

Human Breast Tumor Cells Induce Self-Tolerance Mechanisms to Avoid NKG2D-Mediated and DNAM-1-Mediated NK Cell Recognition

Emilie Mamessier¹, Aude Sylvain¹, François Bertucci^{1,3}, Rémy Castellano^{1,2}, Pascal Finetti³, Gilles Houvenaeghel³, Emmanuelle Charaffe-Jaufret^{1,3}, Daniel Birnbaum^{1,3}, Alessandro Moretta⁴, and Daniel Olive^{1,3}

Abstract

Breast cancer is the leading cause of death for women between the ages of 35 to 65. This is mostly due to intertumor heterogeneity and the lack of specific therapies for all subtypes. However, some breast cancers with an unexpected good prognosis are associated with enhanced antitumor immunity *in situ*. We studied whether breast cancer subtypes might have different susceptibilities to natural killer (NK) cells' antitumor immunity. We collected a large public set of microarray data for primary breast tumors and determined NK cell ligand expression. We found that despite heterogeneous levels of inhibitory HLA members, NKG2D ligands and DNAM-1 ligands are expressed in virtually all breast tumor subtypes. Functional experiments in breast cancer subtypes expressing various levels of NK cell ligands showed that NK-mediated cytotoxicity is mainly HLA, NKG2D, and DNAM-1 dependent. In parallel, we showed that cell lines and primary breast tumor cells secrete soluble inhibitory factors that alter NK cell functions. Finally, we showed that these mechanisms of escape occur *in vivo* in the MMTV-Neu model of spontaneous murine breast cancer. Our study shows that breast cancer cells, independent of the subtype, have developed different mechanisms to escape from NK cells' antitumor immunity. These results emphasize the role of NK cells in breast tumor clearance and underlie the importance of devising future therapy aiming at enhancing NK cell-mediated recognition in parallel with the prevention of the tumor-editing process. *Cancer Res*; 71(21); 6621–32. ©2011 AACR.

Introduction

Breast cancer remains the leading cause of death for women of 35 to 65 years old (1). Gene expression studies have shown the heterogeneity of the disease and identified at least 5 clinically relevant molecular subtypes of breast tumors: luminal A, luminal B, ERBB2, basal, and normal-like (2). These subtypes are associated with distinct gene expression patterns and, more importantly, with different clinical outcome. Sub-

types are likely to reflect alterations in specific cellular pathways and/or different cell-of-origins (3). Luminal A and B tumors are of relatively good prognosis and benefit from hormone therapy. The normal-like subtype is of intermediate prognosis but remains poorly defined otherwise. ERBB2 tumors have a poor prognosis but greatly benefit from anti-ERBB2 therapy. Basal breast cancers are highly proliferative tumors of poor prognosis, which frequently do not express hormone receptors and ERBB2 and cannot benefit from any available targeted therapy. However, recent data have shown that basal breast cancers with increased expression of gene associated to immune response displayed better prognosis than their counterparts (4–6).

The importance of antitumor immunity in the outcome of basal breast cancer is now recognized. However, to date, nothing is known about the different susceptibility to antitumor immunity of the different breast cancer subtypes, as well as about the mechanisms involved. Among the actors of antitumor immunity, natural killer (NK) cells are the immune cells specialized in host defense against transformed cells (7, 8). This is due to an innate ability to distinguish normal from "modified" cancer cells (9). This recognition process is controlled by a broad range of activating and inhibitory receptors, acting as sensors of the "malignant-self" (10, 11). The most important activating receptors are the natural cytotoxicity receptors (NCR3 or NKp30, NCR1, or NKp46) and KLRK1

Authors' Affiliations: ¹Centre de Recherche en Cancérologie de Marseille, and ²TrGET, Centre de Recherche en Cancérologie de Marseille, INSERM UMR U891; ³Institut Paoli-Calmettes, Marseille, France; and ⁴D.I.M.E.S., University di Genova, Genova, Italy

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A. Sylvain and F. Bertucci contributed equally to this work.

Current address for E. Mamessier: Laboratory Genomic Instability and Human Hemopathies Centre d'Immunologie Marseille-Luminy (CIML), Parc Scientifique de Luminy, 13288 Marseille, France.

Corresponding Author: Emilie Mamessier, Laboratory Genomic Instability and Human Hemopathies Centre d'Immunologie Marseille-Luminy (CIML), Parc Scientifique de Luminy, 13288 Marseille, France. Phone: 33-4-91-26-94-73; Fax: 33-4-91-26-91-69; E-mail: mamessier@ciml.univ-mrs.fr

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(NKG2D). They recognize ligands specifically expressed by malignant and/or stressed cells (Supplementary Data S1). NKp30 has 2 recently discovered ligands, B7H6 and BAG6 (BAT3), which are restricted to malignant tissues (12). Except for the HA-(hemagglutinin) antigen expressed on virus-infected cells, nothing is known about the malignant ligands of NKp46 that may be involved in the recognition of several malignancies (13). NKG2D binds MICA, MICB, and the members of the ULBPs family; these ligands are frequently involved in antitumor immunity and, consequently, also used by the tumor-editing process (14). In parallel, NK cells express inhibitory receptors, whose main role is to prevent the onset of autoimmune reactions. These are the killer immunoglobulin receptors (KIR) and KLRC1 (NKG2A) receptors (15, 16), which recognize members of the MHC class-I family expressed by normal cells. Finally, the signal could be fine-tuned by coactivators, such as CD226 (DNAM-1), and coinhibitors, such as CD276 (B7-H3). DNAM-1 binds the receptors PVR and PVRL2/Nectin-2/CD112, whereas the ligand of B7-H3 is unknown. The integration of these opposing signals determines whether a given NK cell will eliminate or not a potential target. Once recognized, the target cells are eliminated through the release of cytotoxic enzymes (perforin, granzyme) and/or soluble factors (IFN- γ , TNF- α , and chemokines) that recruit other effector cells.

In this study, we studied the interrelations between NK cells and breast tumor cells from different subtypes. This work is in line with other recent works setting the basis to devise future immunotherapy enhancing NK cells functions in breast tumors (17, 18). We observed that in return breast cancer cells decrease NK cells cytotoxic activity through the acquisition of different mechanisms of escape. These should also be targeted to restore NK cells cytotoxicity against solid breast tumors.

Material and Methods

Breast cancer gene expression data sets

Gene expression profiles of primary breast tumors ($n = 352$) and healthy mammary tissue ($n = 4$ pools of 4 healthy mammary tissues), established by using whole-genome U133 Plus 2.0 Affymetrix oligonucleotide microarrays, were downloaded from the public GEO datasets GSE21653 (6). We also used whole-genome gene expression data of breast cancer cell lines from our laboratory (19). The Robust Multichip Average (RMA) with the nonparametric quantile algorithm was used as normalization parameter. Quantile normalization or RMA was done in R using Bioconductor and associated packages. mRNA values of NK cell receptor ligands in tumors (Supplementary Data S2) were extracted, \log_2 transformed and mean centered on the respective value found in healthy tissue, before submission to a hierarchical clustering software (Cluster, using average linkage clustering of the arrays) and results were visualized with Treeview. Breast cancer molecular subtypes were defined by using the SSP method (20).

Breast cancer cell lines

All cell lines (MCF7, SK-BR-3, SUM225, BrCa-MZ-01, SUM159, and MDA-MB-157) were purchased from American

Type Culture Collection. Main features of these cell lines were checked and summarized in Supplementary Data S3. Breast cancer cell lines were phenotyped for the main NK cell ligands. HLA molecules were detected with a pan-HLA-ABC-FITC (fluorescein isothiocyanate) antibody (Beckman Coulter), B7-H3 with a anti-human B7-H3-PE antibody, NKp30-Ligand (-L) with NKp30-Fc recombinant protein, DNAM-1-L with an DNAM-1-Fc recombinant protein, and NKG2D-L with an NKG2D-Fc recombinant protein or ULBPs antibodies (all from R&D system) or anti-MICA/B antibody (BD Biosciences). Briefly, cells were saturated in PBS 5% BSA before incubation for 30 minutes on ice with the appropriate antibody. Staining involving Fc-proteins were revealed using an anti-human-Biotinylated monoclonal antibody (mAb), followed by a Streptavidin-PE mAb. Samples were extemporaneously analyzed on a BD FACS Canto (BD Biosciences).

Healthy donors

Peripheral blood from healthy donors was obtained from the "Etablissement Français du Sang" (EFS). The EFS established the HLA-C allotype of donors and breast cancer cell lines.

Patients

Patients with breast cancer treated at the Institut Paoli-Calmettes were prospectively recruited on diagnosis between January 2007 and December 2009. Blood and/or tumors were, respectively, sampled before or during the surgical diagnostic. Fresh samples were extemporaneously treated. After analysis of morphologic tumor characteristics by pathologists, patients were retrospectively classified into 3 groups on the basis of presence or not of estrogen and progesterone receptors and or ERBB2 amplification by FISH, as follows: luminal tumors (Luminal), ERBB2-positive tumors (ERBB2⁺), and basal tumors (Basal). This study received the agreement of the Institutional Ethic Committee Review Board (Comité d'orientation Stratégique, COS Marseille, France) from the Institut Paoli-Calmettes. During the inclusion visit, each patient gave a written informed consent for research use.

See supplementary Material and Method for NK cell isolation and breast tumor processing.

Analysis of NK cytotoxicity by flow cytometry

Allogeneic NK cells were tested for direct cytotoxic activity against the leukemic HLA class-I-negative K562 cell line or HLA-C-matched breast cancer cell lines. CD107-FITC antibodies and Golgistop (BD Biosciences) were added at the beginning of the culture. After 4 hours at 37°C, NK cells were labeled, fixed, and permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) before adding intracellular antibodies (IFN- γ PE from Beckman Coulter and TNF- α -APC from BD Biosciences). Evaluation of NK cells cytotoxicity was done in TrueCount beads tubes (BD Biosciences) allowing the absolute quantification of dead cells with the LiveDead-Red reagent (Invitrogen).

⁵¹Cr experiments

Blocking mAbs directed against NK cell receptors or related ligands were produced in Pr Moretta's laboratory: anti-NKp46

(KL247, IgM), anti-NKp30 (F252, IgM), anti-DNAM-1 (F5, IgM), anti-NKG2D (BAT221, IgG1), anti-B7-H3 (5B14, IgM), and anti-HLA class-I (A6-136, IgM). NK cells were incubated at various effector:target (E:T) ratios (20:1, 10:1, and 5:1), in triplicate, with ^{51}Cr -labeled breast tumor cell lines, in the presence of blocking mAbs or saturating concentrations of appropriate isotype controls. Four hours later, supernatants were harvested and assayed for the release of ^{51}Cr . Spontaneous and total lysis were measured by incubating target cells in the absence of NK cells and in the presence of NP40, respectively. The percentage of specific lysis was measured as follows: $(\text{experimental lysis} - \text{spontaneous lysis}) / (\text{maximum lysis} - \text{spontaneous lysis}) \times 100$.

Phenotypic alterations of NK cells by breast tumors

Peripheral blood NK cells were coincubated with breast cancer cell lines or primary breast cancer cells, either directly or using bicameral chambers (Transwell, 5 μm diameter), using a 2:1 E:T ratio. After 48 hours, NK cells were harvested and phenotyped for NK cell receptors (NKG2D, DNAM-1, and NKp30).

NK cell phenotype of breast cancer patients

Hundred microliters of fresh whole blood were incubated with the appropriated antibodies (NKp30, NKp46, NKG2D, and NKG2A all from Beckman Coulter and DNAM-1 from BD Biosciences) on a rocking platform for 30 minutes. Red blood cells were lysed with OptiLyseB (Beckman Coulter). Samples were extemporaneously analyzed on a BD FACS Canto (BD Biosciences). The gating strategy consisted in the elimination of the doublets based on the FCS-A/FCS-H parameters then the dead cells. NK cells population was selected based on the following phenotype: $\text{CD45}^{\text{pos}}\text{CD3}^{\text{neg}}\text{CD56}^{\text{pos}}$.

Functional alterations of NK cells by breast tumor supernatants

NK cells were activated overnight in IL-2 (100 U/mL; Chiron) and IL-15 (5 $\mu\text{g}/\text{mL}$; R&D Systems), then incubated with supernatants of breast cancer cell lines or primary breast cancer for 24 hours. NK cells were then used in a functional CD107 assay against K562 cells.

The inhibitory effect of breast supernatants was investigated using blocking antibodies against PGE2 (5 $\mu\text{mol}/\text{L}$), IDO1 (1 mg/mL), LGALS3 (5 mmol/L), IL-10 (5 $\mu\text{g}/\text{mL}$), MICA/B (2.5 $\mu\text{g}/\text{mL}$), TGF- β 1 (5 $\mu\text{g}/\text{mL}$), and IL1RA (20 $\mu\text{g}/\text{mL}$) preincubated for 30 minutes with breast tumor supernatant before exposition to NK cells.

MMTV-Neu mice

FVB/N-Tg(MMTVneu)202 Mul/J mice (MMTV-neu mice) were obtained from the Jackson Laboratory. All mice were maintained on an FVB background. Mice were palpated weekly beginning at 5 months of age and, once mammary gland nodules were detected, tumor diameter was measured twice weekly with calipers. Mouse mammary tumor virus (MMTV)-Neu mice ($n = 6$) and FVB control mice ($n = 6$) were compared and a Kaplan–Meier analysis was done to determine tumor-free survival. Monthly blood samples

were collected under isoflurane (5%) inhalation anesthesia and analyzed by flow cytometry for NKG2D and DNAM expression (both antibody were purchased from E-bioscience). At the end of the protocol, mice were sacrificed and breast tumors, peripheral blood, and lymph nodes were collected. NK cells were isolated with EasySep Mouse NK cell Enrichment Kit (StemCell Technologies) according to manufacturer's instructions, then cultured overnight in rIL-2 (R&D system). NK cells were then used in a functional CD107 assay against RMA H2–negative cell line (E:T ratio of 2:1) to evaluate their killing ability. The CD107 assay was done as described in the "Analysis of NK cytotoxicity by flow cytometry" section, except that all antibodies were purchased from e-Bioscience. All animal procedures were done in accordance with protocols approved by the local Committee for Animal Experimentation (CAE) of Marseille, France.

Statistical analyses

All statistical analyses were done with StatView software. Differences between nonexposed and exposed NK cells were evaluated using 2-tailed nonparametric paired Wilcoxon *t* test. The differences between breast patients and controls were evaluated with a 1-way ANOVA and Dunn multiple comparison tests. Differences between FVB and MMTV-Neu mice were evaluated with the 2-tailed nonparametric unpaired Mann and Whitney *t* test. In all figures with histograms, data are represented using the mean value \pm SEM. Only *P* values inferior to 0.05 were considered as significant.

Results

Breast tumor cells express mRNA ligands for both activating and inhibitory NK cell receptors

We extracted the mRNA expression values of 15 known ligands of NK cell receptors from a public DNA microarray database of breast cancers, including 98 luminal A, 64 luminal B, 37 ERBB2, 43 normal-like, 110 basal tumors, and 4 healthy (pool of 4 each, i.e., 16 healthy donors) mammary tissues. mRNA values were mean centered on healthy tissues (Fig. 1A), allowing for a measure of relative expression of these genes in breast cancer subtypes compared with healthy mammary tissues. The expression of HLA class-I transcripts tended to be lower in luminal subtypes, notably HLA-C ($P = 0.0014$), whereas HLA-G expression was decreased in all subtypes ($P = 0.003$). The expression of B7-H3 was upregulated in some tumors compared with healthy mammary tissue, but without correlation to any subtype.

With regard to activating ligands, all types of tumors expressed at least 1 ligand for NK cells activator receptor. Some ligands, such as MICB ($P = 0.002$) or B7-H6 ($P = 0.01$) were upregulated in most tumors compared with healthy mammary tissues, independently of the subtype. ULBP2 ($P = 0.0087$), PVR ($P = 0.02$), and BAG6 ($P = 0.045$) were specifically upregulated in basal tumors, whereas PVRL2 was upregulated in luminal cases ($P = 0.0017$). ULBP-1 and ULBP-3 expression in tumors was not different from healthy mammary tissues.

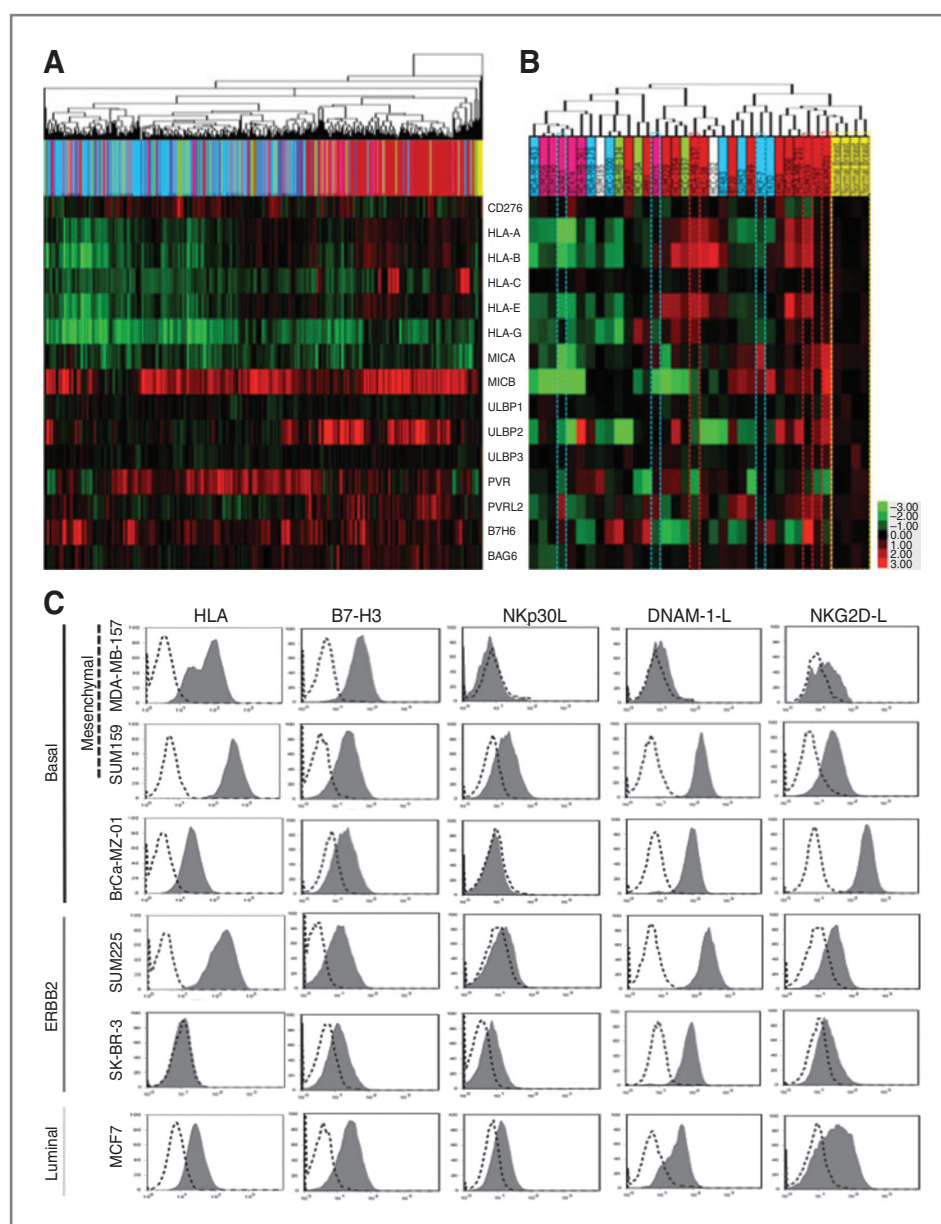


Figure 1. mRNA and protein expression of NK cell receptor ligands in primary breast cancers and breast cancer cell lines. A, hierarchical clustered representation of ligand expression in tumors ($n = 352$; A) and in breast cancer cell lines (B). The expression level of each gene was mean centered on the expression in healthy mammary tissues and is depicted according to a color scale (\log_2 scale) shown at the bottom. Red and green indicate expression levels, respectively, above and below the median. The magnitude of deviation from the median is represented by the color saturation. The dendrograms of samples (above matrix) represent overall similarities in gene expression profiles. The molecular subtypes are indicated under the dendrograms: Healthy mammary tissues ($n = 4$ pools of 4 tissues) are represented in yellow, basal ($n = 110$) tumors in red, ERBB2-positive tumors ($n = 37$) in pink, normal-like tumors ($n = 43$) in green, luminal-B tumors ($n = 64$) in dark blue and luminal-A tumors ($n = 98$) in turquoise. In cell lines, luminal A and B tumors cannot be distinguished and are grouped in blue. C, surface expression of the main NK cell ligands expressed on breast cancer cell lines were measured by flow cytometry. HLA molecules were detected with a pan-HLA-ABC antibody, B7-H3 with an anti-human B7-H3 antibody, NKp30-Ligand(-L) with NKp30-Fc recombinant protein, DNAM-1-L with a DNAM-1-Fc recombinant protein and NKG2D-L with an NKG2D-Fc recombinant protein. On each histogram, dashed lines represent the isotype controls and the shaded line the marker of interest.

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Altogether, these mRNA profiles suggested that NKG2D ligands and DNAM ligands might be the most common ligands for NK cells antitumor immunity in all the breast tumor subtypes and, particularly, in luminal breast tumors that also expressed lower level of inhibitory HLA members receptors.

The same analysis was done on breast cancer cell lines (Fig. 1B). As with primary breast tumors, breast cancer cell lines were highly heterogeneous with regard to NK cell ligands expression. For further analyses, we selected cell lines that illustrated the heterogeneity of the subtypes found *in vivo*: luminal (MCF7), ERBB2 (SK-BR-3, SUM225), and basal (BrCa-MZ-01, SUM159, and MDA-MB-157; ref. 19) and that expressed different levels of transcripts of ligands for NK receptors.

Breast tumor cells express protein ligands for both activating and inhibitory NK cell receptors

In the selected cell lines, we measured the effective protein level of the main NK cells antitumor ligands by flow cytometry (Fig. 1C). HLA class-I molecules were expressed on all cell lines, except for SK-BR-3. B7-H3 expression was present on all cell lines at a low-to-medium level. NKG2D-L expression was heterogeneous, ranging from dull (SK-BR-3) to high level (BrCa-MZ-01). Ligands for the coactivating receptor DNAM-1 were always highly expressed, except in MDA-MB-157. SK-BR-3, MCF7, SUM159, and SUM225 cell lines expressed low to dull levels of NKp30-L. Altogether, the protein levels observed in cell lines were concordant with the gene expression profiles and illustrated the heterogeneity observed in primary tumors.

The frequent expression of NKG2D ligands and DNAM-1-ligands also suggested that these 2 molecules are important in breast tumor cells recognition.

Peripheral NK cells can kill breast cancer cell lines

We next studied the ability of allogeneic NK cells to exert cytotoxic functions against breast cancer cell lines, using human K562 leukemic cells as control. HLA-C interactions with the major inhibitory killer cell Ig-like receptors (KIR) KIR2DL2/3 and KIR2DL1 mostly control the development and response of human NK cells (21). We thus carried out HLA-C typing of both the donors and the cell lines, and matched them accordingly. SK-BR-3 displayed the C1C1 allotype and was matched with 5 donors; MCF7 and MDA-MB-157 were C2C2 and were matched with 5 donors; SUM225, BrCa-MZ-01 and SUM159 were C1C2 and were matched with 8 donors. In such a controlled system, we observed that allogeneic peripheral (p)-NK cells killed breast cancer cell lines with various efficiencies, ranging from 17% to 40% \pm 5%, but significantly less than the K562 control (58% \pm 6%; Fig. 2A). This was also true for the degranulation ability of NK cells, measurable through the expression of CD107, and the synthesis of IFN- γ and TNF- α (Fig. 2B–D). These observations suggested that NK cells cytotoxic response needs to be enhanced to favor breast tumor cells eradication. This is enforced by the fact that activated p-blood NK cells can also kill autologous tumor cells (Supplementary

Data S4). Altogether, these data underlined the importance of identifying the main receptor(s) involved in breast tumor cells recognition to reach a cytotoxic response as efficient as the one observed against K562 cells. A Kruskal–Wallis ANOVA did not show any difference in NK cell-mediated cytotoxicity efficiency between breast cell lines, despite the different profiles of expression of NK cell receptor ligands (data not shown).

To identify the different activating and inhibiting receptors triggered on NK cells upon target recognition, we used commercially available phosphoimmunoreceptor arrays which permit the measure of the relative phosphorylation levels of most ITAM/ITIM-associated immunoreceptors by hybridization of lysates of NK cells preincubated with each breast cancer cell line. We observed different combinations of engaged activating and inhibitory receptors when exposed to the different breast cancer cell lines, whereas most activating receptors were phosphorylated after exposition to the K562 cell line (Supplementary Data S5). Altogether, these data illustrated the complexity of interactions between NK cells and breast tumor cells, and confirmed that NK cells cytotoxicity could be enhanced through the control of these interactions.

NK cell receptors involved in the killing of breast cancer cell lines

We next studied the specific implication of the major NK cell receptors during the recognition of breast tumor cells. Specific

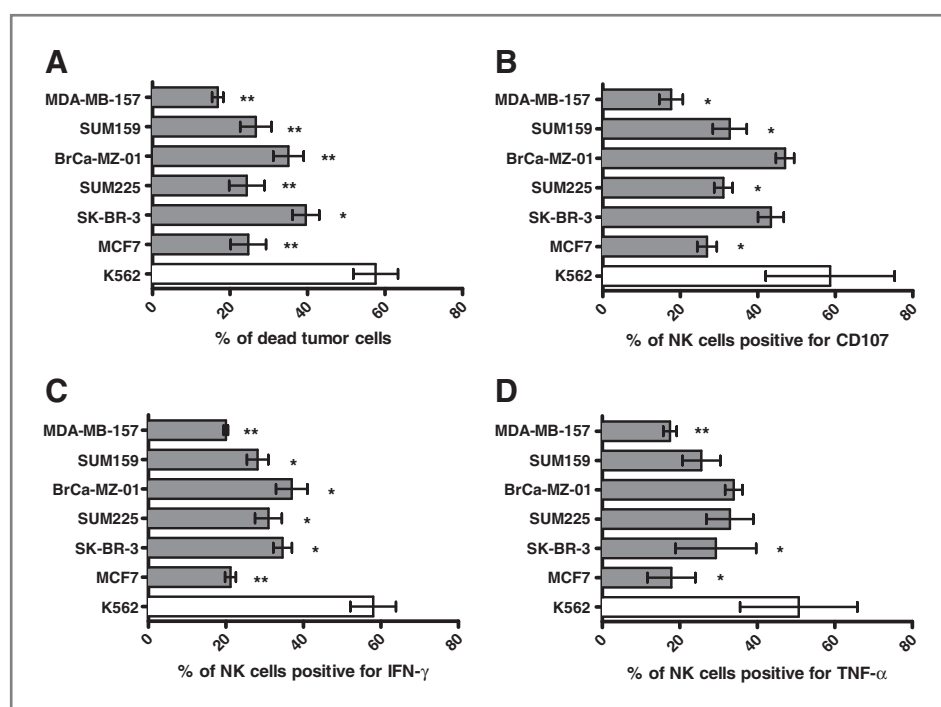


Figure 2. NK cells can kill breast cancer cell lines expressing different pattern of ligands. Allogeneic HLA-C matched NK cells (after an overnight incubation in IL-2/IL-15) were tested for direct cytotoxic activity against the leukemic HLA-negative K562 cell line and 6 breast cancer cell lines according to a 4:1 E:T ratio: MCF7 (C2/C2), SK-BR-3 (C1/C1), SUM225 (C1/C2), BrCa-MZ-01 (C1/C2), SUM159 (C1/C2), MDA-MB-157 (C2/C2). Five donors were used against the C1/C1 and C2/C2 breast cancer cell lines and 8 donors were used against the C1/C2 cell lines. A, percentage of dead tumor cells. B, percentage of NK cells positive for CD107. C, percentage of NK cells positive for IFN- γ . D, percentage of NK cells positive for TNF- α . The cytotoxicity obtained against K562 cells and each of the breast tumor cell lines was evaluated with a nonparametric Mann and Whitney *t* test. Only *P* values < 0.05 were considered significant. *, *P* < 0.05; **, *P* < 0.005.

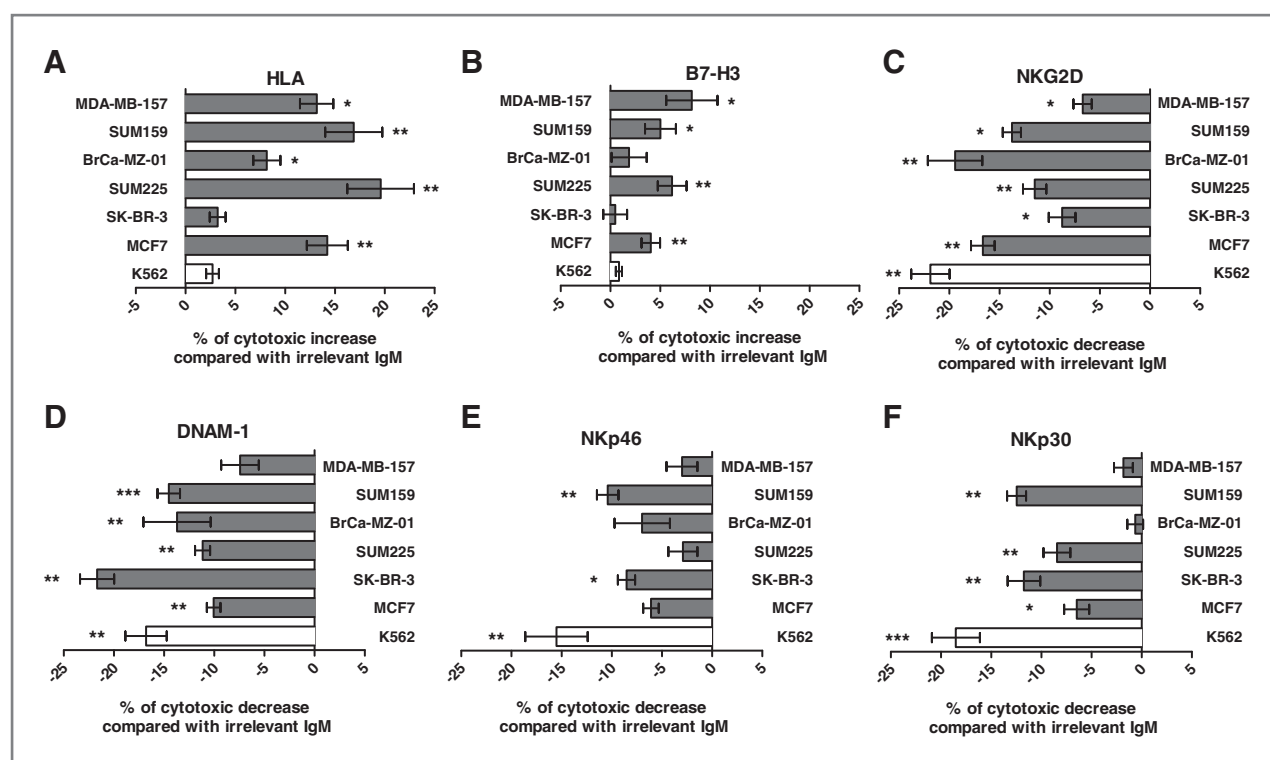


Figure 3. Receptors involved in breast cancer cell line recognition by NK cells. We measured nonactivated NK cells mediated cytotoxicity in 4-hour assays in the presence of blocking antibodies for HLA (A), B7-H3 (B), NKG2D (C), DNAM-1 (D), NKp46 (E), and NKp30 (F) or irrelevant IgM. E:T = 20:1. Target cell lysis was measured by Cr51 release. The cytotoxic effects induced by the respective blocking antibody (increased or decreased) compared with the irrelevant isotype control mAbs were evaluated with a nonparametric Wilcoxon *t* test. Only *P* values < 0.05 were considered significant. *, *P* < 0.05; **, *P* ≤ 0.005; ***, *P* ≤ 0.0005.

blockade of interactions with a pan-HLA mAb increased NK cell cytotoxicity, notably in cell lines expressing high levels of HLA molecules (Fig. 3A). Blocking B7-H3 receptor slightly enhanced NK cell-mediated killing, confirming its role as a negative regulator of NK cell cytotoxicity, coherent with its expression (Fig. 3B). Conversely, blocking NKG2D receptor decreased NK cell cytotoxicity, especially against BrCa-MZ-01 and MCF7, the 2 cell lines expressing the highest levels of NKG2D-L (Fig. 3C). DNAM-1 was also involved in the killing of most cell lines (Fig. 3D). The blockade of NKp46 decreased NK cell cytotoxicity against SUM159 and SK-BR-3, suggesting that these cells might express low levels of the unknown NKp46 ligand (Fig. 3E). Finally, the NKp30 receptor played a minor role in the killing of SUM159 and SK-BR-3, as well as SUM225 and MCF7, corroborating NKp30 ligand detection at the protein level (Fig. 3F).

These results identified HLA (KIRs and/or NKG2A), NKG2D, and DNAM-1 as the most common and important receptors regulating the recognition of breast tumor cells by normal NK cells. NKp30, NKp46, and B7-H3 receptors were also involved, but less systematically. It thus seems that a combination of several interactions, rather than one particular receptor, might be important for the recognition of a given breast cancer cell line by NK cells. This recognition tightly depends on the repertoire of ligands expressed by the targeted cell. To this regard, the educated targeting of 2 activating receptors could

almost completely abolish the killing of the targeted cell line (Supplementary Data S6).

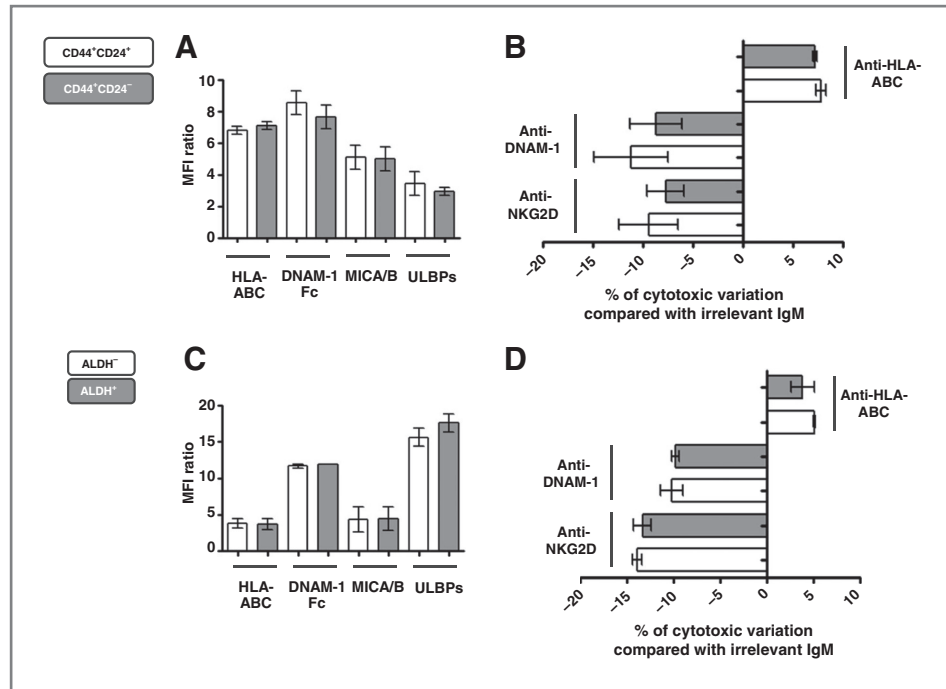
We next wondered whether, in return, breast cancer cells could adopt strategies to evade from NK cells recognition.

Susceptibility of breast cancer stem cells to NK cell killing

A first mechanism of escape from the immune reaction could be that breast cancer stem cells might not be susceptible to NK cell cytotoxicity. We thus studied progenitors of one luminal cell line (MCF7) and one basal (BrCa-MZ-01) cell line. Isolation of luminal stem cells was based on the CD44⁺CD24⁻ phenotype (22) (Supplementary Data S7A). No difference with regard to the expressions of HLA class-I molecules, DNAM-1-L, and NKG2D-L (MICA/B, ULBPs) were observed between the CD44⁺CD24⁻ stem cells and the more differentiated CD44⁺CD24⁺ cells (Fig. 4A). NK cell cytotoxicity against the 2 populations was also equivalent (Fig. 4B). Basal stem cells were isolated from BrCa-MZ-01 based on a differential aldehyde dehydrogenase activity (ref. 23; ALDH⁺; Supplementary Data S7B). As for the luminal cell line, HLA class-I molecules, DNAM-1-L, and NKG2D-L were not differentially expressed in ALDH⁺ and ALDH⁻ populations (Fig. 4C), and there was no difference in NK cell cytotoxicity against both targets (Fig. 4D).

These results show that NK cells similarly recognize and kill breast cancer precursors and their differentiated counterparts, at least in the 2 molecular subtypes tested.

Figure 4. NK cells recognize both differentiated and precursor cells of breast cancer cell lines. Analysis of differentiated (open bars) and precursor (grey bars) cells isolated from MCF7 luminal (A and B) and BrCa-MZ-01 basal (C and D) breast cancer cell lines. Phenotype of HLA-ABC, DNAM-1-L, and 2 NKG2D ligands, MIC-A/B and ULBPs, on differentiated and precursor cells (A and C). B and D, we measured nonactivated NK cell-mediated cytotoxicity against differentiated and precursor cells in 4-hour Cr51 release assays in the presence of blocking antibodies for HLA, DNAM-1, and NKG2D. NK cell-mediated cytotoxicity involving each of these receptors was expressed as a difference from the respective negative control. E:T = 20:1.



Breast cancer cells alter NK cell phenotype and function

A second mechanism of escape consists in the downregulation of activating receptors and/or upregulation of inhibitors of NK cell functions. We compared NK cell phenotype after a 48-hour direct or indirect (transwell cultures) exposition to breast cancer cell lines, representative of breast tumor subtypes heterogeneity, MCF7 (luminal), SK-BR-3 (ERBB2+), and SUM159 (basal), or primary breast tumor cells. NKG2D and Nkp30 were downregulated on p-NK cells after direct or indirect contact with MCF7, SUM159, or primary breast tumor cells, suggesting that soluble molecules are involved in the regulation of these receptors (Fig. 5A and B and Supplementary Data S8). DNAM-1 tended to be downregulated only when p-NK cells were in direct contact with SUM159 cells or with 2 of 5 primary tumor cells, but these observations were not statistically significant; the regulation of DNAM-1 expression was likely to involve cell-cell contact mechanisms, but not systematically.

We thus measured the expression of the main NK cell receptors in the peripheral blood of breast cancer patients, according to the subtype of their tumor. NKG2D, Nkp30, and DNAM-1 were all highly affected independently of the subtype of breast cancer, whereas other receptors were not altered, such as Nkp46, or enhanced, such as the inhibitory receptor NKG2A (Fig. 5C and data not shown).

We next evaluated the impact of cell lines and breast tumor supernatants on NK cells function. When NK cells were pre-exposed to these supernatants, their CD107 activity against K562 cell line were profoundly impaired (Fig. 6A and B). Blocking antibodies against educated inhibitors (24) partially reversed these effects and indicated that PGE2, LGALS3, TGF- β 1, and, occasionally, MICA/B should preferentially be targeted to facilitate NK cells cytotoxicity (Fig. 6C).

In summary, we showed that breast tumor cells could escape from NK cells antitumor immunity by altering the phenotype of NK cells (downregulation of activator receptors allowing their recognition) and by decreasing their cytotoxic potential. This induction of self-tolerance could be mediated by cell-cell contacts but most likely involved the production of soluble inhibitory factors.

The MMTV-Neu transgenic mouse model reproduces some of the NK cell alterations seen with breast cancer cell lines

To show that breast cancer cells alter NK cell functions *in vivo*, we used the immune-competent MMTV-Neu transgenic mouse crossed on an FVB background (FVB-neuN), which spontaneously develops mammary gland tumors. FVB-neuN mice expressed the ERBB2 oncogene under the control of the *MMTV* promoter. The pathology of the developed tumors is considered to be similar to that of human invasive luminal breast tumors (25).

Every month until tumor occurrence, we followed 6 FVB controls and 6 FVB-neuN mice and phenotyped the main NK cell receptors in the peripheral blood of each mouse (Supplementary Data S9). Before tumor occurrence, we did not find any phenotypic difference between transgenic mice and their respective controls. In contrast, at overt tumor stage, expression levels of NK cell activation receptors NKG2D and DNAM-1 (Nkp30 is not functional in the mouse) were decreased on tumor-infiltrating NK cells (Fig. 7A and B) and p-NK cells of the MMTV-Neu mice, compared with their age-matched controls. NKG2D receptor was also decreased in lymph nodes infiltrating NK cells. Concordant with these phenotypic alterations, p-NK cells and NK cells isolated from the mammary tumor of the FVB-neuN transgenic mice displayed decreased cytotoxicity

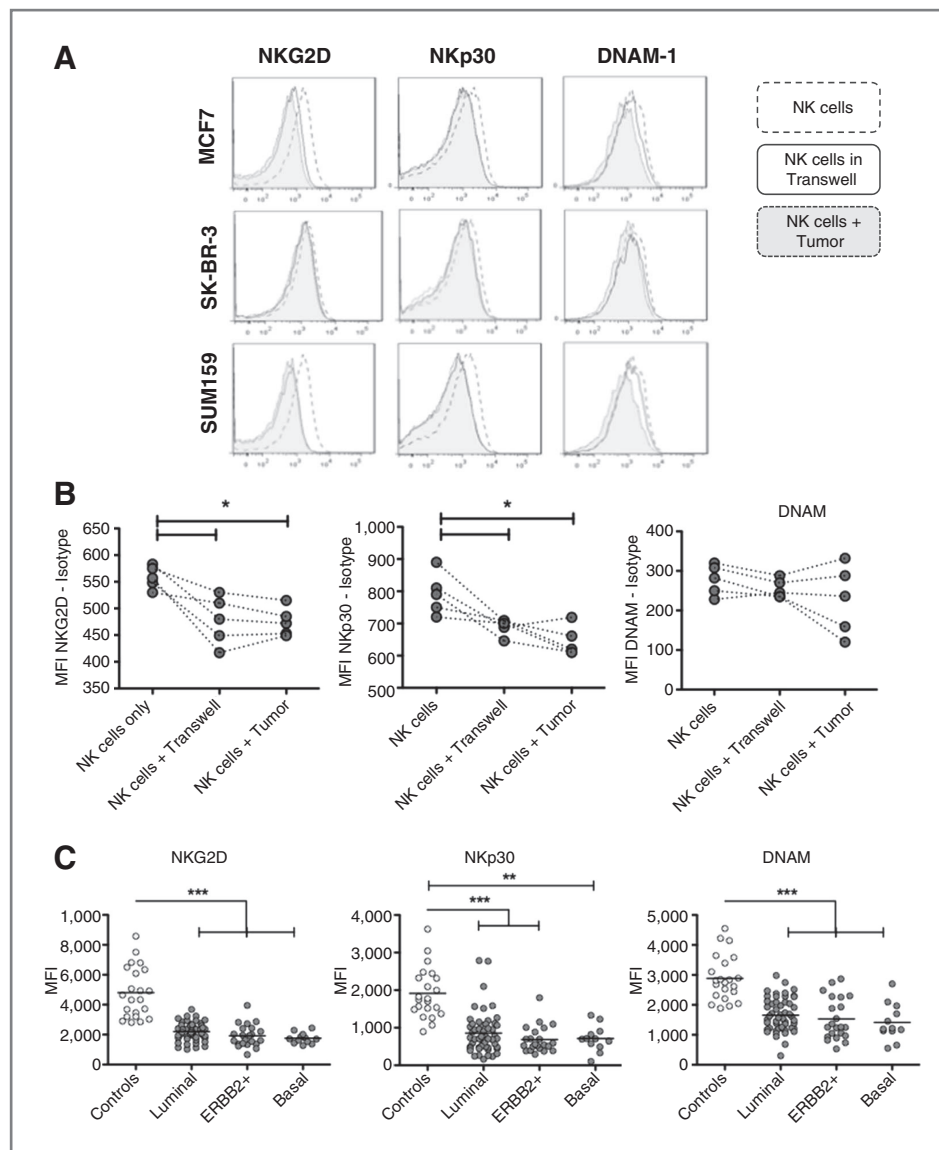


Figure 5. Breast cancer cells use multiple mechanisms to alter NK cells' activating receptors phenotype. Freshly isolated NK cells were exposed to breast cancer cell lines or primary breast tumor cells, directly or indirectly through a transwell, according to a 2:1 and 5:1 E:T ratio, respectively. After 48 hours, NK cells were harvested and phenotyped for NK cell receptors. A, NKG2D, NKp30, and DNAM-1 expression on NK cells exposed to MCF7, SK-BR-3, and SUM159 breast cancer cell lines by comparison with nonexposed NK cells. B, NKG2D, NKp30, and DNAM-1 expression on NK cells exposed to primary breast tumor cells by comparison with nonexposed NK cells ($n = 5$ donors). C, phenotype of NKG2D, NKp30, and DNAM-1 on peripheral blood NK cells from breast cancer patients with different breast tumor subtypes and healthy individuals as controls. The differences between patients and controls were evaluated with a 1-way ANOVA and Dunn multiple comparison tests. Only P values < 0.05 were considered significant. *, $P < 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

against the RMA H2 negative cell line (equivalent to the human K562 cell line), as compared with NK cells isolated from FVB control mice (Fig. 7C). NK cells isolated from FVB-neuN transgenic mice also produced less inflammatory cytokines than their respective controls (data not shown).

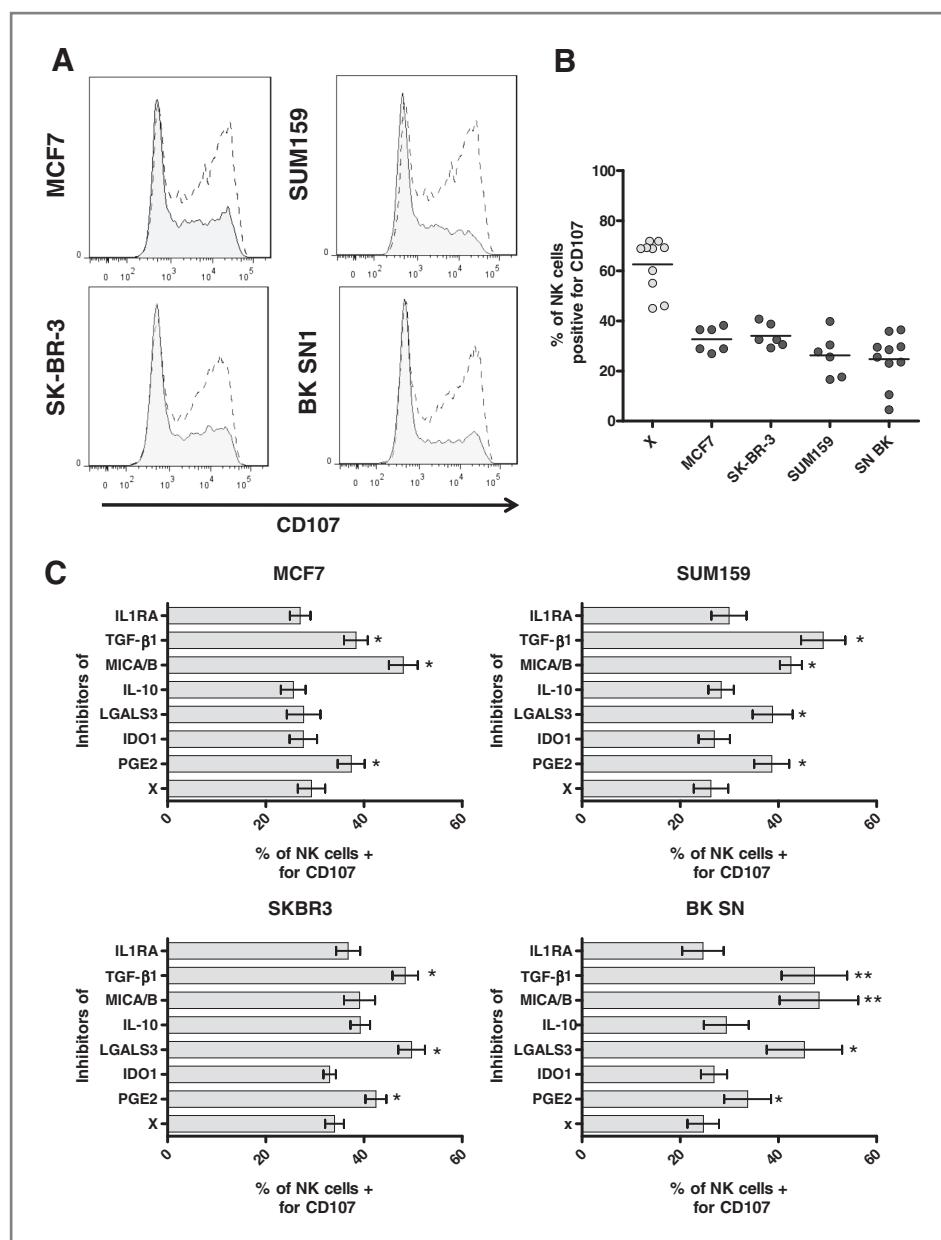
These results showed that breast tumors that develop in an immune-competent individual elicited an altered NK cell cytotoxic function that might promote their own growth.

Discussion

NK cells activation depends on cell surface receptors, which are selected during NK cells ontogeny, and results from a balance between incoming inhibitory and activating signals. Compared with healthy mammary tissues, we showed that breast tumors from all the major subtypes differentially modulate the expression of the various NK cell ligands. For example,

most luminal tumors are MHC class-I^{low}, a status frequently observed in transformed cells resistant to adaptive immunity, but express NK cell activating ligands, suggesting a potential role of innate immunity in tumor control. In contrast, class-I^{normal} or ^{high} basal tumors are more susceptible to properly present malignant antigens to specific immune T cells, thus facilitating tumor clearance. This is in perfect agreement with the *in situ* adaptive response recently identified in basal tumors with good outcome (4, 6). However, MHC class-I molecules might engage the inhibitory KIR receptors and thus prevent NK cell activation unless they express high levels of ligands for activating receptors. In this situation, adoptive transfer of haploidentical NK cells seems as a very attractive strategy to mediate both differentiated and breast cancer stem cells residual tumor clearance (26, 27), and the basal subtype might be the best candidate to test this strategy, as some of these tumors are MHC class-I^{high} and express high

Figure 6. Breast cancer cells synthesize soluble factors that alter NK cell function. NK cells were activated overnight in IL-2/IL-15, then incubated with breast cancer cell line supernatants (MCF7, SK-BR-3, SUM159) or primary breast cancer (BK SN1) for 24 hours. NK cells were then used in a functional CD107 assay against K562 cell line to evaluate their killing ability. A, histogram representing the effect of the various breast tumor supernatants on CD107 activity. Nonpreexposed NK cells are represented with a dashed line. NK cells exposed to breast tumor supernatants (cell line or primary tumor) are represented with a plain line and shaded histogram. B, representation of the $n = 6$ donors tested. C, the NK cells inhibitors contained in breast tumor supernatants could be identified using blocking antibodies against PGE2 (5 $\mu\text{mol/L}$), IDO1 (1 mg/mL), LGALS3 (5 mmol/L), IL-10 (5 $\mu\text{g/mL}$), MICA/B (2.5 $\mu\text{g/mL}$), TGF- β 1 (5 $\mu\text{g/mL}$), and IL1RA (20 $\mu\text{g/mL}$) preincubated 30' with breast tumor supernatant before being added to NK cells. Data were compared with the condition without blocking mAb using nonparametric paired Wilcoxon t test. Only P values < 0.05 were considered significant. *, $P < 0.05$; **, $P \leq 0.005$.



level of activating ligands, thus allowing optimal activation of both adaptive and innate immunity, respectively. The current phase II study of allogeneic NK cell therapy in the treatment of breast cancer, however, still needs to improve NK cells persistence and *in vivo* or *ex vivo* expansion strategies (18, 28). This has already been achieved in acute myeloid leukemia (29, 30).

In addition to CMH class-I modulation, we also showed that NKG2D-L and DNAM-1-L, whose receptors are both found on T and NK lymphocytes, are broadly expressed on the different breast tumor subtypes. NKG2D-L is particularly interesting because it specifically marks stressed or transformed cells. In gastrointestinal tumors, but not always in breast and lung cancers, NKG2D-L levels are systematically increased com-

pared with adjacent normal tissues (31). We showed that NKG2D-L is involved in most tumor cell lines killing, but we and others also showed that soluble MICA/B is a major mechanism inhibiting NK cells activation against tumor cells (32, 33). sMICA expression is even considered as a poor prognosis factor in breast cancer (34). Increasing NKG2D and NKG2D-L expression or preventing NKG2D-L shedding might favor tumor clearance and overall survival, as reported in leukemias (35) and solid tumors (36). NKG2D-L expression can be increased with different drugs, such as retinoic acid or the HDAC inhibitor valproic acid, to render cells more sensitive to NKG2D-mediated cytotoxicity (35, 37). The shedding of NKG2D-L, and other receptors involved in NK cell activation

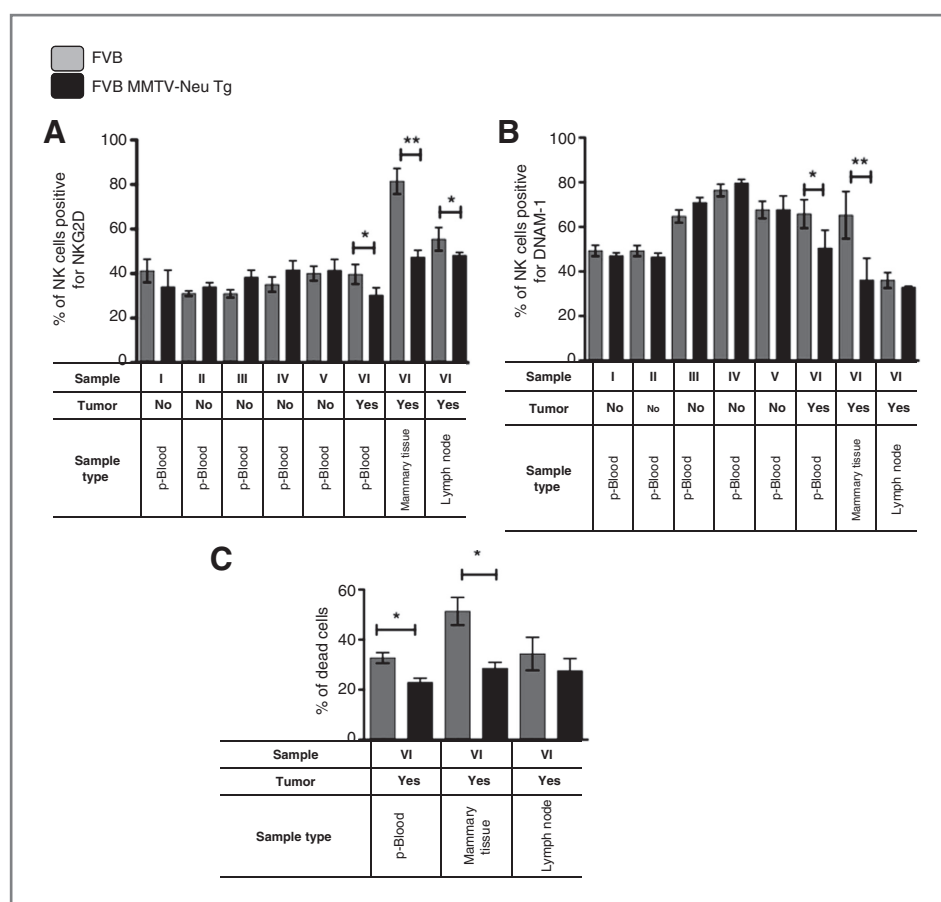


Figure 7. Breast tumor cells alter NK cells' phenotype and function in the MMTV-Neu murine model of breast cancer. FVB control mice ($n = 6$, light grey bars) and MMTV-Neu mice ($n = 6$, dark grey bars) were followed from 3 months of age (samples I to V) to tumor occurrence (samples VI). NKG2D (A) and DNAM-1 (B) expression were followed on NK cells from FVB and MMTV-Neu mice at stage VI against the RMA H2 negative cell lines (E:T of 2:1). FVB and MMTV-Neu mice were compared using nonparametric Mann and Whitney t test. Only P values < 0.05 were considered significant. *, $P < 0.05$; **, $P \leq 0.005$.

and recruitment, could be prevented with metalloprotease inhibitors (such as anti-ADAM10 and ADAM17; ref. 38, 39). More interestingly, a recent study has shown that an anti-ERBB2-NKG2D ligand fusion protein, simultaneously targeting ERBB2⁺ tumors and triggering NKG2D⁺ lymphocytes, allows for a rapid regression of the tumor and the development of a specific memory antitumor response. Both effects are abrogated by NK cells or CD8 depletion (40). Such a therapy displays the advantage of simultaneously targeting the tumor (anti-ERBB2 mAb), inducing NK cells activation through NKG2D (or at least preventing sNKG2D-L to inhibit NK cells) and activating the powerful ADCC (antibody-dependent cell-mediated cytotoxicity) function through the engagement of the CD16 receptors with the Fc portion of anti-ERBB2 mAb.

DNAM-1 has been less studied than NKG2D. However, DNAM-1 interaction with its ligands is also strongly involved in tumor immune surveillance *in vivo*: DNAM-1-deficient mice present accelerated tumor growth (41) and DNAM-1/PVR interactions promote NK cell-mediated suppression of poorly immunogenic melanoma metastases (42). In humans, PVR expressing neuroblastomas are the tumors with the highest susceptibility to lysis by NK cells (43). Our data showed that DNAM-1 facilitates breast tumors clearance mediated by NK cells. Interestingly, DNAM-1-L expression could also be stabilized on tumor cells by HDAC inhibitors (44).

Finally, we found that NKp30 and NKp46 receptors are only occasionally involved in the recognition of breast tumor cell lines. These two ligands are involved in *in vitro* recognition of tumor cells in several malignancies (45, 46). NKp46 is a specific NK cell killer receptor that recognizes influenza hemagglutinins and still-unknown tumor-associated ligands. NKp46 expression is frequently deregulated (45) and involved in tumor editing (47). Our study indicated that NKp46 is poorly involved in the recognition of breast cancer cell lines, underlying the absence of NKp46 ligand on breast tumor cells, potentially as a consequence of a previous tumor editing process. NKp30 has 2 known ligands but only B7-H6 has been involved in tumor clearance due to its restricted expression on human malignant cell lines (12). We found that NK cell activation is concordant with NKp30-L expression, but its expression is clearly not ubiquitous. We have recently reported that the expression of NKp30, NKG2D, and DNAM-1 receptors on NK cells and NK cells functions are mostly altered by soluble factors both *in vitro* and *ex vivo* (24). Some of these inhibitors were identified as TGF- β 1, PGE₂, sMICA, and LGALS3 (24). These inhibitors are all known to dramatically impair NK cell lytic activity, notably, via the downregulation of NK cell activating receptors in other malignancies (48–50) and to be largely produced in malignant breast tissue (24). These data suggest that any NK cell-based therapy should simultaneously

address the question of NK cells inhibitors synthesis, with peptide inhibitors of TGF- β 1, for example, and/or be conducted as a complement of the surgical removal of the tumor, more or less chemotherapy or radiotherapy, to minimize the effects of the inhibitors.

In conclusion, our study shows that breast tumor subtypes heterogeneously express different combinations of NK cell activating receptors that sense malignant cells and further contribute to their heterogeneity: NKG2D and DNAM-1 receptors are involved in the recognition of most breast tumor cells by NK cells, whereas the involvement of the NCRs rather relies on an "à la carte" profile. This suggests that enhancing NK cell efficiency is a good strategy to promote breast tumor cells clearance. However, any immunotherapy might remain poorly efficient without also countering the mechanisms developed by most malignant cells to escape from NK cell antitumor immunity.

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Disclosure of Potential Conflicts of Interest

The authors do not disclose any commercial affiliations as well as consultancies, stock and equity interests, or patent-licensing arrangements that could be considered a conflict of interest, with the exception of A. Moretta who is founder and shareholder of Innate-Pharma (Marseille, France). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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