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Peripheral Blood NK Cells from Breast Cancer Patients Are Tumor-Induced Composite Subsets

Emilie Mamessier,*† Lydie C. Pradel,*‡ Marie-Laure Thibult,*‡ Charlotte Drevet,† Atika Zouine,‡ Jocelyne Jacquemier,‡ Gilles Houvenaeghel,‡ François Bertucci,*‡ Daniel Birnbaum,*‡§ and Daniel Olive*‡§

Human NK lymphocytes are involved in antitumor immunity. The therapeutic potential of this population against cancers has stimulated their study and led to the discovery of several NK cell subsets, each of which is endowed with different immunoregulatory functions. We have previously reported that NK cell functions are profoundly altered in advanced breast cancer patients. In this study, we show that these tumor-mediated alterations also variably affect NK cell subsets. We found that in addition to the known human CD56dimCD16−, CD56brightCD16−, and CD56−CD16+ NK cell subsets, two additional subsets, namely the CD56brightCD16− and CD56dimCD16− subsets, were increased in the peripheral blood of patients with advanced invasive breast cancers. These subsets corresponded to the main two subsets found at the tumor site. The extensive phenotype of these subsets revealed an “à la carte” pattern of expression for the various NK receptors, functional molecules, adhesion molecules, and chemokine receptors, depending on the subset. We next compared these subsets to known NK cell populations endowed with specific phenotypic characteristics, but also with functional properties. Our data show that advanced breast cancer patients have an increased proportion of more immature and noncytotoxic NK cell subsets in their peripheral blood, which might account for at least part of the low cytotoxic functions observed in these patients. They reveal a major heterogeneity and plasticity of the NK cell compartment, which are both tightly linked to the microenvironment. The identification of NK cell subsets endowed with particular functional capabilities might help monitor residual antitumor NK cell-mediated responses in breast cancer patients. The Journal of Immunology, 2013, 190: 2424–2436.

Natural killer cells are innate immune cells specialized in the detection and clearance of “modified-self,” such as cancer cells (1). In peripheral blood (pB), NK cells comprise 10–15% of all lymphocytes. The NK cell phenotype is defined upon their expression of NCAM1 (CD56) and lack of expression of the T cell marker CD3 (2, 3). Among pB NK cells, two distinct populations of human NK cells were initially described in healthy individuals based on their cell surface density of CD56 and CD16, the low-affinity receptor for FcγRIII (4). CD56 may mediate interactions between NK cells and target cells (5), whereas the cross-linking of CD16 receptors is responsible for Ab-dependent cellular cytotoxicity, the most powerful way to initiate NK cell–mediated killing (6, 7). Two circulating major subsets have been described so far, based on the expression of CD56 and CD16: ~5–10% of NK cells are CD56brightCD16−, whereas most (~90–95%), the so-called CD56dimCD16+ NK cell subset, have low-density expression of CD56 and express high levels of CD16 (4).

During the past decade, a number of phenotypic and functional properties of these populations have been characterized. Both subsets have distinct immunoregulatory roles. CD56brightCD16− cells might be more immature cells and might have the ability to progressively differentiate into CD56dim cells, as they appear first after hematopoietic stem cell transplantation or IL-2–driven in vivo therapy (4, 8, 9). This theory was recently supported by looking at telomere length and at the monitoring of additional maturation markers such as CD94, CD62L, and CD57 (10–12). Gene expression profiling of purified nonactivated CD56bright CD16− and CD56dimCD16+ subsets showed major differences, indicating that these subpopulations should also be considered as phenotypically and functionally distinct subsets of mature human NK cells (13). The expression pattern of surface molecules and the genetic program of CD56brightCD16− NK cells allow them to rapidly proliferate, produce high levels of chemokines and proinflammatory Th1 cytokines, home to secondary lymphoid organs, and cross-talk with other innate and adaptive immune cells (14–16). CD56dimCD16+ NK cell functions are orchestrated upon a precise hierarchy with the engagement of surface receptors and notably a tight interaction with a given target that will favor immediate killing, whereas a loose interaction is sufficient to allow...
a rapid production of proinflammatory cytokines and chemokines (11, 17). CD56<sup>d+</sup>CD16<sup>+</sup> NK cells also have preferential homing ability for inflammatory sites. Finally, there is now convincing evidence showing that CD56<sup>d+</sup>CD16<sup>+</sup> cells can change their phenotypic properties and continue to further differentiate throughout their lifespan (18, 19). Additional minor NK cell subpopulations have been found recently to expand under specific pathological conditions, such as CD56<sup>dim</sup>CD3<sup>-</sup>CD16<sup>+</sup> NK cells in HIV- (20) and hepatitis C virus–infected patients (21–24) or CD56<sup>dim</sup>CD16<sup>-</sup> or CD56<sup>bright</sup>CD16<sup>-</sup> cells (25, 26). However, many questions still remain regarding the developmental state and functional relevance of these subsets in vivo (18, 27–30). The identification of NK cell subsets represents an important advance in our understanding of NK cell biology and has an important implication in the monitoring of NK cells in pathologic conditions. Indeed, these effectors may contribute to defenses against tumor cells or possibly reflect the extent of the tumor editing process occurring in situ.

We and others have reported that pB NK cells isolated from breast cancer patients display phenotypic and functional alterations that are more pronounced when the tumor is advanced (31). These alterations reflect those found within the tumor and are reversed upon remission (32, 33). Having demonstrated that pB NK cells were deficient at all functional levels in breast cancer patients, we thus decided to study whether those tumor-mediated alterations also affect NK cell subsets composition in relationship to breast tumor progression. We next compared each of these subsets to purified NK cell populations isolated from different compartments and with dedicated phenotypic and functional characteristics. Finally, we confirmed the phenotypic observations at the functional level and provide evidence showing that the tumor might be responsible for those alterations. This allowed us to confirm the status of the different subsets and to highlight the plasticity of the NK cell compartment in the context of a malignant microenvironment.

### Materials and Methods

#### Patients

Benign breast tumor and breast cancer patients treated at the Institut Paoli-Calmettes were prospectively recruited on diagnosis between January 2007 and December 2009. Blood and/or tumor were sampled before or during the surgical diagnostic or therapeutic act, before administration of any treatment related to tumor progression. Fresh samples were extemporaneously treated before the determination of the diagnosis. After analysis of morphological tumor characteristics by pathologists, the patients were retrospectively classified into four groups as follows (Table I): patients with a benign tumor (the B group), patients with invasive localized cancer (pT1N0 or pT2N0, referred to as the LOC group), patients with invasive locally advanced cancer (pT2N1–2 to pT4, referred to as the LA group), or patients whose initial breast tumor had given rise to metastases in distant organs (referred as the M group).

The study received the agreement of the Ethic Committee Review Board (Comité d’Orientation Stratégique, Marseille, France) from the Institut Paoli-Calmettes. Each patient gave written informed consent during the inclusion visit. Benign hyperplasia, tonsil, and bone marrow biopsies came from carefully informed patients who also gave written consent.

#### NK cell phenotype by flow cytometry

Two hundred microliters of fresh whole blood or 1 × 10<sup>6</sup> cells isolated from benign hyperplasia, healthy, or malignant mammary tissues and bone marrow were extemporaneously (within <2 h after blood puncture and/or tumor removal) incubated with the appropriate Abs (Supplemental Table I) on a rocking platform for 30 min. RBCs were lysed with OptiLyse B (Beckman Coulter). Samples were extemporaneously analyzed on a BD FACSCanto (BD Biosciences). Before and after analyzing the samples, fluorescence intensities from the FACSCanto were standardized over time with photomultiplier tube seven-color setup beads (BD Biosciences) to prevent fluorescence intensity variability related to external or intrinsic factors. The gating strategy consisted in eliminating doublets based on the forward scatter area/forward scatter height parameters, then selecting for the CD45<sup>+</sup>CD3<sup>-</sup>CD16<sup>+</sup> NK cells.

#### Generation of activated NK cells from pB samples

NK cells were negatively isolated from PBMCs with the NK cell StemSep system (StemCell Technology) according to the manufacturer’s instructions. The purity and viability of sorted cells were established and were

### Table I. Clinical features of the breast cancer patients included in the study at diagnosis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Benign Tumor</th>
<th>Localized Tumor</th>
<th>Locally Advanced Tumor</th>
<th>Metastatic Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>55</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>56</td>
<td>57</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Invasive margin</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sample uptake</td>
<td>Before surgery</td>
<td>Before surgery</td>
<td>Before surgery</td>
<td>Before therapy</td>
</tr>
<tr>
<td>Scarff–Bloom–Richardson grade</td>
<td>I</td>
<td>nd</td>
<td>37.0%</td>
<td>3.8%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>nd</td>
<td>38.8%</td>
<td>34.6%</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>nd</td>
<td>24.0%</td>
<td>53.8%</td>
</tr>
<tr>
<td>pN*</td>
<td>No</td>
<td>34.0%</td>
<td>81.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Median pT, mm</td>
<td>nd</td>
<td>26.4</td>
<td>57.1</td>
<td>50.0</td>
</tr>
<tr>
<td>Perivascular invasion</td>
<td>No</td>
<td>16.6%</td>
<td>26.9%</td>
<td>9.3%</td>
</tr>
<tr>
<td>Lymphoplasmocytic stroma</td>
<td>Absent</td>
<td>nd</td>
<td>37.5%</td>
<td>47.7%</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>nd</td>
<td>56.2%</td>
<td>38.1%</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>nd</td>
<td>6.3%</td>
<td>14.2%</td>
</tr>
<tr>
<td>Immunohistochemistry expression</td>
<td>RP*</td>
<td>nd</td>
<td>62.9%</td>
<td>53.8%</td>
</tr>
<tr>
<td></td>
<td>RE*</td>
<td>nd</td>
<td>75.9%</td>
<td>69.2%</td>
</tr>
<tr>
<td></td>
<td>ERBB2+</td>
<td>nd</td>
<td>7.4%</td>
<td>15.3%</td>
</tr>
<tr>
<td></td>
<td>RH ERBB2-</td>
<td>nd</td>
<td>18.5%</td>
<td>15.3%</td>
</tr>
<tr>
<td>Histological type</td>
<td>Ductal</td>
<td>nd</td>
<td>57.4%</td>
<td>65.4%</td>
</tr>
<tr>
<td></td>
<td>Lobular</td>
<td>nd</td>
<td>18.5%</td>
<td>19.2%</td>
</tr>
<tr>
<td></td>
<td>Tubular</td>
<td>nd</td>
<td>14.8%</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>nd</td>
<td>9.2%</td>
<td>15.3%</td>
</tr>
</tbody>
</table>

nd, Nondetermined; pN, % of invaded lymph nodes; pT, median tumor size; RP, progesteron receptor; RE, estrogen receptor.
always $>94\%$. NK cells were incubated 15 d in RPMI 1640/10% FCS complemented with IL-2 (1000 U/ml; Proleukin; Chiron) and PHA (1/1000; Life Technologies) on irradiated PBMCs used as feeder cells. A third of the media was replaced with fresh RPMI 1640/10% FCS complemented with IL-2 at days 6 and 10.

**NK cells isolation from mammary tissues**

A section made within the core of the removed tissue (healthy or malignant) was selected by the pathologist and extemporaneously cleared from pieces of fat, weighed (0.47 ± 0.31 g for healthy mammary tissue; 1.98 ± 1.47 g for breast tumor tissue), and an equal volume (w/v) of medium (RPMI 1640) was added to the tumor. The tumor was mechanically disrupted and then the supernatant was harvested and centrifuged at high speed to remove residual cellular elements. This supernatant, referred to below as the “supernatant of dissociation,” was aliquoted and frozen at $-20^\circ$C until further use. The mean volume “tumor supernatant of dissociation” was of 0.640 ± 0.420 ml. After mechanical disruption and the removal of the supernatant of dissociation, the tumor was digested for 1 h under agitation with collagenase Ia (1 mg/ml) and DNase I (50 kU/ml; Sigma-Aldrich).

**NK cells isolation from lymph nodes/tonsils**

A section made within the core of the removed benign lymph node or tonsil was selected by the pathologist and extemporaneously mechanically disrupted. Cell suspension was filtered and used for flow cytometry staining or NK cell isolation when viability was $>80\%$.

**Functional experiments**

NK cells were tested for cytotoxic activity against the leukemic HLA class I- K562 cell line (direct cytotoxicity). This test was performed in 4 h assays after overnight incubation of purified NK cells with IL-2 (100 U/ml) and IL-15 (5 µg/ml). The measured parameters were the degranulation (CD107a and CD107b) and cytokine production (IFN-γ and TNF-α) by NK cells or the percentage of dead K562 cells. The respective E/T ratios are indicated in the figure legends.

For flow cytometric experiments measuring the degranulation and cytokine production of NK cells, CD107a and CD107b mAb plus GolgStop, all purchased from BD Biosciences, were added in each well at the beginning of the culture. At the end of this incubation, NK cells were stained with CD45, CD36, and Live/Dead Aqua reagent (a viability marker; Invitrogen) and then permeabilized with Cytofix/Cytoperm (BD Biosciences). Intracellular Abs were next added (IFN-γ and TNF-α) before cell analysis.

For the study of alterations induced by breast tumor supernatants, purified NK cells were cultured with or without tumor supernatants (dilution 1/ 2) before staining 24 h later.

For the study of TGF-β1 involvement in breast tumor-mediated alterations, NK cells were cultured in the presence of breast tumor supernatants (dilution 1/4) preincubated with blocking TGF-β1 (20 µg/ml).

Finally, for the study of chemotaxis, we used Boyden chambers (5-µm pore) with breast tumor supernatant in the lower compartment and then added NK cell suspension (without serum) in the upper compartment. After 4 h incubation, we phenotyped NK cells in the top compartment.

**Statistical analyses**

All statistical analyses were done with Prism. NK cell subset proportions in the different patient groups were evaluated using the nonparametric unpaired Mann–Whitney U test. NK cell subset proportions in paired tumor and PB samples were compared with nonparametric Wilcoxon tests. Finally, the comparisons of marker expressions between each NK cell subset were performed using a parametric paired t test. The equality of the population medians among the different groups was triggered with the nonparametric ANOVA (Kruskal–Wallis) for each marker. A p value $<0.05$ was considered statistically significant.

**Results**

**Benign and malignant breast cancer patients have identical absolute numbers of PB NK cells**

The absolute numbers of lymphocytes per cubic millimeter of blood and the absolute numbers of PB NK cells per cubic millimeter of blood were not altered in breast cancer patients compared with the B group (Fig. 1A). The absolute number of lymphocytes per cubic millimeter of blood was lower in the M group than in the LOC and LA groups (Fig. 1B). However, because the percentage of PB NK cells was higher in M patients, the absolute numbers of PB NK cells between all breast tumors patients were similar. On a quantitative level, the PB NK cell compartment was not decreased in any of the patient groups compared with benign tumors.

**The proportion of PB NK cell subsets are altered in advanced stages of malignant breast cancer**

We looked at PB NK cell subsets based on their expression of CD56dim and CD16. We identified five NK cell subsets, namely CD56dimCD16+, CD56dimCD16−, CD56brightCD16−, CD56brightCD16+, and CD56−CD16−, whose proportions varied with disease progression. This was particularly evident in the LA and M groups (Fig. 2). At diagnosis, these patients displayed more CD56dimCD16− and less CD56−CD16− than did B and LOC patients. CD56dimCD16− and CD56brightCD16− circulating cells were also increased in the LA and M patients as compared with the B and LOC groups. The CD56−CD16− NK cell subset was not altered in any of the groups compared with patients with benign tumors.

**The proportion of PB NK cell subsets correlates with NK cell subsets infiltrating mammary tumors**

Because these modifications of PB NK cell subsets occurred in malignant conditions only, notably in patients with invasive breast cancers, we wondered whether they could be induced by the tumor. We thus looked at the composition of NK cell subsets within healthy and malignant paired mammary tissues (Fig. 3A–C). Malignant tissues had less CD56dimCD16− cells but more CD56−CD16− cells than did healthy mammary tissues ($p < 0.05$ for both) and PB ($p < 0.005$ for both). Percentages of
CD56\textsuperscript{bright}CD16\textsuperscript{+} and CD56\textsuperscript{bright}CD16\textsuperscript{−} were also increased in tumor compared with pB (\(p < 0.05\) for both) or with mammary tissue (\(p < 0.05\) for CD56\textsuperscript{bright}CD16\textsuperscript{−} only), but they were similar between healthy mammary tissues and pB (Fig. 3C). When looking at a larger cohort of patients (\(n = 15\)), we observed a positive correlation between the two major NK cell subsets that were found to be increased in mammary tumors, namely CD56\textsuperscript{bright}CD16\textsuperscript{+} and CD56\textsuperscript{dim}CD16\textsuperscript{+} subsets, and their respective counterparts in matched pB samples (Fig. 3D). This observation suggested that the variations of pB NK cell subsets might be related to NK cell affliction at the tumor site. These variations could either be explained by NK cells recirculating from the tumor or induced at a distance by the malignant environment. Because the extraction yield of NK cells from mammary tissue did not allow an extensive analysis of all tumor-infiltrating NK cell subsets, we studied these tumor-induced subsets from the pB of locally advanced and metastatic breast cancer patients.

### Table: NK cells subsets

<table>
<thead>
<tr>
<th>NK cells subsets</th>
<th>Patients groups</th>
<th>Mann&amp;Whitney T-Test between the different groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistics</td>
<td>B</td>
</tr>
<tr>
<td>CD56\textsuperscript{dim}CD16\textsuperscript{+}</td>
<td>Mean (Std. Dev)</td>
<td>87.3 (3.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B vs LA</td>
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<tr>
<td></td>
<td></td>
<td>B vs M</td>
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<tr>
<td></td>
<td></td>
<td>LOC vs LA</td>
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<tr>
<td></td>
<td></td>
<td>LOC vs M</td>
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<tr>
<td></td>
<td></td>
<td>LA vs M</td>
</tr>
<tr>
<td>CD56\textsuperscript{bright}CD16\textsuperscript{−}</td>
<td>Mean (Std. Dev)</td>
<td>5.8 (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B vs LA</td>
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<tr>
<td></td>
<td></td>
<td>B vs M</td>
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<tr>
<td></td>
<td></td>
<td>LOC vs LA</td>
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<td></td>
<td></td>
<td>LOC vs M</td>
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<tr>
<td></td>
<td></td>
<td>LA vs M</td>
</tr>
<tr>
<td>CD56\textsuperscript{bright}CD16\textsuperscript{+}</td>
<td>Mean (Std. Dev)</td>
<td>2.1 (1.7)</td>
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<td></td>
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<td>B vs LA</td>
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<td></td>
<td>B vs M</td>
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<tr>
<td></td>
<td></td>
<td>LOC vs LA</td>
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<td></td>
<td></td>
<td>LOC vs M</td>
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<tr>
<td></td>
<td></td>
<td>LA vs M</td>
</tr>
<tr>
<td>CD56\textsuperscript{dim}CD16\textsuperscript{+}</td>
<td>Mean (Std. Dev)</td>
<td>2.8 (0.9)</td>
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<td>B vs LA</td>
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<td>B vs M</td>
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<td>LOC vs LA</td>
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<td>LOC vs M</td>
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<td></td>
<td>LA vs M</td>
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<tr>
<td>CD56\textsuperscript{bright}CD16\textsuperscript{−}</td>
<td>Mean (Std. Dev)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B vs LA</td>
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<td>B vs M</td>
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<td>LOC vs LA</td>
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<td>LOC vs M</td>
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<tr>
<td></td>
<td></td>
<td>LA vs M</td>
</tr>
</tbody>
</table>

#### The expression of NK cell receptors differs depending on the NK cells subsets

We next studied the expression of specific receptors in each NK cell subset (detailed statistics are described in Supplemental Fig. 1). The mean fluorescence intensities of NKp30 and NKG2C activating receptors were similar on all NK cells subsets, not allowing their discrimination (Fig. 4A, Supplemental Fig. 1). NKp46, NKG2D, CD94, and NKG2A were highly expressed on CD56\textsuperscript{bright} CD16\textsuperscript{+} and CD56\textsuperscript{bright}CD16\textsuperscript{−} subsets, as compared with each NK\textsuperscript{dim} subset (\(p < 0.05\) for all comparisons), and they displayed a very close pattern regarding each of those receptors. These markers have a low to intermediate expression on CD56\textsuperscript{dim}CD16\textsuperscript{−} and CD56\textsuperscript{−} CD16\textsuperscript{+} cells (Fig. 4A). The strength of interactions between CD56\textsuperscript{bright}CD16\textsuperscript{−} cells with a target and the subsequent activation threshold might thus differ from the other three subsets (CD56\textsuperscript{dim}CD16\textsuperscript{−} and CD56\textsuperscript{−} CD16\textsuperscript{+} cells) in response to a given ligand.

In parallel, we evaluated the pattern of costimulatory molecules, but none of them clearly discriminated one subset from another based on their fluorescence intensities (Fig. 4B, Supplemental Fig. 1). Nonetheless, 2B4 intensity seemed to be stronger on CD56\textsuperscript{bright} CD16\textsuperscript{+} and CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cell subsets compared with CD56\textsuperscript{dim}CD16\textsuperscript{−} (\(p < 0.05\) and \(p < 0.0005\), respectively), whereas CD2 mean fluorescence intensity was lower on the CD56\textsuperscript{−} as compared with the CD56\textsuperscript{bright} subsets (\(p < 0.005\) for both subsets).

We next looked at killer cell Ig-like receptor (KIR) expression, which is associated with NK education and killing potential. Most CD16\textsuperscript{+} NK cells (CD56\textsuperscript{bright}CD16\textsuperscript{+}, CD56\textsuperscript{dim}CD16\textsuperscript{+}, and CD56\textsuperscript{−} CD16\textsuperscript{+} cells) expressed different combinations of KIR (all KIRs except KIR2DL4; Fig. 4C, Supplemental Fig. 1). Thus, part of these subsets have been educated and selected to exert a cytotoxic activity when required. The CD56\textsuperscript{dim}CD16\textsuperscript{−} subset expressed the highest level of KIR2DL4 (\(p < 0.05\) compared with all three CD16\textsuperscript{+} subsets), but few of the other KIRs (\(p < 0.05\) compared with CD56\textsuperscript{dim}CD16\textsuperscript{+}). The CD56\textsuperscript{bright}CD16\textsuperscript{−} subset could be distinguished from all of the other subsets by the lack of inhibitory KIRs and a very low percentage of KIR2DL4. Thus, NK cells displaying the CD56\textsuperscript{bright}CD16\textsuperscript{−} phenotype might display a lesser functional ability (Fig. 4C).

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**FIGURE 2.** Peripheral blood NK cell subsets in breast cancer patients with different stages of tumor progression. (Top) Percentages of NK cell subsets found in the pB of different breast cancer patient groups. NK cell subsets were defined by the surface expression of CD16 and CD56. (Bottom) Mean percentages (SD) of the NK cell subsets found in the blood of breast cancer patient groups and \(p\) values of the Mann–Whitney \(t\) tests performed between the different groups of patients.
In parallel, we monitored the expression of CD57. CD57+ NK cells are highly mature and more terminally differentiated NK cells. Compared to CD57− cells, they displayed higher cytotoxic capacity, higher sensitivity to stimulation via CD16, decreased responsiveness to cytokines, and decreased capacity to proliferate. CD57 was strongly expressed on CD56dimCD16+ and CD56brightCD16+ (p = 0.05 compared with both CD56brightCD16− or CD56−CD16+). CD57 expression on CD56dimCD16− was moderate to low, whereas CD56brightCD16− and CD56−CD16− displayed significantly lower levels of this molecule, suggesting that these last three subsets were less mature and differentiated than the previous ones (Fig. 4C, Supplemental Fig. 1).

NK cell subsets enriched in breast cancer patients are more immature and less functional NK cells

To explore the functions of NK cell subsets we measured the expression of molecules involved in NK cell-mediated cytotoxicity. CD56dimCD16− and CD56brightCD16− expressed lower levels of perforin and granzyme B than did CD56dimCD16+ and CD56brightCD16+ NK cell subsets (Fig. 5A, Supplemental Fig. 1). As expected, the levels of expression of these cytotoxic molecules correlated with the expression of CD57, reflecting NK cell cytotoxic potential (Fig. 4C).

Additionally, we looked at effector molecules of the TNF family involved in the killing activity of NK cells. LIGHT and CD95L were globally poorly expressed, whereas TRAIL and HVEM were expressed uniformly across all subsets (Fig. 5B, Supplemental Fig. 1). However, when we compared the expression of CD117 and CD25, we observed that these molecules, usually expressed on relatively immature peripheral NK cells, were increased on CD56dimCD16−, CD56brightCD16−, and CD56−CD16+ but not or poorly expressed on CD56−CD16− and CD56−CD16+ NK cells subsets. In particular, CD117 was significantly more expressed on CD56brightCD16− compared with CD56dimCD16− (p < 0.005) and CD56−CD16− (p < 0.0005) cells.

Finally, we also investigated CD25, one chain of the IL-2R, at the surface of the different subsets and observed that despite a low mean fluorescence intensity of this marker, CD25 was significantly more expressed on CD56bright and CD56dimCD16− cells than on CD56dimCD16− and CD56−CD16+ subsets. Knowing the role of IL-2 on NK cell activation but more importantly on cell proliferation, those subsets might be more prone to proliferate in an inflammatory context.

These results showed that the most represented subsets in advanced breast cancer patients had either the ones with the lowest level of cytotoxic molecules and/or the most immature phenotype, which might be able to expend locally, in the presence of IL-2. They suggest that breast tumor cells may be able to block or reverse NK cell maturation, favoring the emergence of noncytotoxic NK cells subsets.

NK cell subsets enriched in breast cancer patients can virtually home everywhere

We looked at the expression pattern of chemokine receptors (Fig. 6A) and adhesion molecules involved in NK cells homing (Fig. 6B) of each subset.
Concordant with the literature, CD56dimCD16+ cells displayed the highest intensity of CX3CR1 and CXCR1 receptors, in accordance with their ability to reach inflamed tissues in response to fractalkine and IL-8 gradients. The homing capacities of CD56dimCD16+ NK cells were similar to those of CD56dimCD16+ cells. However, because these two subsets were not the major subsets found within breast tumors, we hypothesized that, once recruited in situ, the tumor might alter NK cell phenotype in one of the other usually minor subsets.

Concordant with the enriched subsets found at the tumor site, CD56dimCD16− cells displayed a specific pattern as compared with other NK cell subsets, with a high expression of CXCR1 (and to a lower extent of CCR4 and CCR5), CXCR4, and CD62L, suggesting a preferential tropism for both inflamed tissues and lymph nodes.

CD56brightCD16− and CD56brightCD16+ subsets displayed similar patterns, with low to intermediate levels of most chemokine receptors expressed by NK cells, with the exception of CXCR1, indicating that they can home virtually everywhere in response to multiple stimuli. Furthermore, compared with other subsets, these cells expressed the highest detectable levels of CCR7, CD117, and CD62L, underlying a favorite tropism for secondary lymphoid organs.

Collectively, these data showed that NK cell subsets enriched in the pB of patients with advanced breast cancers can virtually come from and/or home both to the tumor site and the lymph nodes and affect the local immune response.

**Tumor-infiltrating NK cells are noncytotoxic NK cells**

Phenotype and function are correlated. We were thus interested in comparing the different NK cell subsets to phenotypically and functionally known populations of NK cells isolated from specific compartments. We used bone marrow NK cells, mostly composed of immature NK cells (34); NK cells from hyperplasia and tonsils composed of NK cells displaying 80% of CD56brightCD16− cells and known to exert helper function (strong cytokine production and cross-talk with other cells from the innate and adaptive immune system) (35, 36); NK cells grown on feeder cells in the presence of PHA and IL-2, representing strongly activated NK cells (37); and finally pB NK cells from healthy individuals, composed of >90% of CD56dim NK cells and representing the most mature circulating stage of NK cells.

We submitted all of these phenotypes to a clustering software (Fig. 7). Vertically, NK cells markers classified our subsets in three functional clusters, further designed as follows: “activated and homing cluster” (CD25, CD69, CD62L, CD31, CD27, and CCR7), “activation cluster” (all of the NCRs, DNAM, NKG2A and NKG2D), and “cytotoxic cluster” (CD16, KIRs, PERF, GZM and CD57). Horizontally, and from the left to the right, we observed that in vitro–stimulated NK cells, maintained on feeder cells, clustered with NK cells isolated from healthy mammary tissues (Fig. 7, A group). In this group, the three functional clusters displayed a similar pattern of expression. This suggested that NK cells that have reached the healthy mammary tissue have a strong functional potential, as well as the material required to exert cytotoxic function, if required. The B group comprised a very tight cluster of CD56dimCD16− and pB NK cells. Those two populations shared a similar expression pattern for most molecules found in the cytotoxic and activation clusters (all the cytotoxic cluster molecules, except...
NKG2A and NKp44). Interestingly, CD56 bright CD16+ cells displayed a profile closer to CD56 dim CD16+ cells regarding the cytotoxic and activation clusters (B group). However, they also upregulated receptors belonging to the “homing and maturation” cluster as well as NKG2A, two typical features of CD56 bright CD16+ cells (C group). Collectively, these observations suggested that the CD56 bright CD16+ subset is probably an intermediate subset between CD56 bright CD16+ cells and CD56 dim CD16+ cells.

The C group was composed of CD56 bright CD16+ cells and NK cells from hyperplasia and tonsils. They all displayed a profile deprived of molecules found in the cytotoxic cluster but expressed homing molecules, notably those related to lymph node homing, such as CD62L on CD56 bright CD16+ pB cells, which should allow them to get out of the vessel and reach the core of the lymph nodes. NK cells from hyperplasia and tonsil, which by definition are already in secondary lymphoid organs, expressed CCR7 allowing them to remain in situ. As expected (30), the profile of NK cells from pediatric tonsils had the particularity to express low levels of activating receptors compared with CD56 bright CD16+ cells and NK cells isolated from hyperplasia, suggesting that these cells are still immature compared with adult NK cells. Finally, NK cells isolated from mammary tumors were found in the C group, with a low correlation with the other subsets from this group. Despite the presence of some markers (NKp44, CD69) suggesting that these cells might have been activated at one point, allowing recruitment to the malignant tissue, they would not be able to exert cytotoxic function considering that all molecules of the cytotoxic and activation clusters are very low. In this line, we have previously reported (32) that the affliction of the activation cluster was correlated with the Nottingham prognosis index in breast cancer (38). This phenotype clearly contrasted with the one found on NK cells isolated from healthy mammary tissue, suggesting that the tumor can shape tumor-infiltrating NK cells populations, turning them into anergic or exhausted NK cells. The last cluster regrouped CD56 dim CD16+, CD56− CD16+, and bone marrow NK cells (D group). They all shared low expression of molecules found in the cytotoxic cluster, with the exception of CX3CR1, allowing homing to inflamed tissue, and extremely low expression of the activation cluster; they expressed some molecules of the homing and maturation cluster, confirming that these cells indeed have a strong trafficking potential, certainly to provoke peripheral maturation.

### Functional property of the different NK cells subsets

To further detail the functional properties of those NK cells subsets increased into patients’ pB, we sorted them (n = 6) based on their expression of CD56 and CD16. Unfortunately, we were only able to simultaneously purify four subsets from the same donor, and we chose not to isolate CD56− CD16+ NK cells that seemed to be less pertinent than the other subsets in the particular case of breast cancer.
As long detailed in the literature, we observed that CD56 dim CD16+ cells were the NK cell subset with the strongest cytotoxic activity against K562 cells, with a high degranulation potential as measured through the CD107 positivity (Fig. 8A, 8B). Conversely, CD56dimCD16 cells were poorly cytotoxic, produced low amounts of cytokines (IFN-γ and TNF-α; Fig. 8C and data not shown), and were poorly positive for CD107. CD56 brightCD16+ cells displayed higher cytotoxicity and degranulation efficiency than did CD56 dimCD16+ cells, efficiencies that nevertheless remained significantly lower than the ones observed in CD56 bright CD16+ cells. Indeed, CD56 brightCD16+ cells seemed to behave as an intermediary between CD56 brightCD16+ cells and CD56 dimCD16+ cells, showing the highest degranulation potential in association with strong cytokine production and good cytotoxicity against K562 cells. Collectively, these data highlight the fact that CD56bright/dim CD16 subsets were the less efficient to trigger cytotoxic functions against a malignant target, thus adding more evidence to the previous phenotype observations.

Tumor cytokine environment

Collectively, our data have established a link between modified NK cells subsets and the presence of the breast tumor. We thus wondered whether the tumor was able to preferentially recruit one (or more) specific subsets or induce those modifications. Using breast tumor supernatant at the bottom of a Boyden chamber, and NK cells in RPMI 1640 without FCS at the top, we let NK cells migrate and phenotyped cells that had not migrated in the bottom compartment of the chamber (Fig. 9A). If NK cell subsets had been preferentially recruited, the proportion of each subset might then be altered. However, we did not observe significant change in NK cell subset proportions, suggesting that all NK cell subsets have more or less the ability to migrate at the tumor site, or that the phenotype is induced by the tumor at distance, or that pB subsets are enriched with NK cells circulating from the tumor site after their modification locally (Fig. 9B).

However, and to clarify the link between the presence of the tumor and NK cell subsets, we decided to expose normal NK cells (isolated from pB) to breast tumor supernatants and looked at how this was affecting NK cells phenotype. We observed a decrease of CD16 mean fluorescence intensity due to breast tumor supernatant exposition ($p = 0.0039$), which was due to a significant increased of CD56 brightCD16+ and CD56 dimCD16+ subsets (Fig. 9C–E), in parallel to a loss of functionality (data not shown). Because breast tumor supernatants contain lots of pro- and anti-inflammatory molecules, including TGF-β1 (32), a major actor involved in the proliferation and differentiation of several cell types, we decided to investigate its role on NK cell subset generation. For this purpose, we preincubated the breast tumor supernatants with blocking TGFβ1 before adding it to NK cells and showed that at least part of the effect previously described was prevented (Fig. 9A–C).

Discussion

Recent studies of human diseases have revealed the existence of novel NK cell subsets displaying altered functions (24, 39). Differential expression of NK receptors by these subsets likely enables unique regulation of cytotoxic properties, tissue loca-
zation, and interaction with the local immune cells (4), and 2) might help understand NK cell ontogeny in humans in a given context. Contrary to initial thoughts, the NK cell population is highly heterogeneous and, with the increasing discovery of NK cell molecules, one can find nearly as many populations as marker combinations (40). Initially, human NK cells were divided into CD56dimCD16+ and CD56brightCD16− subsets, which since have been characterized extensively. CD56brightCD16− NK cells are highly proliferative, immature regulatory cells (41) and can interact with neighboring immunocompetent cells in lymphoid tissues (11). In contrast, CD56dimCD16+ NK cells home toward inflammatory sites where they can exert cytotoxic functions and promote immune responses (13). In this study, we have described three additional subpopulations, representing all possible cellular intermediates by using the CD56 and CD16 markers, present in various proportions, at steady-state in breast cancer patients.

CD56brightCD16+ cells, sometimes included in the CD56brightCD16− subset under the CD56bright denomination, have an intermediate maturation profile and are the first to emerge in patients undergoing hematopoietic stem cell transplantation, preceding the development of CD56dimCD16+ cells (9, 42). This subset has a strong antitumor activity (43), presumably because this subset

**FIGURE 7.** Representation of NK cell subsets compared with NK cell subpopulations isolated from various tissues or activated in vitro. Mean expression of NK cell receptors expressed on activated or ex vivo NK cell populations isolated from different organs include in vitro–activated NK cells (Ac-NK cells), blood (p-NK cells), lymph node (HP-NK cells), tonsil (To-NK cells), healthy mammary tissue (Mt-NK cells), malignant mammary tumor (Ti-NK cells), and bone marrow (BM-NK cells) NK cells. These already well-known subpopulations were submitted to clustering software together with the expression profiles corresponding to the five NK cell subsets defined in this study (CD56dimCD16+, CD56brightCD16+, CD56brightCD16−, CD56dimCD16+, CD56brightCD16−). The horizontal cluster classified NK cell subsets according to profile similarity within NK cell subpopulations/subsets. The vertical cluster identified three clusters allowing functional/maturational differentiation within subpopulations/subsets after normalization of the values by rows.

**FIGURE 8.** Function of sorted NK cell subsets in vitro. NK cells were sorted based on their expression of CD56 and CD16. Each subset was tested against the leukemic HLA-I K562 cell line in direct cytotoxicity assays. This test was performed in 4-h assays after overnight incubation of purified NK cells with IL-2 (100 U/ml) and IL-15 (5 μg/ml). The measured parameters were the percentage of dead K562 (A), degranulation (CD107a and CD107b) (B), and IFN-γ production (C). The respective E/T ratios were 1:1. Statistical analysis was performed using a nonparametric paired t test (Wilcoxon). *p < 0.05.
expresses strong levels of NKG2D, as well as natural cytotoxicity receptors and, as we have shown, molecules related to cytotoxicity. Bacterial compounds could induce their proliferation, a specific feature shared with CD56brightCD16− cells, suggesting a role in host defense (44). Altogether, this subset appears as an intermediate stage between CD56brightCD16− and CD56 dimCD16+ cells.

CD56dimCD16+ cells are unconventional NK cells that vigorously proliferate in response to cytokines involved in NK cell maturation, such as IL-2, IL-15, or IL-21. Similar to CD56bright CD16− NK cells, CD56dimCD16+ NK cells can produce large amounts of IFN-γ and FAS ligand when stimulated (26). However, in the case of breast cancer, we found that this subset, prominent at the tumor site, expressed low levels of activating receptors and low amounts of cytolytic molecules, clearly indicating its inability to exert a cytotoxic activity or even to get activated in response to any target encounter. We reported elsewhere that the exposition of normal NK cells to soluble factors produced by the tumor or its stroma induces the decrease of cytokine production, activating and cytotoxic molecules, certainly through the local metalloproteases and/or other inhibitors secreted by tumor cells (32). All of these observations suggest that these cells are noncytotoxic, immature, and may be even exhausted or anergic NK cells. Additionally, because of the CD16+ phenotype, this subset should be devoid of the ability to perform Ab-dependent cellular cytotoxicity functions, including therapeutic Ab-mediated effects.

The presence of CD56−CD16+ NK cells was reported in virus-infected patients and in healthy cord blood (25, 45). In both cases, these highly dysfunctional cells share a similar phenotype but remain poorly characterized (24). We showed that this subset was close to the CD56 dimCD16− subset and NK cells isolated from bone marrow, confirming a relative immaturity and a poor antitumor potential. However, we did not find this subset infiltrating the tumor or being affected in the periphery, re-enforcing its relation to antivirus immune reaction.

The developmental relationships between those five NK cell subsets remain controversial. TGF-β can induce the conversion of a minor fraction of pB CD56brightCD16+ cells into CD56bright CD16− cells, highlighting a possible role of the former as a developmental intermediate and TGF-β as a factor able to influence NK cell ontogeny. TGF-β can indeed repress the development of NK cells from CD34+ progenitors and inhibit their differentiation in more terminally differentiated CD56dimCD16+ NK cells (46). Additionally, we have shown that TGF-β rather promotes CD16−
NK cells subsets over the CD16. Of course, other cytokines can promote NK cell differentiation, pushing CD56_{bright}CD16- NK cells to develop into CD56_{bright}CD16+ NK cells and then further into CD56_{bright}CD16- NK cells (26). Our data demonstrated that pB CD56_{bright}CD16+ NK cells shared some features of CD56_{dim} CD16+ NK cells, notably a strong expression of KIR and other cytotoxic molecules, such as perforin and granzyme B. Such a phenotype is inducible in CD56_{bright}CD16- NK cells upon cytokine activation or during inflammation (10). The five NK cell subsets described in this study thus appear as bona fide sequential steps of NK cell ontogeny.

We also described alterations of circulating NK cell subsets in advanced breast cancer patients, notably an increased proportion of CD56_{bright}CD16- and CD56_{dim} CD16- subsets. These subsets are usually poorly represented in the pB of healthy donors. The predominant subset, CD56_{dim}CD16+, was decreased in the blood and tumor of advanced breast cancer patients, but we could not determine whether it was either through their own decrease or the increase of CD56_{bright}CD16- and CD56_{dim}CD16- cells. Both events might be possible. Indeed, specific expansion of circulating CD56_{bright}dimCD16- NK cells has been reported in metastatic melanoma (47); this increase was associated with elevated plasma TGF-β1. Large amounts of TGF-β1 and other major inhibitors are secreted at the mammary tumor site (48) and are consequently detected in the plasma of metastatic breast cancer patients (49). Modifications of NK cell subsets might thus be induced directly at the tumor site or at distance. However, it is also plausible that de novo NK cells are more immature because of a defect in the maturation process, as reported in metastatic breast cancer (31). Finally, CD56_{dim}CD16- NK cells are preferentially targeted for apoptosis, leading to low levels of NK activity in many cancers, including breast cancer (50, 51). Most probably, several mechanisms, all reported at least once in advanced breast cancer patients, affect NK cell maturation. This alteration may reflect tumor progression and/or residual antitumor immunity (52).

We found that the increased peripheral subsets correlated with NK cell subsets were enriched at the tumor site but not in normal tissues. This observation raises the question of the plasticity, modularity, and reversibility of NK cell maturation in a specific environment (4, 53). We found that NK cells isolated from healthy mammary tissues resemble in vitro-activated NK cells, known to have a strong antitumor cytotoxic potential (see Fig. 7, cluster A), suggesting they have indeed continued their maturation into an effector subset as compared with most pB NK cells. In contrast, the tumor’s microenvironment may inhibit NK cells maturation to avoid NK cell-mediated cytotoxicity and induce its tolerance (35). NK cell developmental programming is not entirely fixed, and mature NK cells can be re-educated by their environment (54–56). Indeed, we found that tumor-infiltrating NK cells did not cluster with healthy mammary NK cells, but clustered between CD56_{bright}CD16- (see Fig. 7, cluster C) and the more immature NK cell subsets (cluster D). This was mainly related to two particular subsets representing ~70% of malignant mammary tumor cells, that is, the CD56_{dim}CD16- subset and the CD56_{bright}CD16- subset. TGF-β seems to be at least partially involved in this event. Such a phenotype was also reported in other cancers, for example, in non-small cell lung cancer (50). We know that the acquisition of a mature phenotype can occur independently of NK cell education, in the periphery, by interactions with self-HLA class I ligands, and is an essential part of the formation of human NK cell repertoires (19, 57). However, in this case, the phenotype of tumor-infiltrating subsets is not simply related to the acquisition of a mature phenotype specific to a tissue location, as differentiation is described by the loss of expression of NKG2A, sequential acquisition of inhibitory KIRs and CD57, and change in their expression patterns of homing molecules (19), whereas we observed the opposite in tumor-infiltrating NK cells as compared with NK cells isolated from healthy mammary tissues (32). We reported that those tumor-infiltrating NK cells have a phenotype that resembles noneducated cells, rather suggesting that the tumor environment may reverse or induce a different transcriptional program of NK cell maturation to generate nonreactive “self”-tolerant NK cells (32, 58, 59). Finally, we demonstrated that these altered NK cells subsets can virtually home everywhere according to their chemokine profile, suggesting that these self-tolerant NK cells did not promote cytokine cross-talk between NK cells and T cells in secondary lymphoid organs and/or convey self-tolerance acquisition to adaptive immunity (60). Breast cancer cells have acquired the ability to divert a mechanism used to generate diversity in NK cell repertoire to escape from NK cell–mediated antitumor immunity.

In conclusion, the potential of NK cells in immune-based therapy for the treatment of malignant diseases has become clear. Additionally, our study highlights that the precise identification of NK cell subsets endowed with particular functional capabilities or specific maturation stages should be of great help to monitor antitumor NK cell–mediated responses in periphery and to determine how far the immuno-editing process has already gone in a given patient. The extensive heterogeneity and the plasticity of NK cell subsets, which appear to be dependent on the microenvironment, should be considered as an advantage in patient monitoring because these cells should be amenable to modulation. This strongly suggests that understanding NK cell educational processes is of major importance to design future therapies based on anti-tumor immunity.

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References


