CD56\textsuperscript{bright}Perforin\textsubscript{low} Noncytotoxic Human NK Cells Are Abundant in Both Healthy and Neoplastic Solid Tissues and Recirculate to Secondary Lymphoid Organs via Afferent Lymph

Paolo Carrega, Irene Bonaccorsi, Emma Di Carlo, Barbara Morandi, Petra Paul, Valeria Rizzello, Giuseppe Cipollone, Giuseppe Navarra, Maria Cristina Mingari, Lorenzo Moretta and Guido Ferlazzo

*J Immunol* 2014; 192:3805-3815; Prepublished online 19 March 2014;
doi: 10.4049/jimmunol.1301889
http://www.jimmunol.org/content/192/8/3805
CD56\textsuperscript{bright} Perforin\textsuperscript{low} Noncytotoxic Human NK Cells Are Abundant in Both Healthy and Neoplastic Solid Tissues and Recirculate to Secondary Lymphoid Organs via Afferent Lymph

Paolo Carrega,* Irene Bonaccorsi,†‡ Emma Di Carlo,*§¶ Barbara Morandi,‖ Petra Paul, † Valeria Rizzello, † Giuseppe Cipollone,§** Giuseppe Navarra, †† Maria Cristina Mingari,‖¶ Lorenzo Moretta,* and Guido Ferlazzo†‡

As limited information is available regarding the distribution and trafficking of NK cells among solid organs, we have analyzed a wide array of tissues derived from different human compartments. NK cells were widely distributed in most solid tissues, although their amount varied significantly depending on the tissue/organ analyzed. Interestingly, the distribution appeared to be subset specific, as some tissues were preferentially populated by CD56\textsuperscript{bright}perforin\textsuperscript{low} NK cells, with others by the CD56\textsuperscript{dim}perforin\textsuperscript{high} cytotoxic counterpart. Nevertheless, most tissues were highly enriched in CD56\textsuperscript{bright}perforin\textsuperscript{low} cells, and the distribution of NK subsets appeared in accordance with tissue gene expression of chemotactic factors, for which receptors are differently represented in the two subsets. Remarkably, chemokine expression pattern of tissues was modified after neoplastic transformation. As a result, although the total amount of NK cells infiltrating the tissues did not significantly change upon malignant transformation, the relative proportion of NK subsets infiltrating the tissues was different, with a trend toward a tumor-infiltrating NK population enriched in noncytotoxic cells. Besides solid tissues, CD56\textsuperscript{bright}perforin\textsuperscript{low} NK cells were also detected in seroma fluids, which represents an accrual of human afferent lymph, indicating that they may leave peripheral solid tissues and recirculate to secondary lymphoid organs via lymphatic vessels. Our results provide a comprehensive mapping of NK cells in human tissues, demonstrating that discrete NK subsets populate and recirculate through most human tissues and that organ-specific chemokine expression patterns might affect their distribution. In this context, chemokine switch upon neoplastic transformation might represent a novel mechanism of tumor immune escape. *The Journal of Immunology, 2014, 192: 3805–3815.

Natural killer cells represent the prototype of innate lymphoid cells (ILCs), a family of developmentally related innate cells that plays relevant roles in mediating innate immune responses, as well as in regulating tissue homeostasis and inflammation (1).

Received for publication July 17, 2013. Accepted for publication February 7, 2014. This work was supported by Associazione Italiana Ricerca sul Cancro IG11650 and Istituto Nazionale per la Ricerca sul Cancro, 16132 Genoa, Italy. Address correspondence and reprint requests to Prof. Guido Ferlazzo, Laboratory of Immunology and Biotherapy, University of Messina, Azienda Ospedaliera Universitaria Policlinico Gaetano Martino, 98125 Messina, Italy; †Laboratory of Immunology and Biotherapy, Department of Human Pathology, University of Messina, 98125 Messina, Italy; † Programs of Terapia Cellulare, Azienda Ospedaliera Universitaria Policlinico Gaetano Martino, 98125 Messina, Italy; † Department of Medicine and Sciences of Aging, “G. d’Annunzio” University, 66013 Chieti, Italy; † Department of Experimental Medicine, University of Genoa, 16132 Genoa, Italy; † Viral Immunobiology, Institute of Experimental Immunology, University of Zurich, CH-8057 Zurich, Switzerland; †† Section of General and Thoracic Surgery, “S. S. Annunziata” Hospital, 66103 Chieti, Italy; †‡ Surgical Oncology Unit, Department of Human Pathology, University of Messina, 98125 Messina, Italy; and (1) Instituto di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliera Universitaria San Martino IST-Istituto Nazionale per la Ricerca sul Cancro, 16132 Genoa, Italy

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

In particular, the main effector functions of NK cells span from killing target cells, mainly tumor and virally infected cells, to the ability to influence various steps of the immune response by editing dendritic cells and influencing T cell polarization (2–5). It is now clear that a division of labor exists among NK cells for performing these distinct functions, and, on the basis of CD56 expression, two populations of human NK cells are classically distinguished: CD56\textsuperscript{dim} cells that also express CD16 (the low-affinity receptor for IgG, Fc\textgamma RI) and express low levels of perforin, no KIRs, and are CD16\textsuperscript{low/neg} are able to secrete large amounts of cytokines (e.g., IFN-\gamma, GM-CSF, and TNF-\alpha), but are devoid of killing activity. Nevertheless, with the appropriate stimulus, CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells are also abundant cytokine producers (6–8).

Differences between these two main human NK cell subsets also include the expression of chemokine receptors, as CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cell subsets largely differ in their responsiveness toward a variety of chemotactic factors (9, 10). CD56\textsuperscript{bright} NK cells constitute express CCR7 (CCL19 and -21 receptors), which justifies their selective presence in secondary lymphoid organs (11) and CCR5 (CCL3-5 receptor), and they share with CD56\textsuperscript{dim} NK cell counterpart both CXCR4 (CXCL12 receptor) and CXCR3 (CXCL4 and -9–11 receptors), although the latter is generally expressed at lower levels on CD56\textsuperscript{dim} cells. CD56\textsuperscript{bright} and a subset of CD56\textsuperscript{dim} cells can bind to high endothelial venules (HEV) thanks to the expression of CD62L (8). Conversely, CD56\textsuperscript{dim} cells uniquely display CX\textgamma CR1 (fractalkine receptor), CXCR2 (CXCL1-3, -5, and -8...
NK cell subsets in human tissues

NK cells express an ample array of chemotactic receptors, the role of the conditions, are almost unknown. Although human and mouse NK organs/tissues as follows. GALT (11), secondary lymphoid organs (11, 16). It has also been demonstrated that tissue-resident NK cells can play roles even unrelated to protection against microbial pathogens and instead display other functions, such as the promotion of vascular remodeling in the uterus (2) or even in cancerous tissues (17). Nonetheless, the true distribution of NK cells across the human body is less than clear, and the few data available on human NK cell distribution in tissues should be carefully evaluated, as they often represent observations deriving from pathological conditions (18–20). Much information about the presence of NK cells in healthy tissues comes from mouse models, where NK cells are more easily traceable, and thus straightforwardly investigated, than in their human counterpart. In mice, NK cells are found to be distributed in many organs such as lung, liver, spleen, bone marrow, and LN (10). This distribution of NK cells seems to be subset specific in mice, in which different NK cell subsets showed organ-specific localizations. Conversely, this issue has been only partially investigated in the human system. Furthermore, contrary to B and T lymphocytes, recirculation and trafficking of NK cells in steady-state conditions, as well as in pathological conditions, are almost unknown. Although human and mouse NK cells express an ample array of chemotactic receptors, the role of the different chemokines in guiding the trafficking of NK cells through the tissues remained poorly dissected (12).

To increase current knowledge regarding the presence and trafficking of NK cells in human organs, we analyzed a wide array of tissues derived from different parts of the human body, investigating the presence of NK cells and the distribution of functionally different NK cell subsets, carefully excluding other ILCs from these analyses. Moreover, we examined the underlying mechanisms that might contribute to guide the distribution of NK cells in tissues and, finally, whether NK cell localization might be perturbed in the case of neo-plastic transformation.

Materials and Methods

Patient selection and samples

Healthy tissues were obtained from both lymphoid organs and nonlymphoid organs/tissues as follows. GALT (n = 4) were derived from biopsies collected during endoscopic follow-up observations after colon cancer resections. LN (n = 5) were collected from patients who underwent surgery for cancer resection. Subcutaneous (n = 6) adipose tissues were obtained after breast or abdominal surgery, and visceral (n = 6) adipose tissues were obtained upon laparoscopic surgery in obese patients. Specimens of liver (n = 7), colorectal (n = 15), lung (n = 15), stomach (n = 5), breast (n = 6), adrenal gland (n = 2), and kidney (n = 6) either from tumor tissues or histologically confirmed nonmural tissues were obtained during surgical procedures of cancer resections. Seroma samples (n = 11) were collected in seven different patients operated upon breast cancer and removal of axillary LN. Seroma was collected using needle aspiration between days 15 and 50 after axillary dissection. In three patients, more than one sample was obtained at 1- to 2-wk intervals, whereas in other patients, only one sample was obtained. PB samples were obtained from healthy donors or, in selected experiments, from cancer patients.

All sample collections were obtained after donor informed consent and approval, when necessary, by the Istituto di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliera Universitaria San Martino/IST Institutional Ethics Committee. Patients with diabetes, HIV/hepatitis C virus/hepatitis B virus infection, overt chronic inflammatory conditions, previously treated with chemotherapy or radiotherapy, or those iatrogenically immunosuppressed or having undergone myeloablative therapies were excluded from the study.

Solid tissue dissociation

All solid tissues were extensively washed in PBS to remove cell debris and RBC aggregates. Samples were then mechanically minced by scissors to obtain small fragments. In order to minimize blood contamination, tissue specimens were extensively rinsed after initial tissue slashing in small fragments. Then, samples were enzymatically digested with a mixture containing DNAse (100 μg/ml), collagenase (1 mg/ml), and hyaluronidase (1 mg/ml) in RPMI 1640 supplemented with penicillin/streptomycin by 1.5 h at 37°C. The suspension was then filtered through cell strainer and, subsequently, washed by centrifugation in PBS to remove residual enzymes. To obtain tissue-resident and blood mononuclear cells (MCNs), tissue-cell suspensions and blood were then isolated by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation.

Flow cytometry

The following mouse anti-human mAbs were used in our study: anti-CD3 FITC, -NKp44 PE, -NKp46 PE, -NKp30 PE, -CD16 PE, -CD3 PE, -CD56 APC, and -HLA-DR PE from Miltenyi Biotec; -CD57 Pacific Blue, and -CD94 FITC from BioLegend; -perforin FITC and PE from Ancell; -KIR2DL1 PE, -KIR2DL2 PE, -KIR3DL1 PE, and -NKGA2 APC from Instrumentation Laboratories; -CD62L PE, -CD45 APC-H7, and -CD27 PE from BD Biosciences; -CD56 BV711 from eBioscience; and -CXCR1 PE from R&D Systems. For indirect immunofluorescence assay: -CCL5 (clone 1F5) (5 mg/ml), -CCR7 (clone 150503), -anti-CXCR2 (clone 48311), -anti-CXCR3 (clone 49801), anti-CXCR4 (clone 44717), and anti-SIP5 (clone 282503) were used (all from R&D Systems). After incubation with the relevant mAbs, cells were washed, and PE-conjugated isotype-specific goat anti-mouse Abs were added and incubated for 30 min at 4°C. Negative controls included directly labeled and unlabeled isotype-matched irrelevant mAbs. Cells were then washed and analyzed by flow cytometry. Intracellular staining with anti-human perforin-PETFITC was performed using the Fix/Perm kit from BD Biosciences according to the manufacturer’s indications. Samples were then run either on an FACSCanto II (BD Biosciences) or Gallios (Beckman Coulter) cytometers and analyzed by FlowJo 9.0.2 software (Tree Star).

Microarray data and analysis

Microarray data used in this study were obtained from the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). GEO series with accession numbers GSE11313, GSE2665, GSE16688, GDS1096, GSE24514, GSE5364, GSE2719, GSE9536, GSE2004, GSE15653, GSE781, GDS3257, GSE7670, GDS3139, GSE16873, GSE5090, and GSE19811 were used to evaluate the chemokine expression levels in the normal and tumor tissues. A detailed description of all samples that were included in their microarray meta-analysis is reported in Supplemental Table I.

All of the used datasets were derived using the Affymetrix GeneChip HG-U133A (Affymetrix) for hybridization. Raw data were normalized using RMA Express software (1.0.5 version http://www.biosoft.com) with the RMA algorithm, applying quantile normalization. Data obtained were expressed as log2. Expression data were then filtered to remove nonexpressed (log2 intensity < 4) genes. For genes with multiple probe sets, we selected the probe sets giving the highest signal. To derive the tissue-chemokine expression profiles, we analyzed in each tissue the gene expression levels of 18 known chemokines for which receptors are expressed by human NK cells (shown in Fig. 2C). Then, we selected only those chemokine genes for which the expression level was above the median value in the tissue. On the contrary, for the comparison between normal and matched tumor tissues, we considered all of the 18 NK-relevant chemokines, and we then selected only those chemokine genes that were significantly up-/downregulated (fold changes ≥ 1) in the tumor specimen.

Hierarchical clustering of tissue gene expression data were realized on the basis of euclidean distance using Multiexperiment Viewer (http://www.tm4.org/mev.html).

Immunohistochemistry

Tissue samples were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E. For fractalkine staining, paraffin-embedded tissue sections were deparaffinized, treated with H2O2/3% for 5 min to inhibit endogenous peroxidase, and then washed in H2O. They were then incubated for 30 min with the first primary Ab (rabbit polyclonal anti-CX3CL1 [ab25088 validated by Western blot]; Abcam), followed by detection with the Bond Polymer Reanine Detection Kit (Leica...
The Journal of Immunology

Microsystems) according to the manufacturer’s protocol. For c-Kit/NKp46 double staining, paraffin-embedded tissue sections were deparaffinized, treated with H2O2/3% for 5 min to inhibit endogenous peroxidase, and then washed in H2O. They were then incubated for 30 min with the first primary Ab (rabbit anti-c-Kit Ab; DakoCytomation), followed by detection with the Bond Polymer Refine Detection Kit (Leica Microsystems), according to the manufacturer’s protocol, and subsequently incubated for 30 min with the second primary Ab (mouse anti-NKp46/NC1R1 Ab; R&D Systems), followed by detection with the Bond Polymer Refine Red Detection Kit (Leica Microsystems) according to the manufacturer’s protocol. Formalin-fixed, paraffin-embedded LN samples (histologic report of LN tissue with follicular hyperplasia and sinus histiocytosis archived in the Anatomic Pathology Institute, “SS. Annunziata” Hospital of Chieti, Italy) were used as positive control tissue.

CD107a and cytokine-release assay

MNCs from blood and autologous colon carcinoma 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 E:T ratio of 1:1 (2.5 x 10^5 effector cells:2.5 x 10^5 target cells in a volume of 200 µl) was used. Anti-CD107a mAb was added in each well before incubation. Effectors and targets were then coincubated at 37°C for 4 h; after the first hour, monensin (Sigma-Aldrich) was added at a final concentration of 2 mM to inhibit cell secretion. At the end of coincubation, cells were first stained for relevant surface markers and finally fixed and permeabilized for intracellular detection of IFN-γ expression by specific mAbs (Miltenyi Biotec).

Statistical analysis

For the statistical analyses of chemokine gene expression data and NK cell subset distribution among different tissues, we performed an unpaired t test analysis using the GraphPad Prism software (GraphPad Software).

Results

NK cells populate human tissues, and the distribution of the two main NK cell subsets is organ-specific

In order to investigate the distribution of NK lymphocytes in the human body, we examined the presence of NK cells as well as the reciprocal proportion of the two major NK cell subsets, namely CD56^bright^perforin^low^ (nontoxic) and CD56^dim^perforin^high^ (cytotoxic) NK cells in a wide array of healthy tissues. They were obtained from both lymphoid organs (GALT of the small intestine and thoracic/mesenteric LN) and nonlymphoid organs/tissues (lung, liver, breast, stomach, colorectal, adrenal gland, kidney, and adipose tissues, both visceral and s.c.). It should be noted that, among human ILCs, NK cells share CD56 expression only with a fraction of natural cytotoxicity receptor (NCR)’ILC3 cells (21), which profoundly differ from NK cells by specifically producing the cytokines IL-17A and/or IL-22 and by depending on the transcription factor retinoic acid–related orphan receptor γt for their development and functions (22). Because NK cells share the CD56 expression also with NCR’ILC3 cells (21), the amount of which can be relevant in some of the tissues analyzed in this study (e.g., colon) (23), ILC3 cells were excluded from the analyses on the basis of the absence of CD94 and perforin.

Our results agreed with the wide distribution of NK cells observed in the mouse model (10), as NK cells (identified as CD45^CD3^CD8^CD49^CD56^ cells) were clearly detectable in all the examined tissues (Fig. 1A, top panel). The frequency of NK cells was the highest in nonlymphoid organs such as kidney (mean: 25.3% of lymphocytes), liver (16.4%), and s.c. adipose tissues (14.2%). NK cells were less frequent in the lung and breast tissues, where they accounted, respectively, for an average of 10 and 6.65% of tissue-resident lymphocytes. Conversely, we found that in colorectal, stomach, adrenal gland, GALT, and visceral adipose tissues, NK cells were rather rare (mean values between 1 and 3.45%) (Fig. 1B). Next, we studied the relative distribution of the two main NK cell subsets in the various compartments. Due to the possible downregulation of CD16 on NK cells extracted from solid tissues (24, 25), we distinguished CD56^dim^ (cytotoxic) from CD56^bright^ (nontoxic) NK cells by using the level of expression of perforin as a marker. Therefore, CD56^dim^perforin^high^ cells identified the cytotoxic NK subset, whereas CD56^bright^perforin^low^ cells identified the nontoxic counterpart (Fig. 1A, bottom panel). Interestingly, NK cell distribution appeared to be subset specific, as most tissues were preferentially populated by CD56^bright^perforin^low^ NK cells, with a few others by the CD56^dim^perforin^high^cytotoxic counterpart (Fig. 1C). CD56^bright^perforin^low^ NK cells were abundantly represented in GALT, for which values matched those detected in LN, known to be mainly inhabited by CD56^bright^perforin^low^ NK cells (11). Among nonlymphoid organs, CD56^bright^perforin^low^ NK cells were abundantly detected in stomach, adrenal gland, colorectal, liver, and visceral adipose tissues. In all of these tissues, the percentage of CD56^bright^perforin^low^ NK cells was consistently higher than in PB, with mean values ranging from 83.75 to 40% of the whole NK cell population. Conversely, in lung, breast, and s.c. adipose tissues, most NK cells showed a CD56^dim^perforin^high^ phenotype, and the CD56^bright^perforin^low^ cells (mean values ranging from 20 to 9% of total NK cells) were represented at values comparable to PB or slightly higher (Fig. 1C). Kidney, among the organs analyzed, showed an intermediate percentage of CD56^bright^perforin^low^ cells (mean: 37%).

NK cell subset distribution is consistent with organ-specific chemokine expression patterns

To assess whether tissues might influence the specific distribution of NK cells, we performed a meta-analysis of data gathered from a variety of normal solid tissues. This method allowed us to analyze the gene expression of 18 known chemotactic factors specific for chemokine receptors expressed by NK cells (Fig. 2C). In order to derive a chemokine expression pattern for each tissue, among all of the chemotactic factors for which receptors are expressed on NK cells (9, 10), the six most expressed factors were considered for each tissue analyzed (for details, see Materials and Methods). The gene expression level of the most expressed chemotactic factors in a given tissue was congruent with the expression of chemokine receptors of the NK cell subset predominantly infiltrating the tissue (Fig. 2A). As is already known, most LN NK cells belonged to the CD56^bright^cell subset; indeed, the LN expression profile showed CCL21, CCL19, and CXCL12 as the most expressed chemokines in this organ. Of note, LN appeared as the organ where CCL21 and CCL19 exhibited the highest expression levels compared with the other analyzed organs. High frequency of CD56^bright^ NK cells was also found in colorectal, stomach, and liver. Indeed, the chemokine expression profile of colorectal tissue showed high levels of CXCL12, CCL19, and CCL21, as well as CCL4, CXCL10, and CXCL9, thus being clearly oriented to recruit CD56^bright^ cells. The stomach showed very similar results, even if we had to rely on a smaller set of samples for this analysis. The liver tissue contained less CD56^bright^ NK cells compared with the previous organs (mean 61% of total NK cells), and this might also be explained by the presence of CXCL2 (relevant for CD56^dim^ NK cells) among the group of chemokines expressed in the tissue, which were, however, mainly specific for CD56^bright^ NK cells. A similar pattern was found for the kidney tissue, where another chemokine specific for CD56^dim^ cells (CXCL1) was significantly expressed, thus likely contributing to the intermediate frequency of CD56^bright^ cells (mean: 37% of total NK cells) populating this organ. On the contrary, an opposite chemokine expression profile was derived from the body regions that were characterized by the lowest proportions of CD56^bright^ NK cells (i.e., lung and breast tissues). Accordingly, breast tissue showed high levels of CXCL2, CXCL1,
CXCL1, and CXCL12, a chemokine pattern well oriented to attract CD56dim cells. The lung revealed a similar profile, as CXCL2 and CX3CL1 appeared to be the most expressed NK cell–related chemokines in this organ. Despite significant differences between visceral and s.c. adipose tissues in NK cell subset distribution, the chemokine expression profile in part overlapped in these two tissues. Nevertheless, CCL21, a ligand for CCR7, was strongly expressed in visceral but absent in s.c. adipose tissues, thus possibly accounting for the preferential homing of CD56bright NK cells observed in visceral adipose tissue.

Recently, chemerin has emerged as another chemotactic factor for NK cell recruitment to peripheral tissues, especially CD56dim cells. It is present in healthy tissues as a poorly active precursor, prochemerin, that can be rapidly converted into the active form during inflammation. Therefore, prochemerin represents a ready-to-use chemotactic factor for NK cell recruitment (14).

In conclusion, although hierarchical clustering of the pooled microarray data did not show a very pronounced pattern regarding chemokine expression of different tissues (Fig. 2D), our results suggest that the production of these factors may influence the distribution of NK cells in human solid tissues.

CD56bright perforinlow NK cells recirculate via afferent lymph in the human body

The presence of NK cells in many organs raised the question of whether NK cells eventually exit the organs or terminally reside in the tissues. To address this issue, we investigated the presence of NK cells in seroma fluid, which represents an accumulation of afferent lymph (26). Lymph represents the extracellular fluid produced continuously in peripheral tissues by blood, later enriched with peripheral tissue catabolites, cells, and debris, collected in afferent lymphatic vessels, and conveyed into secondary lymphoid organs, such as LN. It then leaves secondary lymphoid organs via efferent vessels (efferent lymph) before emptying ultimately into the subclavian vein, where it mixes back with the blood.

Interestingly, among the CD45+ leukocytes present in seroma, we observed a fraction of CD3−CD56bright NKP46+ cells (Fig. 3A, 3C), which accounted for >2% of the whole lymphocytic population (Fig. 3B). Notably, all seroma NK cells expressed low levels of perforin, thus resembling blood CD56bright NK cells (Fig. 3A). This observation was further confirmed by the very low expression of CD16 and KIRs on their surface (Fig. 3C). We have recently shown that cells contained in seroma fluids are in general not contaminated.
by either surgery-induced exudate or blood (27). In addition, seroma NK cells did not express the activation markers HLA-DR and NKp44, therefore indicating that they should not merely represent either activated cells recruited in seroma fluid or NCR^ILC3, which express

![Chemokine-expression pattern and NK cell subset frequency in healthy human tissues. Gene expression of chemokines relevant for the recruitment of NK cells were derived from the meta-analysis of publicly available expression data of normal healthy tissues.](http://www.jimmunol.org/)

**FIGURE 2.** Chemokine-expression pattern and NK cell subset frequency in healthy human tissues. Gene expression of chemokines relevant for the recruitment of NK cells were derived from the meta-analysis of publicly available expression data of normal healthy tissues. (A) For each tissue, the six most expressed chemokines are displayed. Relative distribution of CD56^{bright}perforin^{low} (black bars) and CD56^{dim}perforin^{high} (white bars) among total NK cells are also shown for each tissue. (B) Prochemerin gene expression levels in healthy human tissues. (C) Chemotactic receptors expressed by NK cell subsets. For each receptor, the related ligands are indicated. (D) Hierarchical clustering of the microarray data for chemokine gene expression levels of human tissues. Chemokines attracting CD56^{bright}noncytotoxic NK cells are grouped in the left part of the figure. CXCL12 can evenly attract both subsets of human NK cells. --, not expressed; +, expressed; ++, expressed at high levels.
NKp44. As expected in a lymphocytic population migrating to a secondary lymphoid organ, seroma NK cells were strongly positive for CCR7 and CD62L (Fig. 3C). NK cells present in afferent lymph from seroma fluids also expressed the CXCR4, CXCR3, and CCR5 receptors but were negative for CXCR2, S1P5, and CX3CR1 receptors (Fig. 3D), thus matching our current observations on the most expressed chemokines in the LN (Fig. 2A). These findings reveal, for the first time in humans to our knowledge, that subsets of NK cells might even exit peripheral tissues and recirculate to secondary lymphoid organs, and eventually blood, via afferent lymph.

Cancer tissues are enriched in CD56bright perforinlow noncytotoxic NK cells

We then investigated whether NK cell migration and homing to the tissues might be altered in the case of neoplastic transformation. Compared to normal matched tissues, we could not find significant differences in the frequency of total NK cells (CD3negCD94+ CD56+ cells) among the tumor-infiltrating lymphocytes. However, we observed a trend toward a lower percentage of NK cells in the neoplastic tissues of lung and kidney, but a higher percentage in gastric cancers compared with normal counterparts (Fig. 4A). As we previously reported in the case of lung cancer (24), we found that NK cells infiltrating tumors originated from different tissues were, in general, highly enriched in the CD56bright perforinlow NK cell subset. Interestingly, at least for some tumor types, the frequency of CD56bright perforinlow NK cells among the total NK cell compartment was significantly higher than matched normal tissues, such as in the case of lung and breast cancers (54 versus 9.2 and 30 versus 4.1%, respectively) (Fig. 4B). In order to clarify whether the tumor microenvironment might play a role in this specific accumulation, we compared the chemokine expression patterns derived from tumor samples with those derived from matched healthy tissues (Fig. 4C). In agreement with the different distribution of NKp46 on CD56dim perforinhigh cytotoxic NK cells (25), we stained with anti–NKp46 mAbs to discriminate between cytotoxic and noncytotoxic NK cells in tissue sections. In our experimental conditions, and as previously reported by others (23), NCR1+ ILC3 were distinguishable by bona fide NK cells because of their lower expression of NKp46. In addition, because c-Kit is not expressed on CD56dim perforinhigh cytotoxic NK cells (25), we stained with anti–c-Kit mAbs to discriminate between cytotoxic and noncytotoxic cells in tumor infiltrate (Fig. 5). Immunohistochemistry analysis of normal lung tissue and lung adenocarcinomas revealed that NKp46+/c-Kitneg cytotoxic cells were mainly represented in the stromal lymphocytic infiltrate rather than inside the neoplastic epithelial compartment. Infiltrating ductal carcinoma, the most common histotype of invasive breast cancer (32), also showed, in the
stromal compartment, a distinct infiltrate of NKp46+/c-Kit+ cells, which were rarely found in normal breast tissue. By contrast, comparative analyses of colon adenocarcinomas and normal colonic mucosa revealed that both tissues were endowed with a distinct NKp46+/c-Kit+ cell infiltrate located, respectively, in the lamina propria, within intestinal crypts, and in the cancer-associated stroma (Fig. 5).

As a whole, these data showed that neoplastic transformation does not determine an increased migration of NK cells within the tissues. Rather, possibly as a consequence of switches in chemokine expression patterns, cytotoxic NK cells are significantly decreased, compared with healthy tissues, in frequently occurring cancers such as breast and lung carcinomas.

An alternative explanation for these results might be that, rather than causing a distinct recruitment of NK cell subsets, the cancer microenvironment might affect NK cell developmental axis, and, therefore, phenotypical differences may reflect this imprinting.

FIGURE 4. NK cell distribution in cancer tissues. Neoplastic and corresponding healthy tissues from an array of human organs were comparatively analyzed for the presence of NK cells, the frequency of the two main NK cell subsets, and the production of NK-related chemotactic factors. (A) Comparative analyses of the percentage of total NK cells among CD45+ lymphocytes in healthy and neoplastic tissues of the indicated human organs. No statistically significant differences were detectable. (B) Comparative analyses of the percentage of CD56bright/perforinlow noncytotoxic NK cells among total NK cells isolated from healthy and neoplastic tissues of the indicated organs. Histogram bars in both (A) and (B) represent mean values and SDs of data obtained analyzing several specimens from distinct patients. The number of cancer and normal specimens respectively analyzed for each organ are indicated above bars. (C) Gene expression levels of chemokines relevant for the recruitment of NK cells were analyzed in lung and breast cancer tissues and compared with the levels in matched normal tissues. For each tissue, only genes that were either up- or downregulated (at least 1 log) with statistical significance (p < 0.002) are shown. (D) Immunohistochemical detection of CX3CL1 (fractalkine) in both normal and cancerous tissues of lung and breast. In the normal lung tissue (Da), CX3CL1 was expressed by free-floating alveolar leukocytes, mostly endowed with macrophage-like feature, whereas in the neoplastic counterpart (Db), the chemokine was expressed by leukocytes and also by neoplastic epithelial cells. In the normal breast tissue (Dc), CX3CL1 expression was found in the cytoplasm of epithelial cells forming the terminal ductal lobular units, whereas in the ductal adenocarcinoma of the breast (Dd) CX3CL1 expression was scarce to absent and barely detected in the cytoplasm of neoplastic epithelial cells. (Da) Original magnification ×400; (Db–Dd) original magnification ×630. *p < 0.05. u.d., under the level of detection.
caused by tissue milieu. We thus analyzed the phenotype of tumor-infiltrating NK cells in lung and colon carcinomas, for which normal tissue counterparts are mainly populated by, respectively, CD56dimperforin high and CD56bright perforin dim NK cells. The phenotypes of the two NK cell subsets, in both non–small cell lung (NSCL) and colon cancers, were analogous to autologous PB, thus indicating a similar differentiation axis and corroborating the notion that a different NK cell recruitment occurs upon neo-
plastic transformation (Fig. 6). In detail, no major differences were detectable in the expression of the inhibitory receptors KIRs and NKG2A. Differentiation markers such as CD27 and CD57 did not show significant differences between tumor NK cells and autologous PB. In contrast, CD62L was downregulated in both subsets of NK cells infiltrating lung and colon cancers: because CD62L appears downregulated also in CD56bright perforinneg NK cells infiltrating the tumors, this is likely to reflect a functional downregulation upon migration within tissues rather than a terminal stage of differentiation, as described for NK cells harbored in PB (8). Remarkably, as previously described in NSCL cancer (24), CD16 was consistently strongly downregulated in CD56dim perforinh high NK cells isolated from colon cancers.

Nevertheless, as similarly reported in NSCL cancers (24), NK cells infiltrating colon adenocarcinomas were competent in cytokine secretion but displayed an apparent reduced ability in the release of lytic granules, as assessed by IFN-γ production and CD107a surface expression upon coculture with the NK-sensitive K562 cell line (Supplemental Fig. 1). Further and more focused studies, beyond the aim of the present one, are needed to fully

**FIGURE 5.** Immunohistochemical localization of NKp46+/c-Kit+ cells in normal and cancerous lung, mammary and colon tissues. Immunohistochemical detection of NKp46+/c-Kit+ cells in normal lung tissue (a, d) and lung adenocarcinomas (b, c, e, f) with associated stromal lymphocyte aggregates (c). Double immunostaining with anti-NKp46 (red) and anti-c-Kit (brown) Abs shows the presence of rare NKp46+/c-Kit+ cells (dark red) (d) within the alveolar walls (insets show magnification of two dark red-stained NKp46+/c-Kit+ double-positive cells on the top left and one red-stained NKp46+ cell on the bottom right) in the healthy tissue. These cells were more frequently detected in the cancerous tissue, particularly in the stromal compartment (e) (insets in (e) show magnification of two dark red-stained NKp46+/c-Kit+ double-positive cells on the bottom right) and within the small lymphocyte aggregates (f) associated with lung cancer. (a–f) Original magnification ×400; insets in (d) and (e), original magnification ×1000. Immunohistochemical detection of NKp46+/c-Kit+ cells in normal breast tissue (g, i) and in breast cancer (h, j). Double immunostaining with anti-NKp46 (red) and anti-c-Kit (brown) Abs shows that NKp46+/c-Kit+ cells (dark red) [arrowhead in (i)] were rare to absent in the normal breast tissue, but easily detectable in the stroma of breast cancer (j) (g–j) original magnification ×400. Immunohistochemical detection of NKp46+/c-Kit+ cells in healthy colon (k, m) and in colon cancer (l, n). Double immunostaining with anti-NKp46 (red) and anti-c-Kit (brown) Abs shows a distinct infiltrate of NKp46+/c-Kit+ cells (dark red) both in the lamina propria of the normal colonic mucosa (m) and in the stromal compartment of a moderately differentiated colon adenocarcinomas (n) (k–n) original magnification ×400.
elucidate the functional properties of tumor-infiltrating NK cells and to confirm whether a reduced ability to degranulate might represent a general feature of tumor-associated NK cells.

**Discussion**

Most of the current knowledge on human NK cells has been derived from studies restricted either to PB analyses or to cells isolated from PB. As a matter of fact, information on NK cell distribution across human tissues was limited by methodological shortcomings, as earlier analyses often relied on the use of markers that were not adequately suited for the detection of NK cells (i.e., either not sufficiently specific for NK cells [e.g., CD57 and CD56] or expressed only by subsets of NK cells [e.g., CD16]). In addition, previous studies investigating NK cells in human tissues were, for the most part, not technically able to distinguish between the two main NK cell subsets (i.e., CD56bright/CD16low/neg/KIRneg NK cells [nontoxic] and CD56dim/CD16+ NK cells [cytotoxic]).

Given the substantial functional differences between these two main human NK cell subsets, the lack of information regarding the relative distribution of CD56bright and CD56dim NK cells has so far represented a major limitation for a comprehensive understanding of human NK cell biology as well as for their most advantageous exploitation in view of novel attempts of NK cell–based adoptive immunotherapies in both cancer and organ transplantation settings.

In this study, we showed that NK cells populate, and may recirculate through, most human peripheral tissues, and we provided an assessment of the amount of NK cells in the different tissues as well as of their subset distribution, although, because of minor blood contaminations, a partial bias of the NK cell assessment cannot be excluded for those tissues more abundantly perfused, such as liver and kidney.

Despite the fact that the array of chemotactic receptors expressed by NK cells has been previously elucidated (9), the role of different chemokines in guiding in vivo trafficking of NK cells through tissues had been poorly investigated. The meta-analysis of data employed in the current study offered us a unique opportunity for analyzing 18 chemokines produced in a large variety of human tissues, allowing a comprehensive identification of the chemokines potentially involved in NK cell recruitment and a comparison among different tissues, including malignancies, for the presence of factors that may specifically attract either one or the other of the two functionally distinct human NK cell subsets.

In this study, we analyzed an ample set of chemokine genes and derived our chemokine expression pattern from the most expressed ones, discarding the less expressed/undetectable chemokines from each single tissue. Our results are indeed supported by recent data obtained in the mouse system, in particular regarding the relevant role played by CXCL1 in the homing CD11bhigh CD27low NK cells to the normal lung tissue, because this mouse NK cell subset should represent the equivalent of human CD56dim NK cells (33, 34).

Data collected during this study indicate an association between gene expression level of chemotactic factors in the different tissues and the chemokine receptor expression of the NK cell subset infiltrating each individual tissue, thus emphasizing the role of locally released chemokines in the recruitment of NK cells toward the organs.

Although it has recently been shown that conventional NK cells represent the majority of CD56+ cells in most normal nonlymphoid tissues (23), interpretation of studies regarding NK cells in solid tissues might be complicated by the use of markers that did not allow the distinction of bona fide NK cells from other cells belonging to the growing population of ILCs. In our analyses, we employed a combination of markers specifically aimed at discriminating between NCR+ILC3 and NK cells, including CD94, a hallmark of human NK cells that is not expressed on ILC3 (21, 23). In addition, we safely gated on CD56+/perforinlow/high cells, carefully excluding CD56+ cells not expressing perforin, which might include other ILC subsets.

Another novel piece of information provided by this study is the finding that NK cells are present in human afferent lymph, thus implying that NK cells can recirculate from peripheral solid tissues to secondary lymphoid organs via lymphatic vessels, as recently
suggested in a bovine model of skin-draining lymph (35). We have previously reported that seroma fluids represent an accumulation of human afferent lymph consequent to a surgical interruption of lymphatic vessels draining lymph from interstitial spaces (26). We also recently demonstrated that this fluid can accumulate without major contamination by either surgery-induced exudate or leaky blood-derived cells (27), thus confirming the lymph-associated origin of the cells contained in seroma. Interestingly, CD56bright/CD16low/−/KIR−/− noncytotoxic NK cells were almost exclusively detectable in afferent lymph from seroma fluids and appear therefore able, similar to naïve T cells, to recirculate via afferent lymph. This is in agreement with their constitutive expression of CCR7, for which ligands are highly expressed in lymphatic vessel endothelium, LN, and other secondary lymphoid organs. Nevertheless, although CD56dim cytotoxic NK cells, like effector T cells, should not migrate via afferent lymph in steady state, it cannot be excluded that they might enter afferent lymphatic vessels upon locoregional tissue inflammation, because stimulation of human CD56dim NK cells with IL-18 can induce the expression of CCR7 on their surface (13). At the same time, immune reactions occurring in draining LN might convert prochemerin in its activated form, on their surface (13). At the same time, immune reactions occurring in draining LN might convert prochemerin in its activated form, which binds with high efficiency to l-selectin physiologic ligands on LN HEV. The identification of NK cells in human afferent lymph clearly indicates that HEV may not represent the only route for NK cell entrance into the LN. Moreover, having previously described a discrete amount of NK cells also in human afferent lymph (25), our current data now highlight, for the first time to our knowledge, that subsets of human NK cells, similar to T cells, can recirculate from peripheral tissues to PB through lymphatic circulation and secondary lymphoid organs.

We were also interested in investigating whether neoplastic transformation might affect NK cell recruitment in tissues, as suggested in previous reports (24, 39–41), and found, remarkably, that a large variety of human malignancies display a lymphocytic infiltrate highly enriched in CD56bright noncytotoxic NK cells. These results were confirmed by immunohistochemistry analyses performed on both healthy and cancer tissues of lung, breast, and colon. For these further analyses, we investigated on tissue sections whether NKp46+ cells expressed c-Kit, the receptor of stem cell growth factor, which is not expressed on the more differentiated cytotoxic NK cells (42). Histochemistry assays revealed that almost all NKp46+ cells in neoplastic, but not in healthy tissues, expressed c-Kit, confirming that cytotoxic NK cells are rare in cancerous tissues. Comparison of chemokine expression profiling data between healthy and matched neoplastic tissues showed that this different distribution of NK cell subsets is in agreement with a chemokine switch occurring upon neoplastic transformation. The production of chemokines able to preferentially recruit NK cells deprived of cytotoxic activity might represent a further mechanism of cancer immunoeediting with implications for both immunosurveillance and tumor escape from immune attack. The recent finding that fractalkine expression in breast cancer is associated with a better prognosis (28), together with our current data showing a downregulation of fractalkine expression upon neoplastic transformation of mammary tissues, are in line with this hypothesis.

Cytotoxic NK cells express on their surface CD16 (also known as FcγRIII), an FcR able to recognize IgG that are bound to the surface of target cells (43). Activation of FcγRIII by IgG causes the release of cytokines, such as IFN-γ, that signal to other immune cells, and cytotoxic mediators like perforin and granzymes that enter the target cell and promote cell death by triggering apoptosis. This latter process is known as Ab-dependent cell-mediated cytotoxicity and represents a major mechanism of action of several targeted therapies, currently employed in cancer patients, aiming to exploit NK cell Ab-dependent cell-mediated cytotoxicity by the administration of tumor-specific mAbs (44). Our current evidence that all cancer tissues analyzed were infiltrated by a large amount of noncytotoxic NK cells, which are devoid of FcγRIII (6, 25), should therefore be considered in planning mAB-mediated targeted therapies for the cure of solid tumors. Nevertheless, because it has been shown that CD56bright noncytotoxic NK cells can differentiate, at least in vitro, in FcγRIII+ killer cells upon activation-induced acquisition of lytic granules (11, 25, 45), the identification of factors able to induce this NK cell differentiation in vivo, as well as factors able to improve the chemotraction of CD56dim/perforinhigh cytotoxic NK cells into the tumors, should now be pursued and implemented in future strategies of NK cell–based immunotherapies for neoplastic diseases.

Another relevant outcome of this study is that CD56bright perforinlow noncytotoxic NK cells, which are almost negligible in PB, where NK cells have so far mainly been investigated, might probably outnumber CD56dim/perforinhigh NK cells in the human body by reason of their higher amount in several organs, including secondary lymphoid organs, liver, visceral adipose tissues, and gastrointestinal tract. If we consider that PB contains only ~2% of the total lymphocytes harbored in an adult human body (46, 47), the amount and relevance of this subset of noncytotoxic NK cells in the human system can be easily envisaged. The functional role of such an abundant noncytolytic, but cytokine-secreting, NK cell subset in solid organs remains to be fully clarified. It might play relevant immunoregulatory roles—for example, for T cell polarization—and indeed already described for NK cells harbored in secondary lymphoid organs (47–49) or being involved, as other ILC subsets, in tissue remodeling (50). However, because, unlike other ILC subsets, CD56bright/perforinlow NK cells can convert in perforinhigh NK cells able to kill virus-infected and cancer cells, cytokine-induced activation by, for example, IL-2 or IL-15 should be sufficient to induce natural cytotoxicity in these abundant NK cells harbored in solid tissues (11, 25). This should be taken into account in planning novel immunotherapeutic strategies for the cure of human diseases such as viral infections and cancer.

Disclosures
The authors have no financial conflicts of interest.

References


