLA-class Ib HLA-E molecules (CD94/NKG2A).[5–9] Accordingly, NK cells lyse target cells that have lost (or express low amounts of) MHC class I molecules. This event occurs frequently in tumors or in cells infected by some viruses such as certain herpesviruses or adenoviruses.

For many years NK cells have been considered a homogeneous lymphocyte population characterized by a strong cytotoxic capability. Today NK cells would appear rather as formed by various subsets that differ in function and, in part, in their surface phenotype. Human peripheral blood mononuclear cells contain around 10% of NK cells.[10] The majority ($\geq$95%) belongs to the $CD56^{dim}CD16^{+}$ cytolytic NK subset[11–13]: these cells carry homing markers for inflamed peripheral sites and express granule-associated perforin to rapidly mediate cytotoxicity.[11,12] A minor NK subset in blood ($\leq$5%) is represented by $CD56^{bright}CD16^{-}$ cells[11–13]: these NK cells lack or express low levels of perforin but secrete on activation both IFNγ and TNF in larger amounts than $CD56^{dim}$ NK cells.[12,13] Notably, the HLA class I allele specific KIR receptors are expressed on subsets of $CD56^{dim}CD16^{+}$ cytolytic NK cells, whereas the immunoregulatory $CD56^{bright}CD16^{-}$ NK subset uniformly expresses CD94/NKG2A and lacks KIRs.[12] Recent reports have shown that the $CD56^{bright}$ NK subset is enriched in all secondary lymphoid organs analyzed so far (lymph nodes, tonsils, and spleen).[13]

Although it is clear that NK cells are capable of recognizing and killing tumor cells in vitro, NK cells infiltrating malignant neoplasms have been poorly characterized. Similarly, the ability of NK cells to migrate in tumors, as well as their antitumor activity in vivo, have not been investigated in detail, especially in humans. In general, histological examinations performed in previous studies have shown that NK cells are not found in large numbers in advanced human neoplasms, with the exception of some renal cell carcinomas.[14–18] The limited number of NK cells infiltrating tumors has hampered more extensive studies of such tumor-associated NK cells. Indeed, most of the previous studies (reviewed in Ref. 2) have been performed using IL-2 activated NK cell lines, which are not equivalent to NK cells freshly isolated from cancer tissues. Nevertheless, the infiltration of NK cells appears to have prognostic value in gastric carcinoma,[15] colorectal carcinoma,[16] and lung carcinomas,[14,19,20] as a relatively higher level of NK cell-infiltrate correlates with a better prognosis, thus suggesting relevant protective roles for NK cell infiltrate.

Lung cancer is currently 1 of the most common malignancies and nonsmall cell lung cancer (NSCLC) represents about 75% to 80% of all cases and its overall 5-year survival rate is less than 12%.[21–23] Identification of new therapeutic strategies, including immunotherapy, is highly required. A deeper understanding of the immune response against NSCLC might pave the way for innovative clinical approaches. Remarkably, in this regard the infiltration of NK cells was found to indicate a favorable prognosis,[19,20] whereas the presence of tumor-infiltrating $CD8^{+}$ T cells was not a predictor of patient survival.[24,25] However, the interpretation of these studies, as well as the development of rational NK cell-based therapeutic attempts, have been hampered by the lack of information on NK cells infiltrating NSCLC.

The purpose of the present study was to analyze NK cells infiltrating human NSCLC and to identify their potential protective role in antitumor immune response.

MATERIALS AND METHODS

Tissue Samples and Cell Isolation

Samples were obtained from 28 untreated patients, without any other concomitant lung disease, who underwent surgical resection of NSCL cancer (Table 1). This study was approved by our Institutional Ethics Committee and patients gave written informed consent according to the Declaration of Helsinki. NSCLC specimens were incised immediately after removal and split into sagittal parts, half of which were paraffin-embedded to perform histology for clinical purposes, whereas the other half was processed either for single cell isolation or immunohistochemistry for NK cell localization within tumor tissue.

Lung tissue samples were obtained immediately after surgical resection of their primary tumors at the Thoracic Surgery Department Ospedale Santa Croce and Carle (Cuneo, Italy) and Ospedale Santi Antonio and Biagio and Cesare Arrigo (Alessandria, Italy). For immunohistochemistry, tissue samples of primary tumors and surrounding normal tissue were snap-frozen in liquid nitrogen. For direct ex vivo isolation of tumor-infiltrating and peritumoral lymphocytes, both primary tumors and normal tissues were washed extensively with phosphate-buffered saline (PBS) to remove peripheral blood lymphocytes and were processed by mincing the tissue with operative scissors. Mechanically dissociated tissues underwent enzymatic digestion with 150 U/mL hyaluronidase and 250 U/mL collagenase type IV (Sigma-Aldrich, St. Louis, Mo). Single-cell suspensions were filtered

TABLE 1
Characteristics of All Patients and Resected NSCLC Analysed

Patient ID	Histology	TNM	Diameter (mm)	% NK	% bright
LC1	adenocarcinoma	$pT_3N_0M_0G_3$	20	10	47,7
LC2	adeno-squamous carcinoma	$pT_2N_1M_0G_2$	80	25,6	66
LC3	squamous cell carcinoma	$pT_3N_1M_0G_3$	65	4,6	43
LC4	squamous cell carcinoma	$pT_1N_0M_0G_3$	20	7	35,7
LC5	adenocarcinoma	$pT_2N_0M_0G_3$	110	13,7	4,7
LC6	squamous cell carcinoma*	$pT_2N_0M_0G_3$	50	5,3	16,8
LC7	squamous cell carcinoma	$pT_2N_1M_0G_2$	40	8	22,6
LC8	adenocarcinoma	$pT_1N_0M_0G_2$	20	3	42
LC9	N.D.	N.D.	N.D.	4,6	N.D.
LC10	squamous cell carcinoma	$pT_2N_0M_0G_2$	50	8,3	46,8
LC11	undifferentiated large cell carcinoma	$pT_3N_0M_0G_3$	40	6,4	N.D.
LC12	adenocarcinoma	$pT_1N_0M_0G_3$	30	4,5	41,7
LC13	adenocarcinoma	$pT_3N_1M_0G_2$	130	10,7	N.D.
LC14	squamous cell carcinoma	$pT_3N_1M_0G_3$	70	5,1	22,9
LC15	squamous cell carcinoma*	$pT_2N_1M_0G_3$	50	3,5	70
LC16	adenocarcinoma	$pT_1N_0M_0G_1$	25	3,4	N.D.
LC17	squamous cell carcinoma	$pT_2N_0M_0G_2$	80	2,1	23,8
LC18	squamous cell carcinoma	$pT_2N_1M_0G_2$	23	1,8	56
LC19	squamous cell carcinoma	$pT_1N_0M_0G_2$	30	1,5	11,8
LC20	squamous cell carcinoma	$pT_1N_0M_0G_2$	30	5,2	14,4
LC21	squamous cell carcinoma	$pT_2N_0M_0G_2$	40	4,3	9,8
LC22	squamous cell carcinoma	$pT_4N_1M_0G_X$	45	N.D	N.D.
LC23	N.D.	N.D.	N.D.	3,2	32
LC24	N.D.	N.D.	N.D.	14,8	74,6
LC25	adenocarcinoma	$pT_1N_0M_0G_1$	45	1,4	39
LC26	squamous cell carcinoma*	$pT_1N_1M_0G_X$	10	1,4	70
LC27	adenocarcinoma	$pT_1N_0M_0G_X$	10	1,1	N.D.
LC28	adenocarcinoma	$pT_1N_0M_0G_2$	25	N.D.	N.D.

* : Poorly differentiated. % NK: Percentage of NK cells in $CD45^+$ MNC infiltrating cancer tissues. % bright: Percentage of $CD56^{bright}CD16^-$ NK cells in $CD45^+$ MNC infiltrating cancer tissues.

through a 100-μm cell strainer (BD Labware, Mountain View, Calif), washed, and mononuclear cells (MNC) were isolated by Ficoll-Hypaque (Sigma) density gradient centrifugation. Similarly, peripheral blood MNC were isolated using Ficoll-Hypaque density gradient. Cells were either directly analyzed by flow cytometry or cryopreserved for further analyses.

Immunohistochemistry

To localize NK cells infiltrating NSCLC, 8-μm-thick slices were cut from frozen surgical samples of tumor. Sections were fixed for 5 minutes at −20°C in 100% acetone. After washing with PBS, endogenous peroxidases were blocked by incubating samples for 15 minutes with the peroxidase block from EnVision Kit (Dako, Glostrup, Denmark). After washing in water for 5 minutes sections were incubated for 30 minutes at room temperature with PBS plus 5% bovine serum albumin (BSA) (Sigma-Aldrich), 0.5 mg/mL human IgG (Sclavo, Siena, Italy), and 0.05% Tween 20. Afterward, sections were incubated for 90 minutes at room temperature with undiluted supernatant of NKp30 or NKp46 hybridomas (respectively, clone AZ20, IgG1, and clone KL247, IgM) produced in our laboratory. Isotype-matched mouse IgG were used as control.

After washing 3 times for 5 minutes with PBS plus 0.05% Tween 20, a biotinylated goat antimouse antibody (BIOSPA, Milan, Italy), diluted 1:800 in PBS plus 1% BSA and 0.05% Tween 20, was added. After 30 minutes at room temperature sections were washed 3 times for 5 minutes with PBS plus 0.05% Tween 20. Afterward, a streptavidin-horseradish peroxidase conjugate at a 1:300 dilution was added and sections were further incubated for 15 minutes at room temperature. After washing 3 times for 5 minutes with PBS plus 0.05% Tween 20, sections were stained with the AEC chromogen and the AEC substrate of the Ultravision Detection System (Lab Vision, Fremont, Calif). Counterstaining was performed with hematoxylin.

For evaluating the expression of HLA class I molecule in the NSCLC specimens, sections of paraffin-embedded tissues were used. Sections were heated at

75°C (4 minutes × 2) and treated with hydrogen peroxide before staining with anti-HLA class I mAb (HC-1O, IgG2a) for 30 minutes and biotin for 8 minutes.

Monoclonal Antibodies and Flow Cytometry

Analysis of cell surface markers was performed using the following mAbs in direct immunofluorescence assay: FITC-conjugated anti-CD3, PC5-conjugated anti-CD56, PE-conjugated anti-NKp30, anti-NKp44, anti-NKp46, and anti-NKG2A (Immunotech-Coulter, Fullerton, Calif); PE-conjugated anti-KIR2DL1, anti-KIR2DL2, anti-DR, anti-CD107a; FITC-conjugated anti-CD45/PE-conjugated anti-CD14 (Leucogate) (BD PharMingen, San Diego, Calif). APC-conjugated anti-CD56, FITC-conjugated anti-CD3, and PE-conjugated anti-CD16 were from Miltenyi Biotech (Bergisch Gladbach, Germany). Anti-NKG2D (clone ECM/217), anti-CD69 (clone FS3) and anti-2B4 (clone MA344) were produced in our laboratory.

Direct immunofluorescence staining was performed diluting fluorochrome-labeled mAb with 1 mg/mL human γ-globulin (human therapy grade from Biotest S.R.L., Milan, Italy), to block nonspecific FcR binding. Cells were then washed and the flow cytometric analysis was performed. For indirect immunofluorescence staining, nonspecific binding sites were saturated with human γ-globulin, and then the relevant mAb was added and incubated for 30 minutes at 4°C. After washing, PE-conjugated isotype-specific goat antimouse Abs (Southern Biotechnology, Birmingham, Ala) were added and incubated for 30 minutes at 4°C. Negative controls included directly labeled and unlabeled isotype-matched irrelevant mAbs. Cells were then washed and analyzed by flow cytometry. Perforin, IFN-γ, and TNF-α expression analysis were performed by PE-conjugated reagents (BD Biosciences-PharMingen) after cells were fixed in 1% paraformaldehyde and permeabilized with 0.1% saponin.

CD107a Assay

MNC from blood, tumor, and peritumoral samples were used as effectors in a CD107a assay; the K562 cell line was used as target. To detect spontaneous degranulation, a control sample without target cells was included.

An effector/target (E/T) ratio of 1:1 (2×10^5 effector cells: 2×10^5 target cells in a volume of 200 μL) was used. Anti-CD107a (PE-conjugated) was added in each well (8 μL/well) before incubation. Effectors and targets were then coincubated at 37°C for 4 hours; after the first hour monensin (Sigma), at a final concentration of 2 mM, was added to inhibit cell secretion, as previously described.[26]

At the end of coincubation cells were washed twice in PBS and stained with mAbs (anti-CD3 and anti-CD56) for flow cytometric analysis and surface expression of CD107a was assessed in NK cells.

^{51}Cr Release Assay

To confirm the different ability of NSCLC-infiltrating NK cells in killing target tumor cells, NK cells from PB, tumor, and peritumoral tissues were compared in a standard ^{51}Cr release assay. Briefly, 10^6 K562 cells were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ for 90 minutes at 37°C, extensively washed, and finally cultured with the different NK cell populations for 4 hours at 37°C. Microculture supernatants were then collected and radioactivity counted on a gamma-counter (Beckman, Milan, Italy). 'Specific' ^{51}Cr release is calculated on the bases of the ratio {(sample release − spontaneous release) / (total release − spontaneous release)}. Assays were performed in triplicate at the indicated effector-target ratios.

Cytokine Production

Paired samples of mononuclear cells from peripheral blood, tumor, and peritumoral tissues were incubated in culture medium alone or in culture medium supplemented with 10 ng/mL PMA and 500 ng/mL ionomycin at 37°C for 6 hours. Monensin was added at a final concentration of 2 mM. Cells were then washed, fixed, and permeabilized as described above, and finally stained with PE-conjugated anti-IFN-γ and TNF-α.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism v. 4.00 for Windows (GraphPad Software, San Diego, Calif). Statistical significance was evaluated by paired Student *t*-test.

RESULTS

NK Cells Infiltrating NSCL Cancers Are Abundantly Represented by Activated CD56^{bright}CD16$^-$ Cells

NK cells were isolated from surgically resected cancer specimens and, as a comparison, from autologous peripheral blood (PB) and, whenever possible, from normal peritumoral lung tissues. After ficoll gradient separation only MNC were collected and analyzed for NK cell content. Whereas no differences in the CD4$^+$/CD8$^+$ T-cell ratio were appreciable between lung tissue and autologous PB MNC, the percentage of B cells was significantly increased compared with both PB and normal lung tissue. The remaining part of the CD3$^-$CD56$^-$ cell fraction in MNC isolated from tumors was mainly represented by large and scattered

cytokeratin$^+$ cells. NK cells were identified as CD3$^-$CD56$^+$CD45$^+$ cells and their total percentage within CD45$^+$ MNC isolated from lung tumors was consistently lower (mean value, 4.6 ± SE: 1.25) than in MNC from the PB counterpart (mean value, 27.8 ± SE: 3) and was comparable to that in MNC from peritumoral lung tissue (mean value, 7.8 ± SE: 2.68) (Fig. 1A, left panel). Interestingly, the percentage of NK cells harbored within NSCLC directly correlated with the size of the resected tumors ($P = .0012$) (Fig. 1B). Nevertheless, NK cells isolated from NSCLC (NSCLC-NK) displayed a peculiar surface molecular pattern: most of the CD3$^-$CD56$^+$ NK cells infiltrating the tumor displayed higher levels of surface CD56 as compared with both PB-NK cells and peritumoral normal lung tissue NK cells (PLT-NK) (mean percentage value ± SE of CD56^{bright} NSCLC-NK: 37.8 ± SE: 4.6; CD56^{bright} PB-NK: 6.3 ± 1.3; CD56^{bright} PLT-NK: 16.5 ± 4.4) (Fig. 1A, right panel, and Fig. 1C), whereas CD16 was consistently expressed on fewer cells (mean percentage value of CD16$^+$ cells in NSCLC: 22 ± 3.3; in PB: 76.9 ± 5.9; in PLT: 55.5 ± 6.42) (Fig. 2). A similar surface phenotype characterized a minor subset of PB and peritumoral NK cells, suggesting a preferred pattern of migration into the tumor for this NK cell subset. A representative phenotypic pattern of NK cells isolated from the 3 different autologous tissues is depicted in Figure 2A. Remarkably, an evident surface expression of KIR was detectable in CD56^{bright}CD16$^-$ tumor NK cells, whereas, as previously reported, the phenotypically identical CD56^{bright}CD16$^-$ NK cells isolated from peripheral blood were KIR-negative. Notably, this was also true for CD56^{bright}CD16$^-$ NK cells isolated from normal peritumoral lung tissue, which did not show KIR expression. However, when the total NK cell population was considered, the percentage of KIR expressing NK cells was significantly lower in NSCLC-NK (KIR2DL1: 16.7 ± 1.75; KIR2DL2: 26.2 ± 6) as compared with peripheral blood NK cells (KIR2DL1: 34.4 ± 3.6 KIR2DL2: 35.8 ± 6.1) and to NK cells from peritumoral lung tissues (KIR2DL1: 33 ± 3.5; KIR2DL2: 31.1 ± 3.5) (Fig. 2). Conversely, CD94/NKG2A, another inhibitory NK cell receptor, was expressed in NSCLC-NK cells in a significantly higher percentage (NKG2A$^+$ cells in NSCLC: 65.9 ± 8.1; in PB: 47 ± 8; in PLT: 49.5 ± 7.8). Regarding activating NK receptors, other than CD16, only NKp44 showed a significantly higher expression (mean percentage value of NKp44$^+$ cells in NSCLC: 18.2 ± 4.4), whereas it was virtually undetectable in both PB-NK and PLT-NK cells. Conversely, neither NKp30, NKp46, nor NKG2D showed significant differences in their surface expression. It is noteworthy that NKp44 is usually induced on PB-NK cells upon cytokine activation and its expression ex vivo has been previously detected only in NK cells derived from inflamed tonsils.[27] Accordingly, NSCLC-NK cells displayed increased levels not only of NKp44 but also of HLA-DR and CD69, both antigens expressed by activated NK cells.

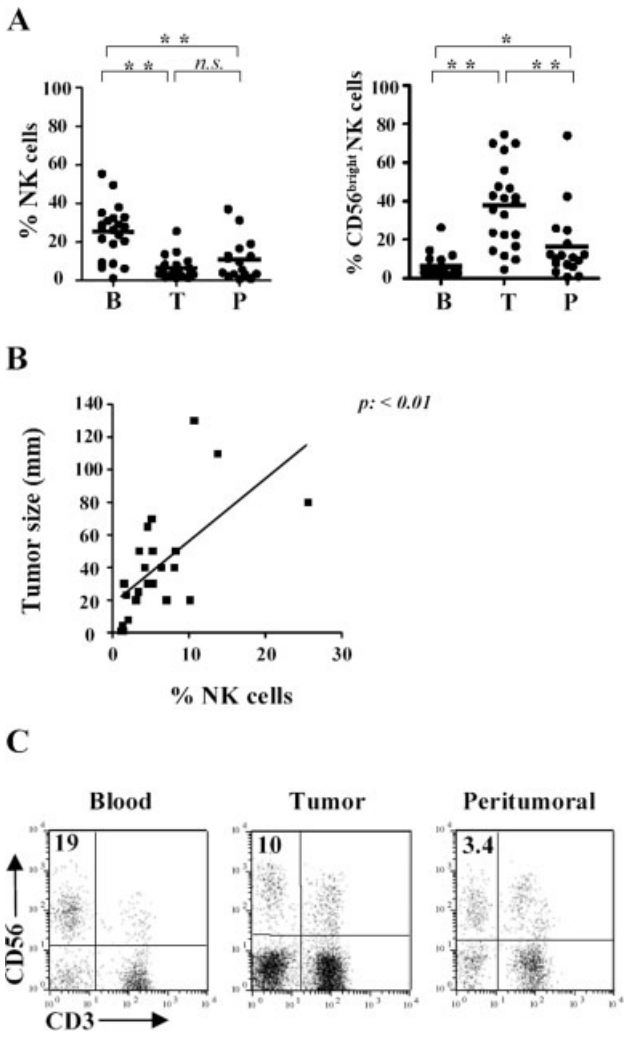

FIGURE 1. CD56^{bright} natural killer (NK) cells are highly enriched within nonsmall-cell lung cancer (NSCLC) tissues. (A) Single mononuclear cell suspensions were obtained from peripheral blood (B), nonsmall cell lung cancer (T), and peritumoral lung tissue (P). After gating on CD45$^+$ cells (ie, lymphocytes), NK cell percentages were valued using anti-CD3 and anti-CD56 mAbs. The different percentages of either the whole infiltrating NK cells or CD56^{bright} subset were analyzed in the 3 different tissues. *$P < .05$; **$P < .01$; n.s., not significant. (B) The percentage of NK cells harbored within tumors directly correlated with tumor size. In the y axis the maximum diameter of the resected tumors is indicated. (C) A representative phenotype of lymphocytes isolated from blood, tumor, and peritumoral tissue is shown. Quadrants depicted are set on isotypic controls.

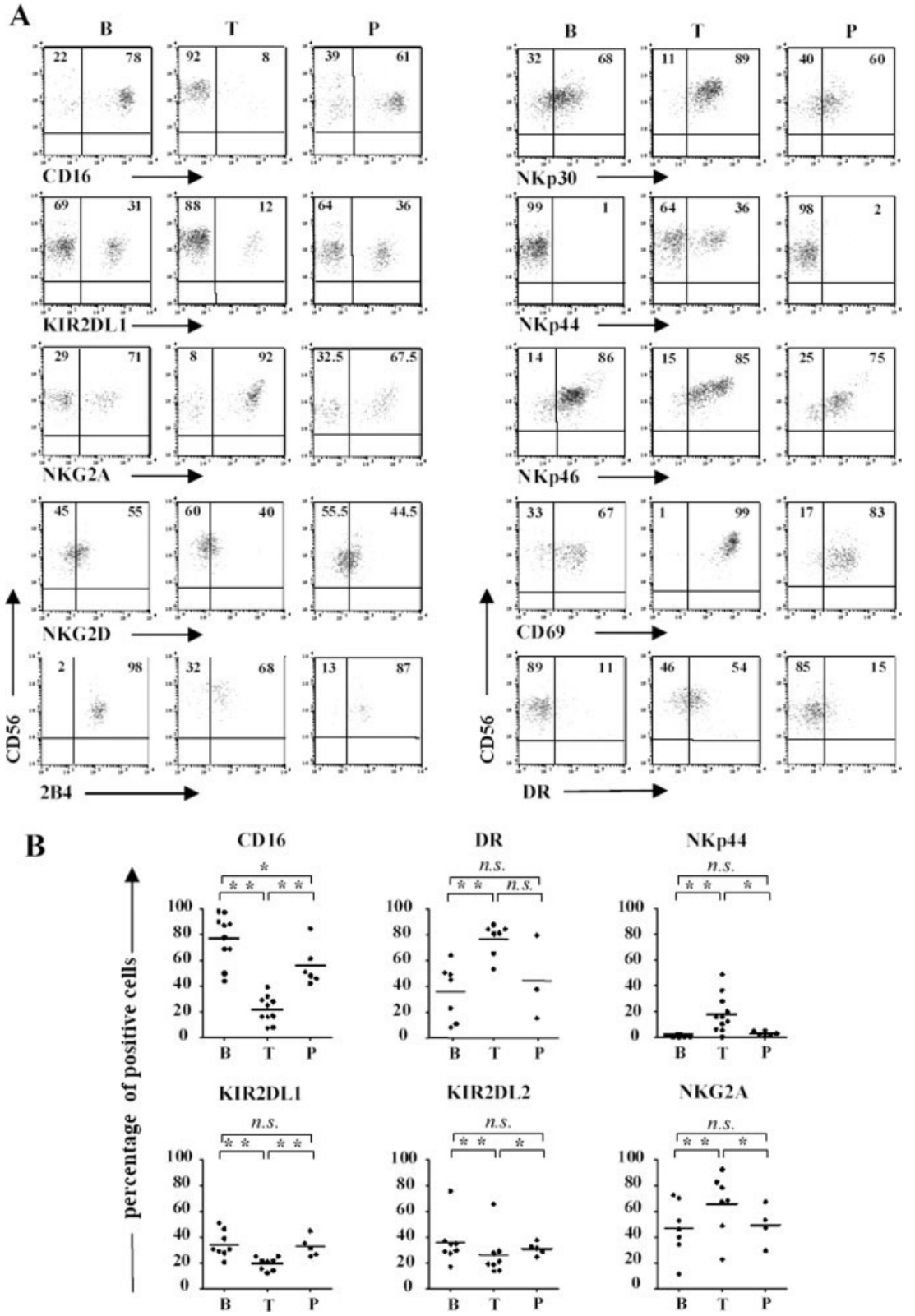

FIGURE 2. Natural killer (NK) cells isolated from nonsmall-cell lung cancer (NSCLC) display a distinctive surface molecular pattern. Autologous mononuclear cells isolated from peripheral blood (B), NSCLC (T), and peritumoral lung tissue (P) were comparatively analyzed by 3-color flow cytometry using mAbs specific for the indicated markers after gating on $CD3^{-}CD56^{+}$ NK cells. (A) The phenotype of NK cells isolated from the tissues of a representative patient is shown. Quadrants depicted are set on isotypic controls. (B) The major phenotypic differences detected in NSCLC-NK cells are summarized. Data shown represent the results obtained in all the patients analyzed. $*P < .05$; $**P < .01$; n.s., not significant.

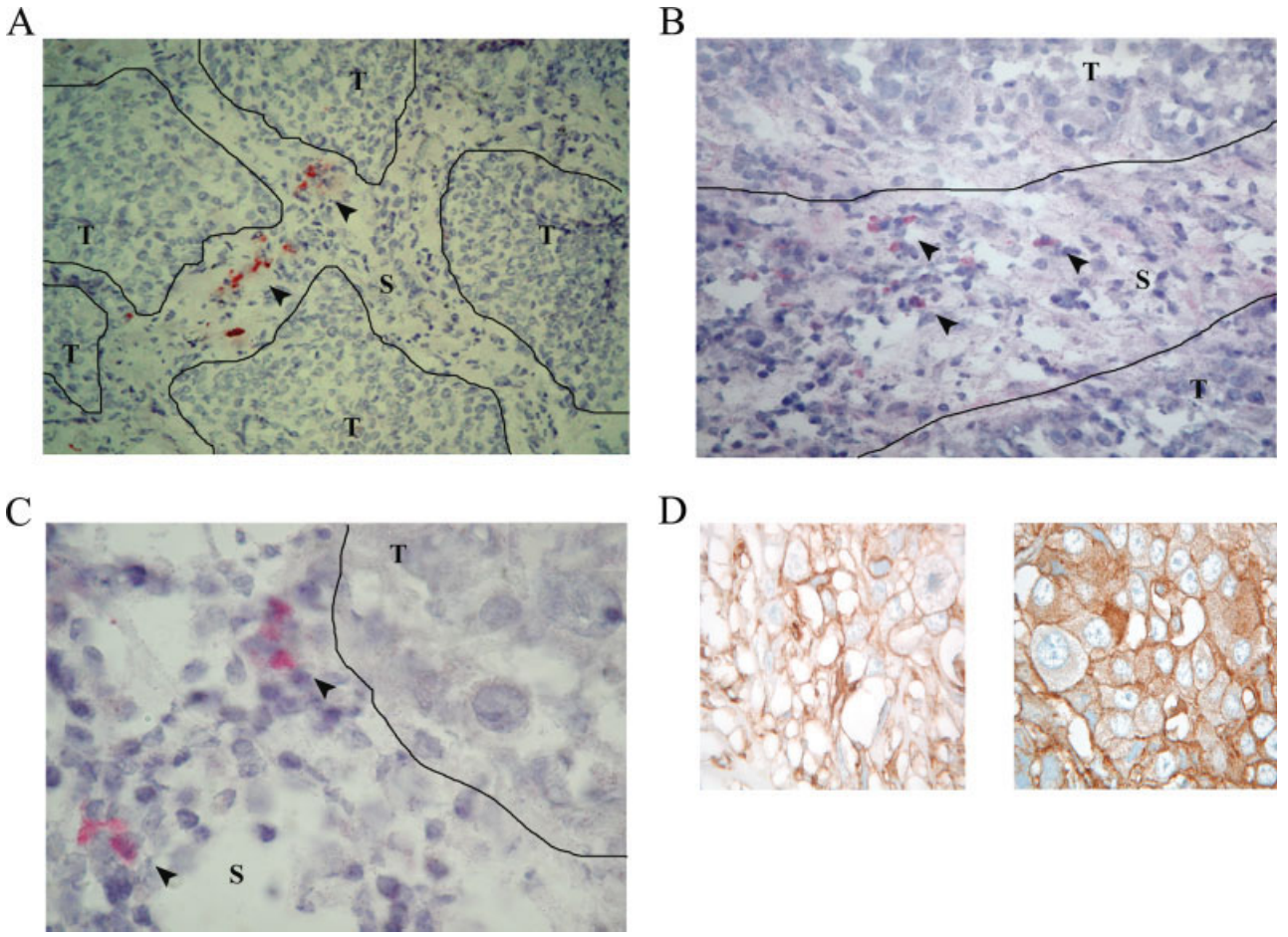

FIGURE 3. Nonsmall-cell lung cancer (NSCLC)-infiltrating natural killer (NK) cells localize in the tumor stroma and at the interface between stromal and tumor cells, which express variable levels of human leukocyte antigen (HLA) class I molecules on their membrane. Eight-μm-thick slices were cut from frozen samples of tumor. Sections were fixed and stained with mAb directed against NKp30 followed by biotinylated goat antimouse antibodies. After a streptavidin-horseradish peroxidase conjugate was added, sections were stained with the AEC chromogen and the AEC substrate (see Materials and Methods). Counterstaining was performed with hematoxylin. Representative sections from 2 different patients are shown in (A) and (B). (C) Higher magnification of tumor tissue is shown. Arrows indicate tumor infiltrating NK cells (in red). S, stroma; T, tumor. (D) Sections of paraffin-embedded cancer tissues were stained with anti-HLA class I mAb and revealed by the biotin-avidin reaction. Representative NSCLC are depicted showing a focal and lower expression of HLA class I molecules (left panel) or a homogenous high expression, in a well-differentiated squamous cell carcinoma (right panel).

NK Cells Localize in the Stroma of NSCLC

The finding that NK cells isolated from NSCLC tissues uniformly displayed a characteristic surface phenotype ruled out that NK cells isolated from cancer tissues might be derived from blood contamination, but rather represented a true cell infiltrate.

Thus, we investigated the localization of infiltrating NK cells within cancer tissues. To this end, NSCLC specimen sections were analyzed by immunohistochemistry using mAbs directed against NKp46 and NKp30, whose expression is restricted to NK cells. As shown in Figure 3, NSCLC-NK consistently presented a peculiar distribution, restricted to the intratumoral fibrous septa and to the interfaces between stromal cells and surrounding tumor cells. Remarkably, NK cells detected within tumor tissues consistently did not appear to be in direct contact with cancer cells.

NSCLC-NK Cells Contain Low Amounts of Perforin and Do Not Express CD107a

Perforin is a potent cytolytic pore-forming protein constitutively contained in cytoplasmic granules of almost all PB-NK cells. The remaining PB-NK cells ($\leq$5%) are represented by $CD56^{bright}CD16^{-}$ cells, expressing low levels of lytic granules. We therefore

analyzed the perforin content of NSCLC-NK cells. In agreement with their surface phenotype, NK cells isolated from cancer tissues displayed a lower content of perforin if compared with autologous PB-NK and PLT-NK cells (Fig. 4, upper panels).

Because cytolytic activity depends not only on the content of lytic granules but also on granule polarization and exocytosis, we further investigated the surface expression of CD107a (LAMP-1). CD107a represents an excellent surface marker, revealing the occurrence of granule exocytosis. Flow cytometry analysis clearly showed that, despite their activated phenotype and their intratumoral localization, NSCLC NK cells were completely negative for CD107a surface expression (Fig. 4, middle panels). This finding might be interpreted either as a peculiar deficiency of CD107a or, alternatively, as an indication that no interaction/recognition of tumor target cells has occurred in vivo. Along this line, in preliminary in vitro experiments we observed that CD107a was detectable on NK cell surfaces up to 18 hours after recognition of cancer cells (not shown).

$CD56^{bright}CD16^{-}$ NK Cells Infiltrating NSCLC Can Express KIR

KIR are expressed on a considerable fraction of $CD56^{dim}CD16^{+}$ NK cells, whereas they are in general absent on $CD56^{bright}CD16^{-}$ NK cells. Surprisingly, $CD56^{bright}CD16^{-}$ NSCLC-NK cells were found to express KIR (Figs. 2A, 4, lower panels). Therefore, we asked whether $KIR^{+}CD56^{bright}CD16^{-}$ NSCLC-NK cells were derived from $KIR^{-}CD56^{bright}CD16^{-}$ PB-NK cells upon migration into tumors. Alternatively, they could derive from a $KIR^{+}CD56^{dim}CD16^{+}$ PB-NK cell subset upon up-regulation of CD56 molecules, possibly induced by local, intratumoral activation. Because $CD56^{dim}CD16^{+}$ express high levels of perforin, whereas $CD56^{bright}CD16^{-}$ do not, we analyzed perforin content in $CD56^{bright}KIR^{+}$ NK cells. Indeed, perforin was expressed at the same low level on either $CD56^{bright}KIR^{+}$ NSCLC-NK cells or on $CD56^{bright}KIR^{-}$ PB and PLT-NK cells, thus suggesting that $CD56^{bright}CD16^{-}KIR^{+}$ NSCLC-NK cells would derive from $CD56^{bright}CD16^{-}KIR^{-}$ PB-NK cells and not from $CD56^{dim}CD16^{+}$ KIR^{+} PB-NK cells (Fig. 4). In addition, similar to the whole NK cells infiltrating NSCLC, also the $CD56^{bright}KIR^{+}$ subset failed to express CD107a, thus ruling out the possibility that the low levels of perforin could be related to recent degranulation that might have occurred in vivo before tumor resection. Conversely, the higher amount of perforin detected in PLT-NK cells excluded that tissue processing might be responsible for the low perforin content.

These data suggested that $CD56^{bright}CD16^{-}$ NK cells can acquire KIR expression within tumor tissues, possibly as a consequence of tumor microenvironment-derived signals.

NSCLC-NK Cells Exert Low Cytolytic Activity But Are Competent for Cytokine Production

Our data indicated that NK cells present in NSCLC, although expressing normal levels of activating receptors, were characterized by a low amount of perforin. In this context the cytokine production was analyzed in parallel with cytolytic activity.

Cytotoxic function has recently been assessed by flow cytometry on the basis of the cell-surface mobilization of CD107a (LAMP-1).[28–31] The CD107a assay has been shown to strictly correlate with the classic Cr^{51} release assay (Ref. 31 and our current data, not shown). By means of the CD107a assay we could comparatively analyze the cytolytic activity of NK cells isolated from NSCLC, PLT, and PB of the same patients. These studies indicated that NSCLC-NK cells display a significantly lower cytotoxic activity against the NK cell susceptible K562 cell line (Fig. 5A). Conversely, no significant differences were observed between PB-NK cells and PLT-NK cells, indicating that the NK cell isolation procedure from solid tissues did not affect cell viability and function. As might be predicted, we found an inverse correlation between the percentage of $CD56^{bright}CD16^{-}$ NK cells in the tumors and the level of cytolytic activity, measured as CD107a surface expression upon incubation with K562 target cells (Fig. 5B). To further confirm that enzymes used in tumor digestion were not responsible for the observed functional difference, PB-NK cells were also exposed to the same enzymes. PB-NK cells exposed or not to the enzymes displayed similar CD107a expression after coculture with K562 target cells.

Finally, we analyzed in 3 patients the cytolytic function of NK cells isolated from the distinct districts by chromium release assay. As shown in Figure 5C, the radioactive assay confirmed that NK cells isolated from NSCLC showed a reduced ability to kill target cells compared with PB- and PLT-NK cells.

Because NK cells isolated from NSCLC could express KIR, we analyzed whether the expression of HLA class I might dictate the functional behavior of infiltrating NK cells. HLA class I molecules were always expressed in the 7 NSCLC analyzed, although different levels of expression were detectable in the distinct cancer specimens. In general, squamous cell carcinomas, particularly the most differentiated histotypes, presented a higher and more homogeneous expression of HLA class I molecules than adenocarci-

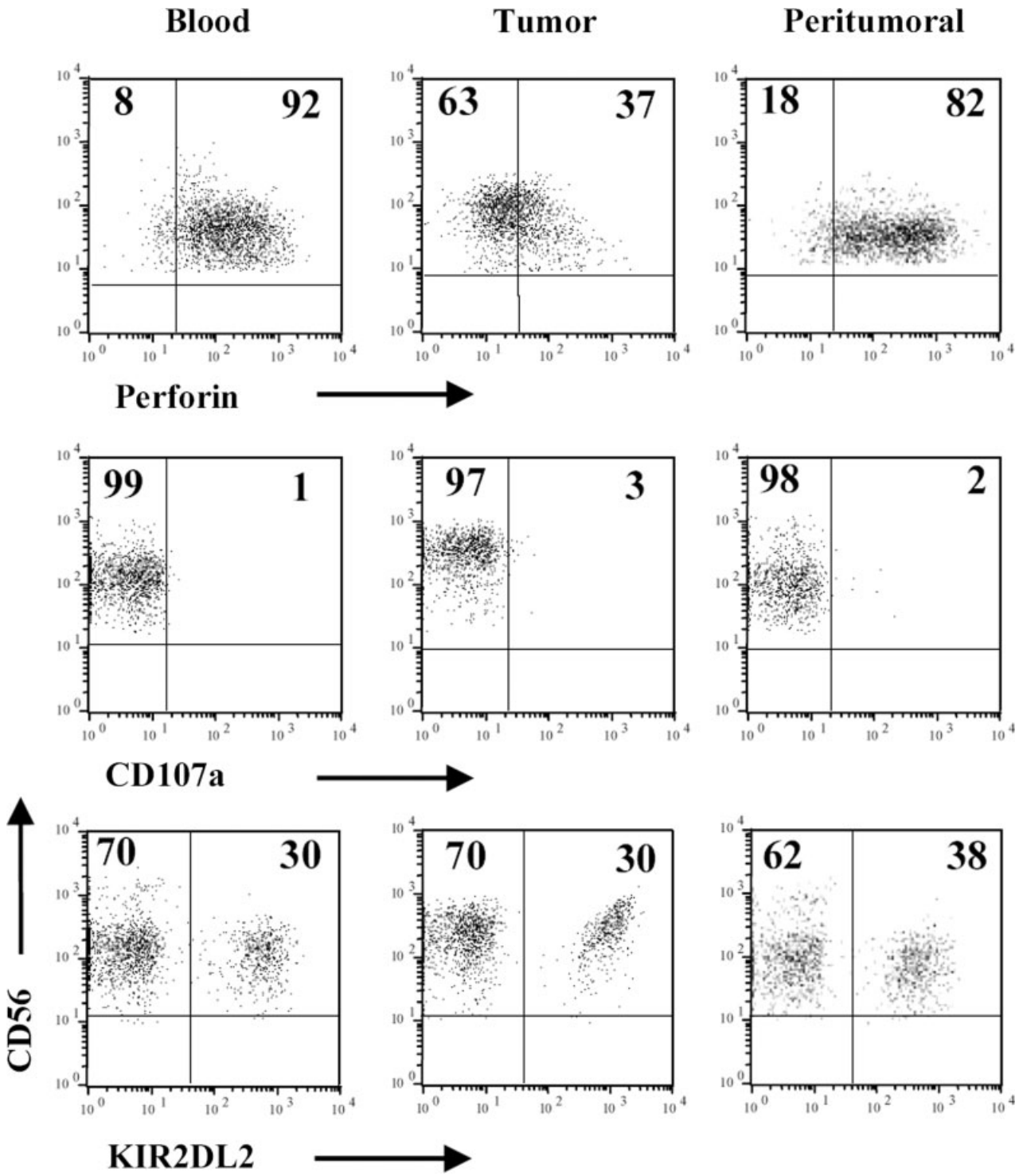

FIGURE 4. Nonsmall-cell lung cancer (NSCLC)-NK (natural killer) cells display a low amount of perforin also in KIR^+ NK cells. Cells isolated from the indicated tissues of the same patient were analyzed for the expression of perforin (upper panels), CD107a (middle panels), and KIR (lower panels). Data from a representative patient of 14 analyzed are shown. Quadrants depicted are set on isotypic controls.

nomas and adenosquamous carcinomas (Fig. 3D). However, the degree of HLA class I expression in the specimens analyzed showed no correlation with the cytolytic ability of NK cells infiltrating the single tumors. Nevertheless, we cannot rule out at this time that deletions of specific HLA class I alleles might hypothetically influence NK cell functions.

On the other hand, when the same NK cell subsets were analyzed for their ability to produce IFN-γ and TNF-α, an efficient cytokine production was observed both in NSCLC-NK cells and in the other NK cell populations analyzed (Fig. 5B). These results suggest that NK cells infiltrating NSCLC are not anergic and are fully competent for relevant cytokine release.

DISCUSSION

In the present study we show that NK cells can infiltrate NSCLC. Our data indicate that these NK cells display particular phenotypic features and that they are poorly capable of tumor cell killing, whereas they release abundant cytokines.

It is frequently assumed that tumor rejection is mainly the result of cytotoxic lymphocyte-mediated killing of the tumor cells. However, recent studies have indicated that the tumor rejection process may be a more complex event. For instance, T cells displaying cytolytic activity but unable to produce IFN-γ rarely reject tumors, whereas IFN-γ or other lymphocyte-derived cytokines such as TNF-α, IL-4, or IL-10 contribute to tumor rejection by inhibiting tumor stroma formation and tumor-induced angiogenesis.[32]

Soluble mediators released by NK cells are also relevant during the crosstalk between NK and dendritic cells (DCs), because it has been widely demonstrated that NK cells are able to induce DC functional maturation and consequently a more effi-

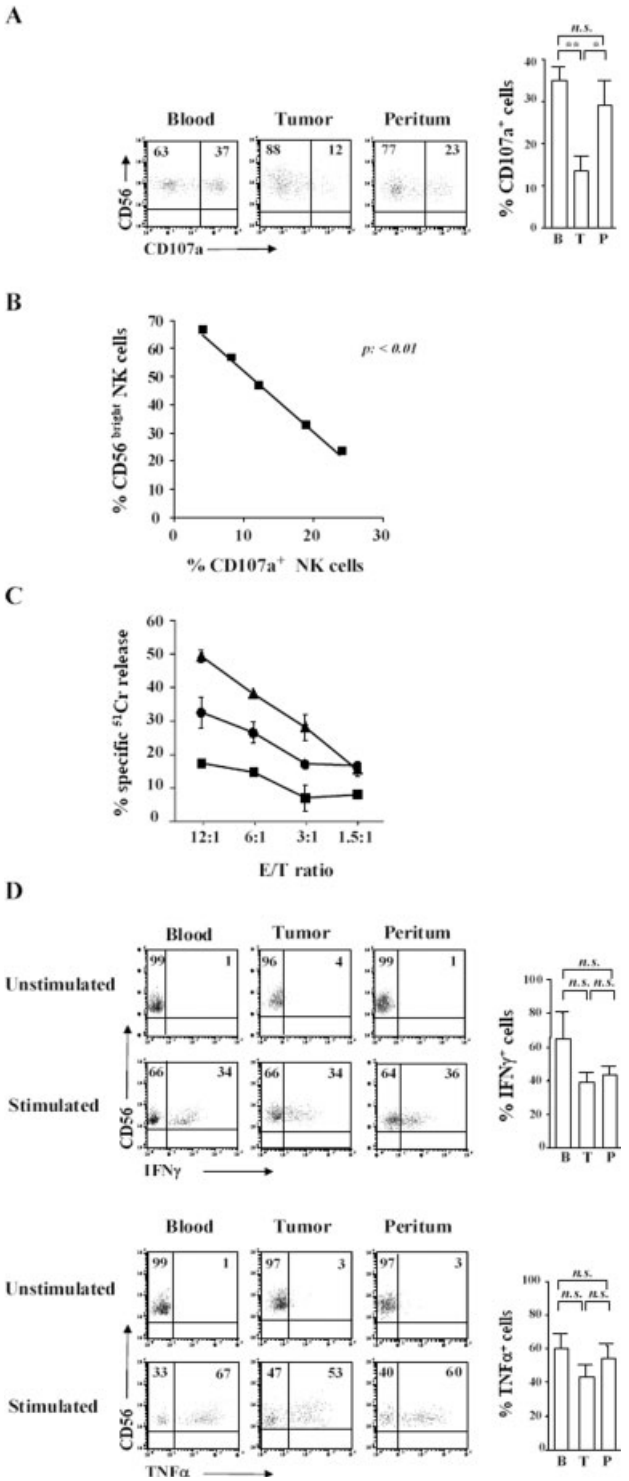

FIGURE 5. Nonsmall-cell lung cancer (NSCLC)-infiltrating natural killer (NK) cells display impaired cytolytic capabilities but can effectively secrete cytokines. Mononuclear cells isolated from peripheral blood, NSCLC, and peritumoral tissue were comparatively analyzed for their cytolytic and cytokine-secreting properties. (A) Cells were cocultured with the NK-sensitive K562 cell line and after 4 hours CD107a expression was assessed gating on $CD3^{-}CD56^{+}$ NK cells. (B) The percentage of $CD56^{bright}CD16^{-}$ NK cells harbored within tumors inversely correlated with the expression of CD107a. (C) The cytotoxic ability of NK cells isolated from peripheral blood (triangle), peritumoral tissue (circle), and NSCLC (square) was also evaluated by a standard chromium release assay. One representative experiment of the 3 performed is shown. (D) To assess cytokine producing capability, cells were stimulated for 6 hours with PMA/ionomycin (Stimulated) or cultured for the same interval of time in complete medium (Unstimulated) and then analyzed for IFN-γ and TNF-α production. Data shown in the dot plots are representative of results obtained in 5 independent experiments. Quadrants depicted are set on isotypic controls. Numbers within quadrants represent percentages of positive cells. Bars represent mean value ± SE of the 5 experiments. $*P < .05$; $**P < .01$; n.s., not significant.

cient adaptive immune response. This might be particularly important for evoking an adaptive immune response against cancer cells. Indeed, the absence of pathogen-related molecules and inflammation, at least in the early phases of cancer development, does not allow DC activation and, as a result, an effective antigen-specific T-cell response.

It has recently become manifest that an intricate communication network between the innate and adaptive immune system exists in vivo that can also activate NK cells, which might function as potent regulators of T-cell responses.[33–38] Thus, a lower cytolytic activity in NSCLC-NK cells does not necessarily mean an ineffective anticancer activity, because NK cell cytokine production might be as relevant as cytotoxic functions in controlling cancer cell growth. Nevertheless, we found that larger tumors contained a higher percentage of NK cells than smaller tumors, thus challenging the idea that NK cells should exert a protective role during tumor cell growth. Recent data have indeed demonstrated that NK cells can also be detrimental during a productive immune response, either via the release of immune suppressive cytokines or by specifically inhibiting T-cell proliferation by direct interaction with T cells.[39–41] Conversely, the infiltration of NK cells had been previously reported to correlate with a more favorable prognosis.[19,20] A more extensive characterization of NK cell-derived cytokines released locally during the antitumor immune response is now required to better elucidate the role of NK cell infiltrate within NSCLC.

A similar pattern of NK cell infiltration, characterized by abundant $CD56^{bright}CD16^{-}$ NK cells with low cytolytic activity, has been reported in renal cell carcinoma,[17,18] suggesting that a prominent regulatory role of infiltrating NK cells might be a common feature of the antitumor immune response. The finding of a comparable amount of NK cells in tumor and peritumoral tissues could be explained by a low capability of NK cells to migrate toward chronically inflamed tissues, such as cancer; however, peritumoral regions might not be considered a true normal tissue, being influenced by the particular, tumor-associated, chemokine microenvironment.

In line with our finding that NK cells localize primarily in tumor stroma, it has been previously postulated that extracellular matrix proteins, present in the stroma surrounding the tumor blood vessels, might act as a barrier preventing direct NK-cell–tumor-cell contact.[42–44] However, ultrastructural studies showed that NK cells display a marked ability to alter their shape, thus facilitating their extravasation and intratumor migration.[45,46] This would question the extracellular matrix proteins as a true physical barrier. Alternatively, it might be possible that a chemokine environment produced by stromal leukocytes might provide anchorage signals to tumor-infiltrating NK cells. For instance, it has been suggested that heterodimerization of the chemokine receptor CCR5, ie, the receptor of MIP-1β, leads to an adhesive signal and to the arrest of leukocytes within tissue.[47] Consistent with our present findings, only $CD56^{bright}$ $CD16^{-}$ PB NK cells express CCR5,[11] which might explain their accumulation in tumor tissues. In fact, like other components of the innate immune system present in the circulation (such as neutrophils and monocytes), all NK cells may be attracted to areas of inflammation. Nevertheless, the chemokine receptor repertoire of $CD16^{+}$ NK cells is similar to that of neutrophils and these cells may thus be similarly attracted to sites of acute inflammation. With the exception of CCR7, the chemokine receptor pattern expressed by $CD56^{bright}CD16^{-}$ NK cells would appear similar to that of monocytes. These chemokine receptors may serve for migration and arrest of the cells into sites of chronic inflammation, such as cancer.[11,48]

Another interesting finding of this study is the presence of KIR on NSCLC-$CD56^{bright}CD16^{-}$ NK cells, because PB as well as secondary lymphoid organ $CD56^{bright}CD16^{-}$ NK cell subset uniformly lack KIR. However, KIR can be promptly up-regulated by cytokine activation on both secondary lymphoid organ NK cells and PB-NK cells.[27,49] Thus, there is increasing in vitro evidence that activation can convert $CD56^{bright}CD16^{-}$ NK cells into effectors analogous to blood $CD56^{dim}CD16^{+}$ NK cells. In line with these findings, our present data suggest that up-regulation of KIR on $CD56^{bright}CD16^{-}$ NK cells can occur also in vivo, most likely because of a proinflammatory cytokine microenvironment due to local immune reactions. It might be argued that KIR expression on $CD56^{bright}$ NK cells might be explained as CD56 up-regulation on KIR^{+} NK cells, rather than the other way around. Indeed, although CD16 can be down-regulated on NK cells upon cytokine activation, as previously demonstrated,[50,51] perforin was absent on $CD56^{bright}KIR^{+}$ NK cells infiltrating the tumors, thus suggesting that perforin, a potent lytic molecule, is acquired after KIR, a self-protective receptor mechanism, during NK cell differentiation. Further investigation, beyond the scope of this study, should aim at clarifying whether inflamed tissues, including cancers, might be colonized by NK cells in an early developmental stage and might thus represent a site for NK cell terminal maturation and self-tolerance acquisition.

In conclusion, we have shed some light on NK cells infiltrating human cancers, analyzing for the

first time NK cells in NSCLC. The finding that NSCLC-infiltrating NK cells are not merely killer cells but can mainly act as producers of relevant cytokines, either productive or detrimental for the antitumor immune response, might open new perspectives for manipulating these innate effector cells during immunotherapeutic strategies. Moreover, because recent findings suggest that activation can convert $CD56^{bright}CD16^{-}KIR^{-}$ NK cells, able to mainly secrete cytokines, into $CD56^{dim}CD16^{+}KIR^{+}$ NK cells with higher cytolytic activity,[49,52] the key to successful NK cell-based therapies might also depend, among other factors, on new tools for a sustained in vivo activation of NK cells.

REFERENCES

1. Trinchieri G. Biology of natural killer cells. *Adv Immunol*. 1989;7:176–187.
2. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol*. 2001;19:197–223.
3. Moretta L, Ferlazzo G, Mingari MC, Melioli G, Moretta A. Human natural killer cell function and their interactions with dendritic cells. *Vaccine*. 2003;21(suppl 2):S38–S42.
4. Moretta L, Bottino C, Pende D, Mingari MC, Biassoni R, Moretta A. Human natural killer cells: their origin, receptors and function. *Eur J Immunol*. 2002;32:1205–1211.
5. Yokoyama WM, Seaman WE. The Ly49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu Rev Immunol*. 1993;11: 613–635.
6. Moretta A, Bottino C, Vitale M, et al. Receptors for HLA-class I-molecules in human natural killer cells. *Annu Rev Immunol*. 1996;14:619–648.
7. Lanier LL. NK cell receptors. *Annu Rev Immunol*. 1998;16: 359–393.
8. Long EO. Regulation of immune response through inhibitory receptors. *Annu Rev Immunol*. 1999;17:875–904.
9. Lopez-Botet M, Perez Villar M, Carretero M, et al. Structure and function of the CD94 C-type lectin receptor complex involved in the recognition of HLA class I molecules. *Immunol Rev*. 1997;155:165–174.
10. Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. *Blood*. 1990;76:2421–2438.
11. Campbell JJ, Qin S, Unutmaz D, et al. Unique subpopulations of $CD56^{+}$ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol*. 2001;166:6477–6482.
12. Jacobs R, Hintzen G, Kemper A, et al. $CD56^{bright}$ cells differ in their KIR repertoire and cytotoxic features from $CD56^{dim}$ NK cells. *Eur J Immunol*. 2001;31:3121–3127.
13. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subset. *Blood*. 2001;97:3146–3151.
14. Villegas FR, Coca S, Villarrubia VG, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer*. 2002;35:23–28.
15. Ishigami S, Natsugoe S, Tokuda K, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer*. 2000;88:577–583.
16. Coca S, Perez-Piqueras J, Martinez D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer*. 1997;79:2320–2328.
17. Schleypen JS, Von Geldern M, Weiss EH, et al. Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. *Int J Cancer*. 2003;106:905–912.
18. Schleypen JS, Baur N, Kammerer R, et al. Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. *Clin Cancer Res*. 2006;12:718–725.
19. Takeo S, Yasumoto K, Nagashima A, Nakahashi H, Sugimachi K, Nomoto K. Role of tumor-associated macrophages in lung cancer. *Cancer Res*. 1986;46:3179–3182.
20. Takanami I, Takeuchi K, Giga M. The prognostic value of natural killer cell infiltration in resected pulmonary adenocarcinoma. *J Thorac Cardiovasc Surg*. 2001;121:1058–1063.
21. Ihde DC. Chemotherapy of lung cancer. *N Engl J Med*. 1992;327:1434–1441.
22. Non-small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. *BMJ*. 1995;311:899–909.
23. Jemal A, Murray T, Ward E, et al. Cancer statistics 2005. *CA Cancer J Clin*. 2005;55:10–30.
24. Mori M, Ohtani H, Naito Y, et al. Infiltration of CD8+ T cells in non-small cell lung cancer is associated with dedifferentiation of cancer cells, but not with prognosis. *Tohoku J Exp Med*. 2000;191:113–118.
25. Wakabayashi O, Yamazaki K, Oizumi S, et al. CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. *Cancer Sci*. 2003;94:1003–1009.
26. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003;281:65–78.
27. Ferlazzo G, Thomas D, Lin SL, et al. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J Immunol*. 2004;172:1455–1462.
28. Rubio V, Stuge TB, Singh N, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med*. 2003;9:1377–1382.
29. Wolint P, Betts MR, Koup RA, Oxenius A. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. *J Exp Med*. 2004;199:925–936.
30. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med*. 2005;202:1001–1012.
31. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25:331–342.
32. Blankenstein T. The role of tumor stroma in the interaction between tumor and immune system. *Curr Opin Immunol*. 2005;17:180–186.
33. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force." *Blood*. 2005;106:2252–2258.
34. Ferlazzo G. Natural killer and dendritic cell liaison: recent insights and open questions. *Immunol Lett*. 2005;101:12–17.

35. Moretta L, Ferlazzo G, Bottino C, et al. Effector and regulatory events during natural killer-dendritic cell interactions. *Immunol Rev*. 2006;214:219–228.
36. Kelly JM, Darcy PK, Markby JL, et al. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat Immunol*. 2002;3:83–90.
37. Kelly JM, Takeda K, Darcy PK, Yagita H, Smyth MJ. A role for IFN-gamma in primary and secondary immunity generated by NK cell-sensitive tumor-expressing CD80 in vivo. *J Immunol*. 2002;168:4472–4479.
38. Mocikat R, Braumuller H, Gumy A, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. *Immunity*. 2003;19:561–569.
39. Zhang C, Zhang J, Tian Z. The regulatory effect of natural killer cells: do "NK-reg cells" exist? *Cell Mol Immunol*. 2006;3:241–254.
40. Trivedi PP, Roberts PC, Wolf NA, Swanborg RH. NK cells inhibit T cell proliferation via p21-mediated cell cycle arrest. *J Immunol*. 2005;174:4590–4597.
41. Johansson S, Berg L, Hall H, Hoglund P. NK cells: elusive players in autoimmunity. *Trends Immunol*. 2005;26:613–618.
42. Hagenaars M, Zwaveling S, Kuppen PJ, et al. Characteristics of tumor infiltration by adoptively transferred and endogenous natural-killer cells in a syngeneic rat model: implications for the mechanism behind antitumor responses. *Int J Cancer*. 1998;78:783–789.
43. Hagenaars M, Ensink NG, Basse PH, et al. The microscopic anatomy of experimental rat CC531 colon tumour metastases: consequences for immunotherapy? *Clin Exp Metastasis*. 2000;18:189–196.
44. Kuppen PJ, Van Der Eb MM, Jonges LE, et al. Tumor structure and extracellular matrix as a possible barrier for therapeutic approaches using immune cells or adenoviruses in colorectal cancer. *Histochem Cell Biol*. 2001;115:67–72.
45. Lardner A. The effects of extracellular pH on immune function. *J Leukoc Biol*. 2001;69:522–530.
46. Hellstrand K. Melanoma immunotherapy: a battle against radicals? *Trends Immunol*. 2003;24:232–233.
47. Buckley CD, Simmons DL. Sticky moments with sticky molecules. *Immunol Today*. 2000;21:601–603.
48. Albertsson PA, Basse PH, Hokland M, et al. NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. *Trends Immunol*. 2003; 24:603–609.
49. Romagnani C, Juelke K, Falco M, et al. $CD56^{bright}$ $CD16^{-}$ KIR^{-} NK cells display longer telomeres and acquire features of $CD56^{dim}$ NK cells upon activation. *J Immunol*. 2007;178:4947–4955.
50. Loza MJ, Perussia B. The IL-12 signature: NK cell terminal CD56+high stage and effector functions. *J Immunol*. 2004; 172:88–96.
51. Mailliard RB, Alber SM, Shen H, et al. IL-18-induced CD83+CCR7+ NK helper cells. *J Exp Med*. 2005;202:941–953.
52. Chan A, Hong DL, Atzberger A, et al. CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J Immunol*. 2007;179:89–94.