Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity

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NK cells are a major component of the antitumor immune response and are involved in controlling tumor progression and metastases in animal models. Here, we show that dysfunction of these cells accompanies human breast tumor progression. We characterized human peripheral blood NK (p-NK) cells and malignant mammary tumor-infiltrating NK (Ti-NK) cells from patients with noninvasive and invasive breast cancers. NK cells isolated from the peripheral blood of healthy donors and normal breast tissue were used as controls. With disease progression, we found that expression of activating NK cell receptors (such as NKp30, NKG2D, DNAM-1, and CD16) decreased while expression of inhibitory receptors (such as NKG2A) increased and that this correlated with decreased NK cell function, most notably cytotoxicity. Importantly, Ti-NK cells had more pronounced impairment of their cytotoxic potential than p-NK cells. We also identified several stroma-derived factors, including TGF-β1, involved in tumor-induced reduction of normal NK cell function. Our data therefore show that breast tumor progression involves NK cell dysfunction and that breast tumors model their environment to evade NK cell antitumor immunity. This highlights the importance of developing future therapies able to restore NK cell cytotoxicity to limit/prevent tumor escape from antitumor immunity.

Introduction

Breast cancer (BC) is the primary cause of cancer deaths in women. The main cause of this mortality is the metastatic spread to other organs (1). Metastasis occurs when tumor cells acquire invasive features (2) and the ability to escape from antitumor immunity (3, 4). Defects in antitumor immunity may also facilitate BC occurrence. Indeed, mice deficient in IFN-γ production spontaneously develop mammary tumors (5). Breast tumor cells transplanted into NOD/SCID mice (which lack adaptive immunity) form noninvasive tumors, whereas the same cells transplanted into NOD/SCID/γ-c^{null} mice (no adaptive immunity and no NK cells) form invasive tumors that metastasize rapidly (6). This effect is strictly dependent on NK cells (7). Similarly, in a highly metastatic model, BC metastasized to the lung only after elimination of NK cells by Tregs (8).

Advanced BC patients show defects in antitumor immunity, such as alterations of DC maturation (9) and an increase in Treg infiltrates (10). Major impairment of peripheral blood NK cell maturation and cytotoxic functions has also been reported in metastatic BC (11). Several gene expression profiling studies have shown that a better outcome is associated with a strong cytotoxic infiltrate containing NK cells (12–15). These data suggest that BC progression is linked to antitumor immunity efficiency and particularly to NK cells. However, the precise relationships between NK cells and BC progression in humans have not been studied so far.

NK cells are innate immune cells that have the natural ability to distinguish normal cells from "modified" cancer cells (16). Once activated, NK cells eliminate their target through the release of cytotoxic enzymes (perforin 1, granzymes, granulysin) and/or soluble factors (chemokines and inflammatory cytokines), which, in turn, recruit and/or activate other effectors (17). Activating and inhibitory receptors present on NK cells are triggered during target cell recognition and induce a positive or a negative cell signaling pathway, respectively. The integration of these opposite signals determines NK cell activation (18). The main activating receptors or coreceptors of NK cells are NKG2D, the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, DNAM-1, CD2, NKp80, 2B4, and NTBA (19–21). These molecules recognize various ligands usually upregulated upon cellular stress (22). NK cells also express the Fc immunoglobulin fragment low-affinity receptor or CD16, which, when cross-linked, induces a powerful reaction called antibody-dependent cellular cytotoxicity (ADCC). Inhibitory receptors include the killer immunoglobulin receptors (KIRs), NKG2A, CD85j, and LAIRs (23–26). They are specific for different HLA–class I molecules. Accordingly, NK cells can kill target cells that have lost (or express low amounts of) HLA–class I molecules, which is frequently the case for tumor cells, including breast tumor cells (27). However, tumor cells also have the ability to impair NK cell "visibility" through the modulation of their receptors (28, 29). Recent studies have shown that several molecules, notably inhibitory factors often found in the tumor microenvironment, such as IDO1 and TGF-β1, can sharply impair NK cells' phenotype and functions (30, 31).

Table 1
Clinical features of BC patients

Groups		B	Tis	LOC	LA	M
n =		19	8	55	26	32
Mean age (years)		56	51	57	52	52
Invasive margin		No	No	Yes	Yes	Yes
Sample uptake		Before surgery	Before surgery	Before surgery	Before surgery	Before therapy
Scarff-Bloom-Richardson (SBR) Grade	I	ND	ND	37.0%	3.8%	
	II	ND	ND	38.8%	34.6%	
	III	ND	ND	24.0%	53.8%	
pN+		No	No	34.0%	81.0%	100.0%
Median pT (mm)		ND	20.2	26.4	57.1	50.0
Perivascular invasion		No	No	16.6%	26.9%	9.3%
Lympho-plasmocytic stroma	Absent	ND	ND	37.5%	47.7%	ND
	Moderate	ND	ND	56.2%	38.1%	ND
	High	ND	ND	6.3%	14.2%	ND
IHC expression	PR+	ND	ND	62.9%	53.8%	46.8%
	ER+	ND	ND	75.9%	69.2%	40.6%
	erbB-2+	ND	ND	7.4%	15.3%	43.7%
	HR−erbB-2−	ND	ND	18.5%	15.3%	18.7%
Histological type	Ductal	ND	100% intraductal	57.4%	65.4%	82.2%
	Lobular	ND		18.5%	19.2%	6.2%
	Tubular	ND		14.8%	0.0%	0.0%
	Other	ND		9.2%	15.3%	12.5%

PR, progesterone receptor; ER, estrogen receptor; HR, hormone receptor, i.e., PR and /or ER+; ND, not determined.

We show here that mechanisms of escape from NK cell–mediated immunity are at play in BC patients. In a cohort of BC patients sampled at different stages of the malignant process, we found that breast tumors have altered NK cell phenotype and function and that invasive tumors build strong inhibitory microenvironments to escape NK cell antitumor immunity.

Results

p-NK cell phenotype is altered in invasive BC patients

We prospectively enrolled patients with different stages of BC at diagnosis. Based on tumor pathologic tumor-node-metastasis (pTNM) classification and tumor margins, groups were composed of noninvasive (in situ) BCs (Tis) (n = 8) and invasive BCs (n = 113) including localized (LOC) (n = 55), locally advanced (LA) (n = 26), and metastatic stages (M) (n = 32). This classification, proposed by the American Joint Committee on Cancer (AJCC), is based on the histoclinical extension of disease, which represents the most important prognostic factor of BC, with decreasing survival from the Tis group to the M group (32). Clinical characteristics of the patients are summarized in Table 1. Due to the prospective nature of our study, the clinical follow-up of the patients was too short for survival analysis. Patients with benign mammary tumors (B) (n = 19) and healthy donors (HD) (n = 22) were included as control groups.

To explore NK cell cytotoxic potential, we first determined the expression of 22 NK cell receptors on cells from fresh blood samples. The MFI of NKp30, NKG2D, DNAM-1, and 2B4 were lower in patients with invasive breast tumors (LOC, LA, and M) than in control groups and patients with Tis tumors (Figure 1, A–D). The expression of CD16 was lower in patients with invasive BC than in control groups (Figure 1E). The expression of other activating mol-

ecules did not vary with tumor progression (Figure 1F). In contrast, 2 inhibitory receptors, NKG2A and CD85j, were upregulated in the M group compared with control groups and the Tis group (Figure 1, G and H). Histograms are shown in Supplemental Figure 1A (supplemental material available online with this article; doi:10.1172/JCI45816DS1). We submitted the normalized expression values of the 22 receptors to TMEV software analysis based on unsupervised hierarchical clustering. The clustering emphasized the similarities between the control groups and Tis tumors on one side and the 3 invasive tumor groups on the other side (Figure 1I). The degree of alterations clearly progressed with tumor stage, notably in the invasive tumors, with underexpression of activating receptors and overexpression of inhibitory receptors in more advanced stages.

p-NK cell functions are altered in patients with invasive BC

We next determined whether these modifications of expression were associated with altered NK cell functions. We assessed peripheral blood NK (p-NK) cells' functionality through their ability to kill a target cell (cytotoxic activity), to degranulate (CD107 positivity, associated with both helper and cytotoxic functions), to produce IFN-γ and TNF-α (helper functions), and to mediate ADCC. We found that effective killing (Figure 2A) and degranulation efficiency (Figure 2B) were altered in the invasive groups compared with the noninvasive ones. IFN-γ and TNF-α levels were also reduced in the M group compared with the noninvasive groups (Figure 2, C and D). Highly responsive NK cells can exert multiple functions with increased potency compared with monofunctional cells (33). We used the Boolean gating strategy to identify this multifunctional potential of NK cells. p-NK cells from the M group were mostly monofunctional (either CD107 or IFN-γ or TNF-α), whereas p-NK cells from the other groups
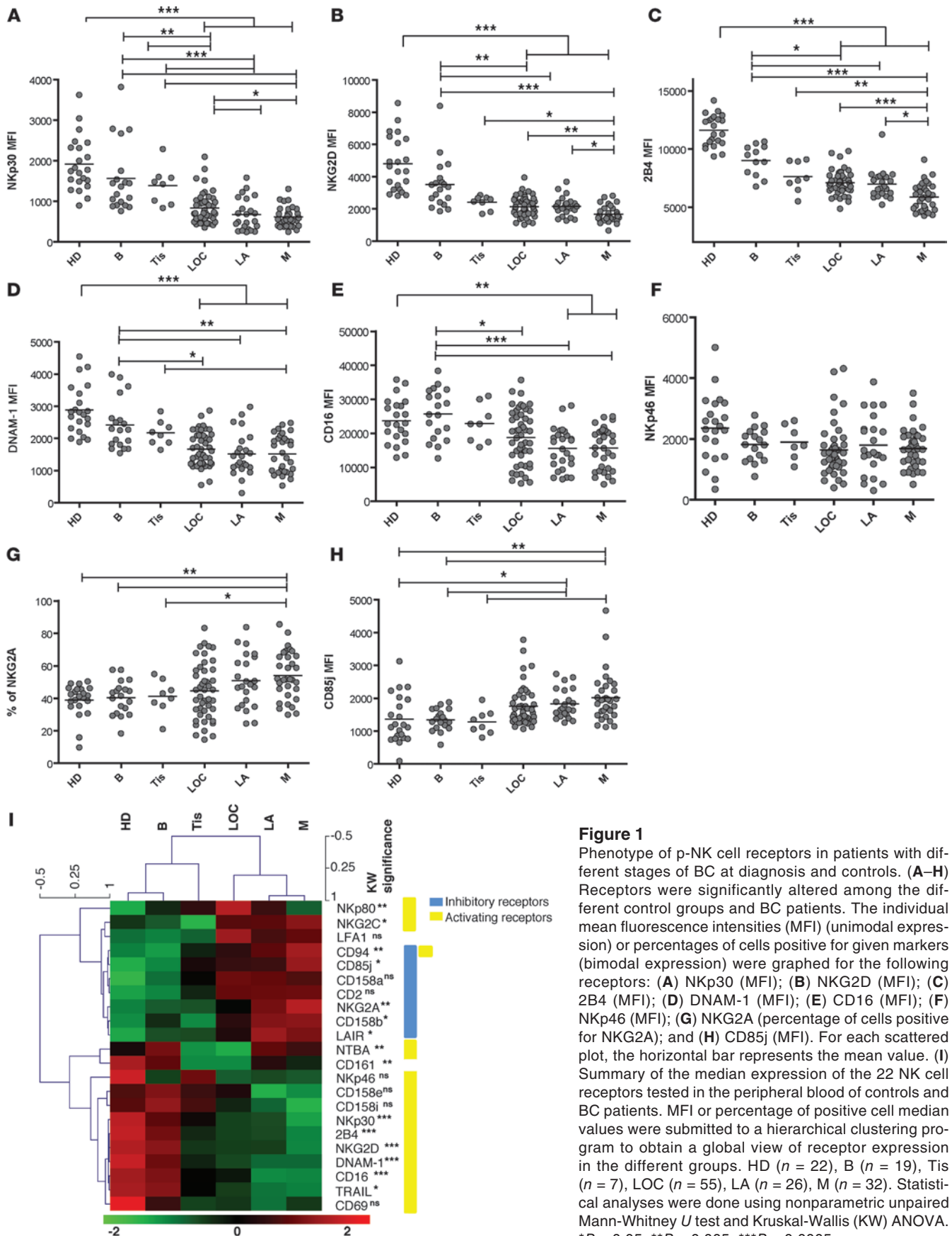
Figure 1
Phenotype of p-NK cell receptors in patients with different stages of BC at diagnosis and controls. (**A–H**) Receptors were significantly altered among the different control groups and BC patients. The individual mean fluorescence intensities (MFI) (unimodal expression) or percentages of cells positive for given markers (bimodal expression) were graphed for the following receptors: (**A**) NKp30 (MFI); (**B**) NKG2D (MFI); (**C**) 2B4 (MFI); (**D**) DNAM-1 (MFI); (**E**) CD16 (MFI); (**F**) NKp46 (MFI); (**G**) NKG2A (percentage of cells positive for NKG2A); and (**H**) CD85j (MFI). For each scattered plot, the horizontal bar represents the mean value. (**I**) Summary of the median expression of the 22 NK cell receptors tested in the peripheral blood of controls and BC patients. MFI or percentage of positive cell median values were submitted to a hierarchical clustering program to obtain a global view of receptor expression in the different groups. HD (*n* = 22), B (*n* = 19), Tis (*n* = 7), LOC (*n* = 55), LA (*n* = 26), M (*n* = 32). Statistical analyses were done using nonparametric unpaired Mann-Whitney *U* test and Kruskal-Wallis (KW) ANOVA. *$P < 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$.

A Effective Killing

B Degranulation

Groups' color code

B
Tis
LOC
LA
M

C IFN-γ synthesis

D TNF-α synthesis

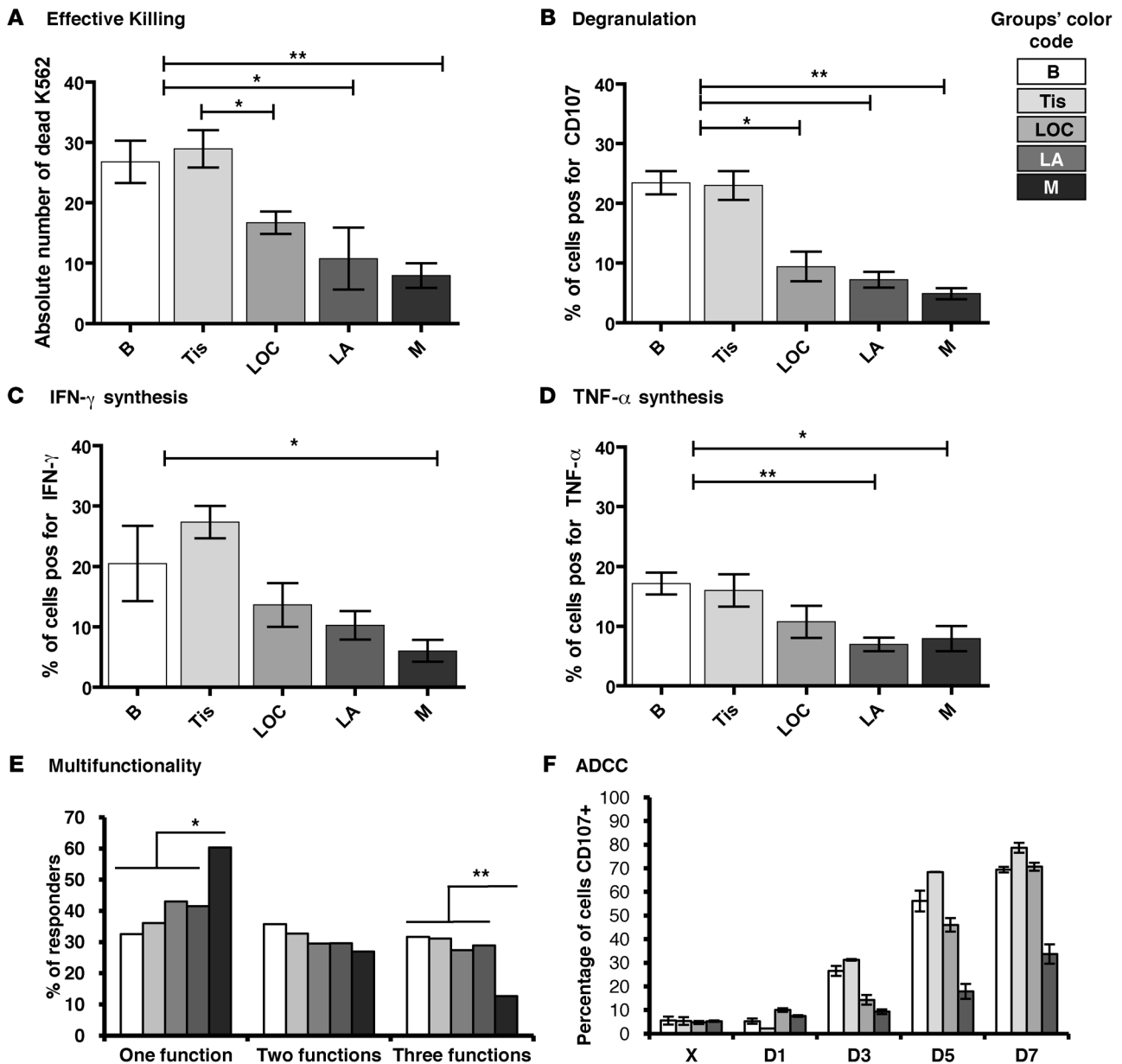**E Multifunctionality**

F ADCC

Figure 2

p-NK cell functions are altered in invasive BC patients. p-NK cells isolated from the different groups of patients were exposed to K562 cells in a direct cytotoxic assay. (**A**) Effective killing of K562 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. (**E**) The multipotentiality of p-NK cells was determined from the number of functions (degranulation as measured by CD107 expression, production of IFN-γ and/or TNF-α) that each p-NK cell was able to simultaneously accomplish against K562 target cells. The E/T ratio was 1:1 in cytotoxic experiments performed against K562 cells. (**F**) ADCC efficiency was measured against the SK-BR-3 BC cell line preincubated without or with increasing therapeutic doses (D1 to D7) of trastuzumab. Activation of NK cells was measured by the expression of CD107. The E/T ratio was 2:1. The numbers of included patients per group were the following: B ($n = 10$), Tis ($n = 7$), LOC ($n = 16$), LA ($n = 16$), M ($n = 12$, except for ADCC experiments where we did not obtain enough cells to perform the test). The statistical differences between groups were established using nonparametric Mann-Whitney U test. *$P < 0.05$; **$P \leq 0.005$. Data are represented as mean ± SEM.

exerted the 3 functions simultaneously (Figure 2E). Finally, we measured the ability of p-NK cells to mediate ADCC against SK-BR-3, a BC cell line sensitive to trastuzumab, an anti–erbB-2 monoclonal antibody known to induce ADCC (3). We were not able to obtain enough p-NK cells to perform this test in the M group. In the other groups, we observed a massive enhancement

of cytotoxicity in the presence of increasing doses of trastuzumab, especially in the noninvasive groups, with more than 80% of cells responding (Figure 2F and Supplemental Figure 2A). NK cells from B and Tis patients were more efficient in mediating ADCC at the lowest doses of trastuzumab than NK cells from LOC and LA patients, which is coherent with their different levels of CD16
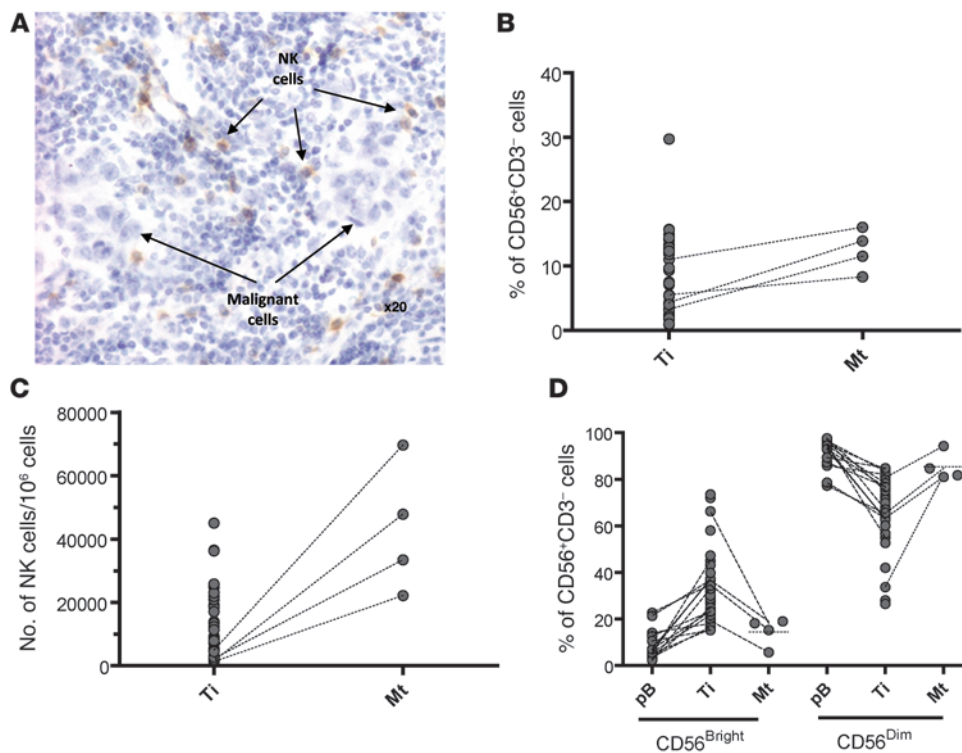
Figure 3
NK cells infiltrating breast tumors (Ti-NK cells) compared with healthy mammary tissue (Mt-NK cells). (**A**) Immunohistochemistry staining of Ti-NK cells on a paraffin section of a breast tumor. NK cells were identified with an anti-CD56 antibody (brown staining). Other lymphocyte populations, mostly T cells, displayed condensate dark blue nucleus, while tumor cells are distinguishable by their larger size. (**B**) FACS analysis of the percentage of CD45+CD56+CD3− cells found in tumor and paired healthy mammary tissue. (**C**) Absolute number of CD45+CD56+CD3− cells per million cells found in tumor compared with healthy mammary tissue. (**D**) Percentage of CD56^{Bright} and CD56^{Dim} NK cells in the peripheral blood, tumor, and healthy mammary tissue of BC patients.

expression. The lower responses observed in these patients were not due to a decreased proportion of the *VV* polymorphism, the *CD16* allotype with the highest affinity for trastuzumab (Supplemental Figure 2B). To evaluate specific effects of the major phenotypic alterations, we performed a number of redirected cytotoxic assays involving NKp30 and CD16. We found that the expression of a given receptor directly correlated with the receptor-triggered cytotoxicity (Supplemental Figure 2, C and D).

Thus, phenotypic alterations of p-NK cells in invasive BC correlate with altered functions. However, even if p-NK cells alterations are associated with disease progression, they might not reflect the situation within the tumor. We thus next studied tumor-infiltrating NK (Ti-NK) cells.

Ti-NK cells are functionally impaired compared with Mt-NK cells
Despite their major role in antitumor immunity and their presence in close vicinity to cancer cells (Figure 3A), the phenotype of mammary-infiltrating NK cells remains unknown. To identify alterations that are tumor induced rather than tissue specific, we compared NK cells isolated from breast tumors (*n* = 24, Ti-NK) with paired p-NK cells (*n* = 11, p-NK) and/or with paired tumor-free NK cells isolated from symmetric normal breast samples (*n* = 4, mammary tissue–NK cells [Mt-NK]). Because of a low lymphocyte extraction yield, we limited our study to NK cells isolated from the largest LOC tumors and LA tumors. The proportion of Ti-NK cells was similar to that found in paired tumor-free mammary tissues (7.9% vs. 12.5%, respectively; Figure 3B). However, because the proportion of CD45+ cells was lower in tumors than in healthy mammary tissues, the absolute number of NK cells per million cells was severely reduced in the malignant tissues (12,320 ± 1700 vs. 43, 265 ± 10 225 in healthy tissue; Figure 3C). Most importantly, the phenotype of NK cells differed in malignant and healthy tissues, at

least with respect to their CD56^{Bright} and CD56^{Dim} phenotype: there were more CD56^{Bright} cells infiltrating the tumor (33.9% ± 14.8%) than in the healthy mammary tissue (14.5% ± 6.1%). This latter level was more comparable to that seen in P blood (8.9 ± 6.5%) (Figure 3D). This increase of Ti-CD56^{Bright} cells suggested either the existence of 2 different maturation stages, as CD56^{Bright} cells are usually recognized as more immature NK cells with poor cytotoxic activity, or 2 activation stages, as the upregulation of CD56 expression is also a characteristic of activated NK cells, independent of their function.

To strengthen this observation, we studied other NK cell markers. The increased expression of CD56 together with NKp44 and CD69 (Figure 4, A and B, and Supplemental Figure 3A) suggested that Ti-NK cells were indeed activated NK cells. Moreover, the increased expression of NKG2A and CD27 and the decreased expression of activating receptors (NKp30, NKG2D, DNAM-1, CD16) and cytotoxicity-related molecules (CD57, PRF1, GZMB, and TRAIL) on Ti-NK cells compared with p-NK cells and Mt-NK cells suggested that Ti-NK cells have a poor cytotoxic potential. We then submitted our NK cells' profiles to TMEV software analysis. BC aggressiveness was evaluated with the Nottingham Prognostic Index (NPI), a prognostic index based on 3 pathological parameters (tumor size, tumor grade, and axillary LN status) in nonmetastatic invasive BC (34). Hierarchical clustering underlined the similarity of the profiles between Mt-NK cells and p-NK cells (Figure 4C). Ti-NK cells profiles were heterogeneous, with the exception of those from a group of patients with poor prognosis (high NPI) that showed underexpression of nearly all NK cell receptors. NK cell markers clustered into 3 groups: group I was composed of the cytotoxic-related effectors, group II, the receptors involved in NK cell activation, and group III, the molecules related to NK cell maturation markers.
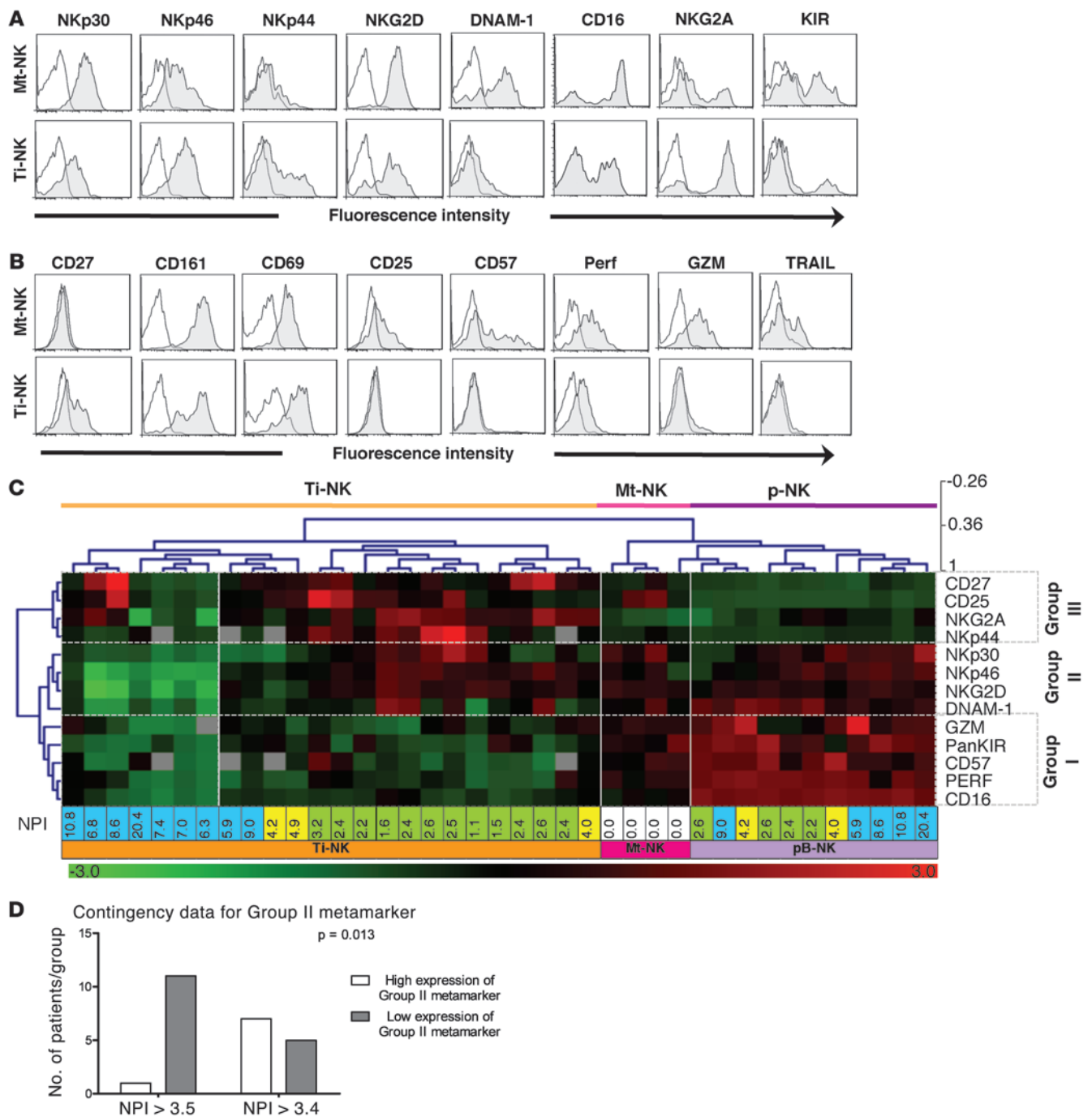
Figure 4
Phenotype of NK cells infiltrating healthy mammary tissue (Mt-NK cells), tumors (Ti-NK cells), and comparison with peripheral blood (p-NK cells) profile. (**A**) Monoparametric histograms of the most important NK cell cytotoxic receptors in paired compartments, respectively, Mt-NK cells and Ti-NK cells. (**B**) Monoparametric histograms of NK cell receptors involved in NK cell maturation and/or cytotoxicity in paired compartments, respectively, Mt-NK cells and Ti-NK cells. (**C**) Hierarchical cluster representation of NK cell receptors expressed on Ti-NK, Mt-NK, and p-NK. The phenotypes of 24 Ti-NK cells, 4 paired Mt-NK, and 11 paired p-NK cells were submitted to TMEV, and data were normalized by row; then the hierarchical clustering was applied to both NK cell markers and patient samples. Markers are represented horizontally while each patient is graphed vertically. Patients' NPI is shown below the clustering. The following color code was used: green, NPI < 3.4 (good-to-excellent prognosis); yellow, 3.4 < NPI < 5.4 (moderate prognosis); blue, NPI > 5.4 (poor prognosis). (**D**) Result of the contingency data for the group II metamarker. The Fisher exact test was significant (*P* = 0.013), and the strength of association was as follows: relative risk: 0.1477, 95% CI: 0.02170 to 1.006; odds ratio, 0.0625; 95% CI: 0.006019 to 0.6490.
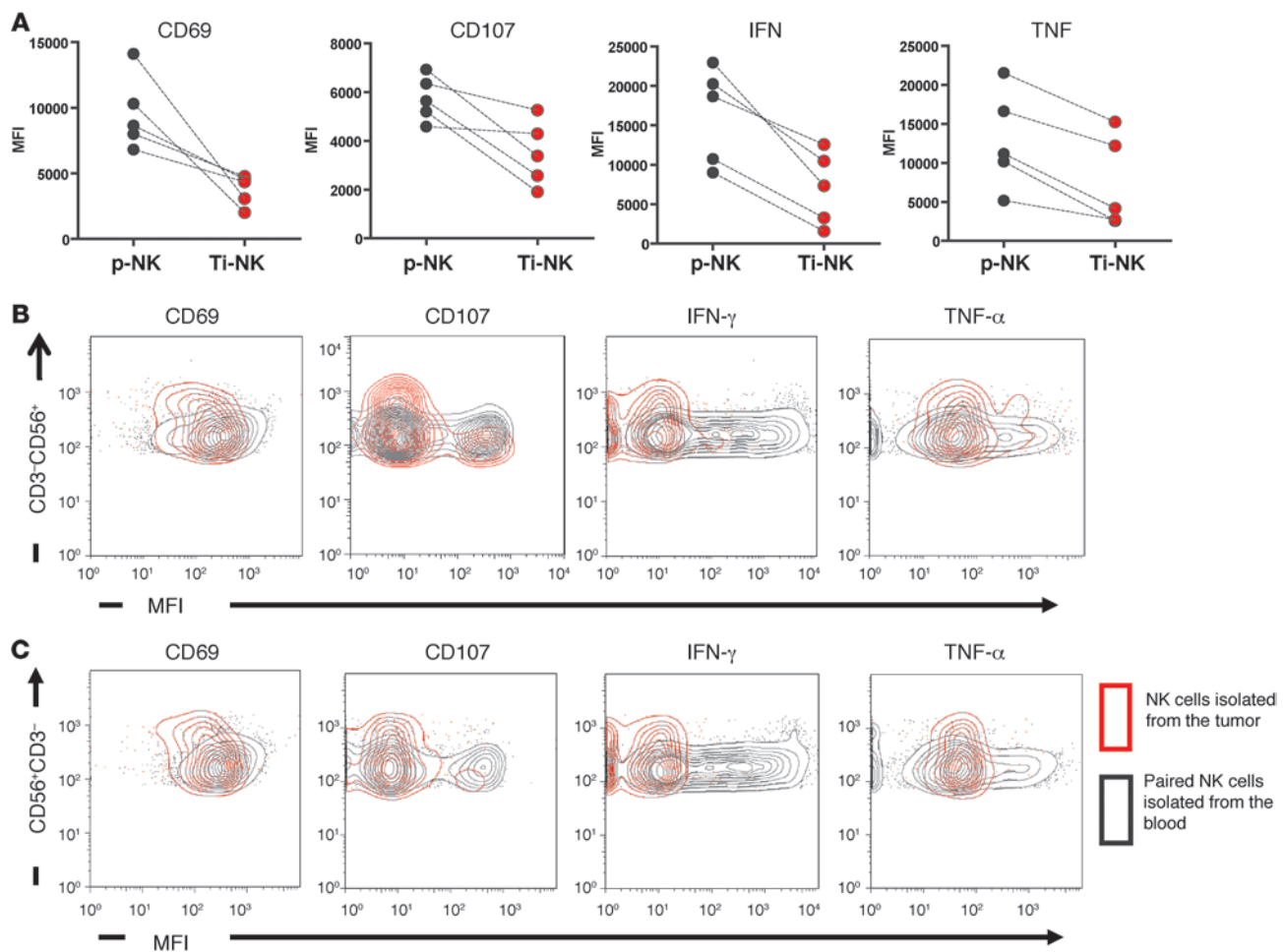
Figure 5
Functionality of NK cells infiltrating tumors (Ti-NK cells) compared with paired p-NK cells. (**A**) Isolated NK cells from malignant tissue (red) or peripheral blood (black) from paired sampled were used in direct cytotoxic assays on 5 paired samples from BC patients. (**B**) Dot plot representation of CD69, CD107, IFN-γ, and TNF-α expression in NK cells after incubation with K652 cells, according to a 1:1 E/T ratio. Isolated NK cells were incubated overnight in medium complemented with suboptimal concentrations of IL-2 and IL-15 before incubation with K562 cells for 4 hours. (**C**) Dot plot representation of CD69, CD107, INF-γ, and TNF-α expression in p-NK versus Ti-NK cells after exposure to SK-BR-3 cells in the presence of trastuzumab. The E/T ratio was of 2:1. These results were obtained on paired samples ($n = 3$).

Downregulation of group I molecules was observed in all Ti-NK cells compared with Mt-NK and p-NK cells, suggesting that it represents an alteration induced by the tumor. We searched for correlations among the expression of these 3 groups (gene clusters) and tumor aggressiveness using a metagene approach (35). The metagene expression value was determined by calculating the mean of the normalized expression values of all genes in the respective gene groups (metagenes I, II, and III). The only clear trend that emerged was that a high expression of metagene II was associated with a good-to-excellent prognosis (NPI value < 3.4) (Figure 4D). Together, our results indicate that most Ti-NK cells are CD56[Bright]NKG2A^{hi}CD16^{lo}KIR^{lo} cells with poor cytotoxic potential (PRF1^{lo}GZMB^{lo}CD57^{lo}), associated with poor outcome in BC. With the exception of the KIRs, Ti-NK cell alterations were very similar to those found in p-NK cells, but dramatically more pronounced. Indeed, we found positive correlations between the MFI of some markers expressed on Ti-NK cells with paired p-NK cells (NKp30 [$R = 0.645$, $P = 0.03$], CD16 [$R = 0.638$ $P = 0.03$],

NKG2A [$R = 0.774$, $P = 0.003$], and NKG2D [$R = 0.531$, $P = 0.07$]) (Supplemental Figure 3B). The alterations detected in peripheral blood might thus come from recirculating Ti-NK cells and/or be induced by soluble factors secreted by the tumor.

We next asked whether compromised Ti-NK cells are nevertheless able to exert a helper function, as suggested by their CD56^{bright} phenotype. CD107 functional assays comparing paired Ti-NK and p-NK cells showed that Ti-NK cells were activated more slowly (CD69 as early activation marker), had a decreased degranulation potential (CD107), and displayed strong alterations of IFN-γ and TNF-α production (Figure 5, A and B). Moreover, Ti-NK cells were unable to fulfill their ADCC function, which was not surprising considering the low proportion of CD16^{pos}CD56[Dim] cells within the tumor (Figure 5C).

In conclusion, we showed that NK cells are strongly altered within a breast tumor compared with the equivalent tumor-free environment. Alterations detected in peripheral blood are less marked but nevertheless likely to reflect what occurs in the
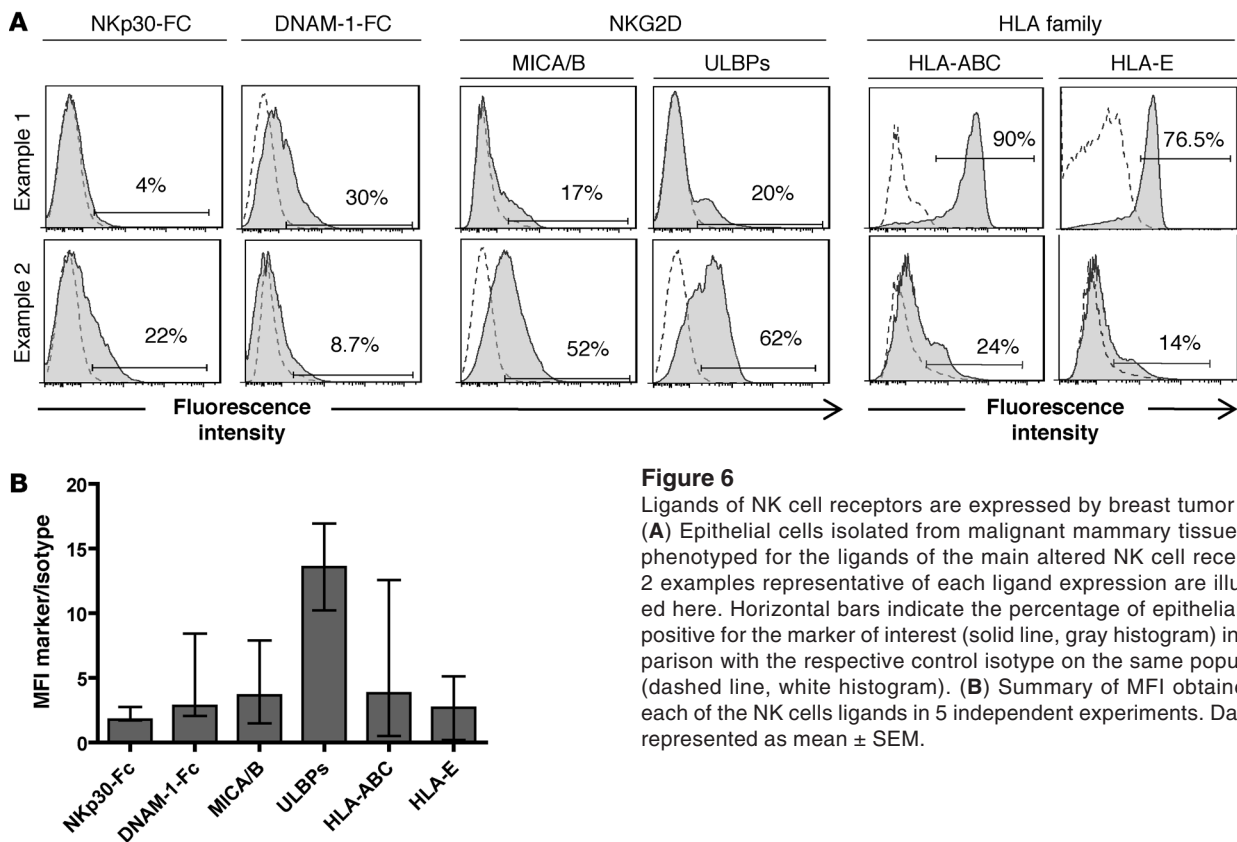
Figure 6
Ligands of NK cell receptors are expressed by breast tumor cells. (**A**) Epithelial cells isolated from malignant mammary tissue were phenotyped for the ligands of the main altered NK cell receptors. 2 examples representative of each ligand expression are illustrated here. Horizontal bars indicate the percentage of epithelial cells positive for the marker of interest (solid line, gray histogram) in comparison with the respective control isotype on the same population (dashed line, white histogram). (**B**) Summary of MFI obtained for each of the NK cells ligands in 5 independent experiments. Data are represented as mean ± SEM.

tumor. These changes are indicative of a sustained escape from NK cell antitumor immunity, probably induced by the malignant cells. This latter assumption will be assessed below.

NK cells are modulated by the tumor microenvironment

Cancer cells express ligands for NK cell receptors. NK cell alterations represent a mechanism of tumor escape or tumor immuno-editing only if the affected receptors are involved in cancer cell recognition. We thus measured the expression of NK cell ligands on breast tumor cells. We found that breast tumors express heterogeneous levels of ligands for NK cell receptors, notably regarding HLA members and NKp30-ligand (L) (Figure 6, A and B). Breast tumors also frequently expressed high levels of DNAM-1–L and NKG2D-L (Figure 6, A and B). Most importantly, we found deregulated transcriptional expression of NK cell ligands in a large set of BC ($n = 250$) compared with healthy mammary tissues ($n = 5$ pools of 5) previously profiled using DNA microarrays, suggesting that an editing process was indeed involved (Supplemental Figure 4).

Tumor cells synthesize soluble factors affecting Ti-NK cells. To determine whether the malignant cells were responsible for the observed NK cell alterations, we exposed normal NK cells to breast tumor supernatants. Tumor supernatants, but not supernatants from normal mammary tissue, induced alterations of NK cell receptors concordant with the phenotype observed on Ti-NK cells. The most prominent effect was observed on GZMB ($P < 0.05$), NKG2D ($P < 0.05$), and NKG2A ($P < 0.005$) (Figure 7A). When we exposed p-NK cells to these supernatants and then used them in cytotoxic assays, we observed a profound inhibition

of all NK cell functions, particularly IFN-γ secretion, compared with nonexposed p-NK cells (Figure 7B). These results suggest that soluble factors released by tumors (epithelial or stromal components) affect both NK cell phenotype and functions. We determined the levels of soluble factors known to be increased in other cancers and to alter lymphocyte functions, such as TGF-β1, PGE$_2$, LGALS3, and sMICA. We also measured ADAM17, a metalloproteinase involved in the shedding of MICA and other molecules modulating NK cell functions (Supplemental Figure 5). We correlated these factors with the respective expression of receptors on mammary NK cells. The resulting R coefficients were submitted to TMEV to obtain a correlation matrix (36). The levels of soluble inhibitory factors positively correlated with NKG2A expression (Figure 7C), whereas they negatively correlated with molecules related to NK cell cytotoxicity (GZMB, PRF1, CD57, KIR, CD16). The highest negative correlations were obtained with TGF-β1 and PGE$_2$. TGF-β1 also displayed the highest correlation with the NPI, showing that more aggressive breast tumors contain more TGF-β1–producing cells (Figure 7D). Blocking TGF-β1 in tumor supernatants partially restored NK cell functionality, by 20% on average (Figure 7E).

Reversion of p-NK cell alterations under remission

As a final proof that NK cell alterations are tumor induced, we established the phenotype of p-NK cells from 7 former patients with invasive BC (Ex-BC) who had undergone surgery more than 5 years ago and had not relapsed since. We matched these cases for age and pTNM classification with the BC patients from our series. Patients from the benign group (B group) were used as the
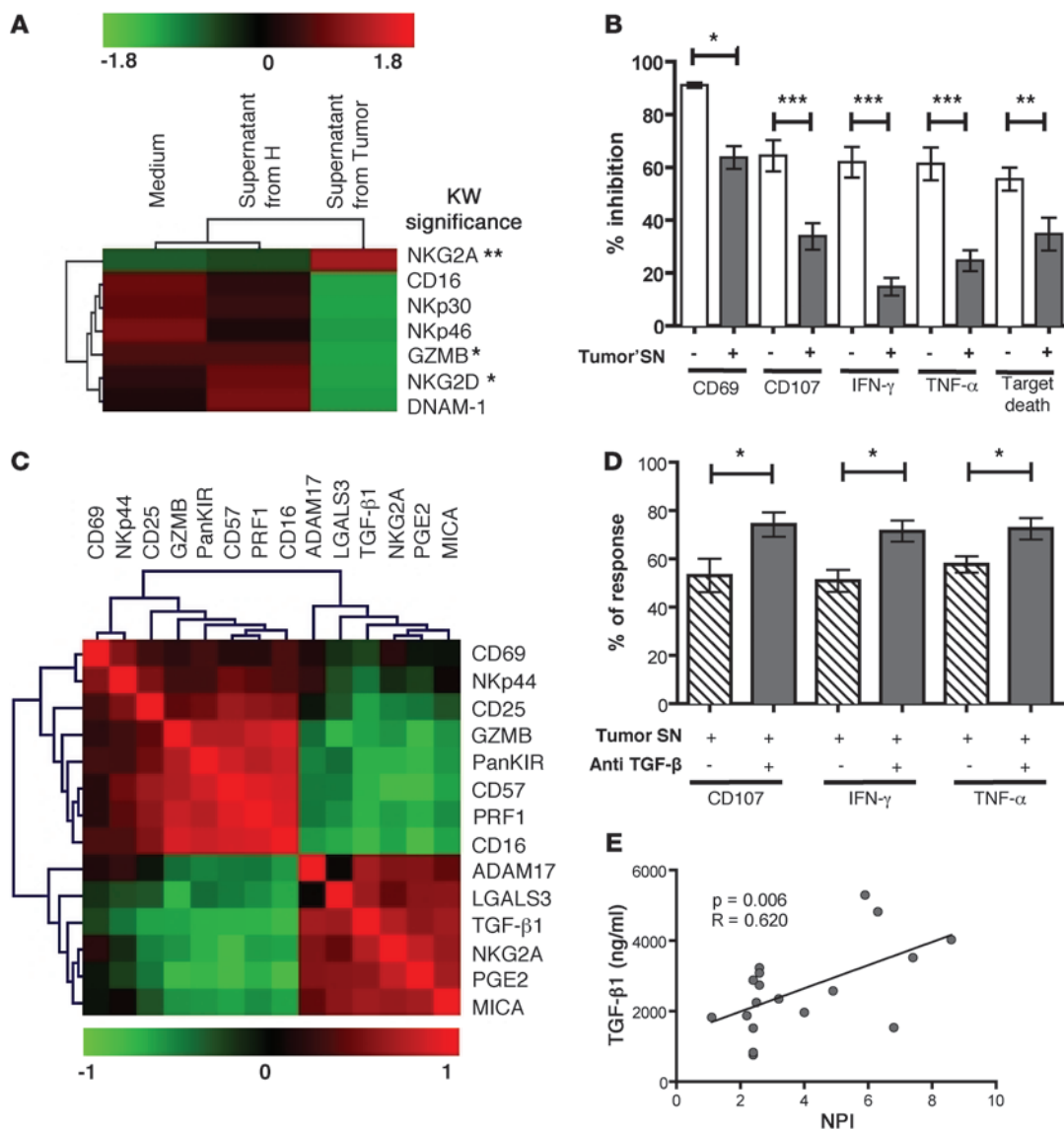
Figure 7
Impact of breast tumor stroma on p-NK cells. (**A**) p-NK cells were cultured for 48 hours in complete medium, dissociation supernatant from tumor-free samples (H, $n = 4$), or dissociation supernatant from malignant samples (T, $n = 12$). The main NK cell receptors were then phenotyped. The resulting expressions were submitted to TMEV. The significance of the observed variation was measured with a Kruskal-Wallis (KW) test. (**B**) Alterations of NK cell functions were measured from 10 different tumor supernatants. CD69 expression, CD107 degranulation, IFN-γ, and TNF-α synthesis and percentage of absolute dead K562 cells were measured from p-NK cells cultured in complete medium (white bars) and p-NK cells exposed to tumor supernatant (gray bars). (**C**) The correlation matrix of the R coefficients was obtained with a Spearman's test between the quantitative levels of tissue-associated soluble factors and paired NK cell receptors expression. (**D**) Correlation between TGF-β1 levels found in breast tumor supernatants and the respective NPI. (**E**) Partial restoration of NK cell functions after preincubation of tumor supernatants ($n = 10$) with blocking TGF-β1 antagonist antibody. Variations were evaluated with a nonparametric Mann-Whitney U test. *$P < 0.05$; **$P \le 0.005$; ***$P \le 0.0005$. Data are represented as mean ± SEM.

control for a nonmalignant situation. NKp30, CD16, NKG2D, and NKG2A receptors were expressed at similar levels in the B group and Ex-BC patients, respectively up- and downregulated compared with matched NK cells isolated from the BC groups, indicating that a normal NK cell phenotype was restored in the Ex-BC patients after tumor removal (Figure 8). In conclusion, our data show that BC cells can shape a strong inhibitory microenvironment able to inhibit NK cell antitumor immunity.

Discussion
Understanding how the immune system affects the development and progression of solid tumors remains one of the most challenging questions in immunology. It has been previously observed that mice with defects in IFN-γ signaling or antitumor immune cells are more likely to develop primary tumors (5, 37, 38). Accordingly, antitumor immunity is frequently deficient in patients with solid tumors (28, 29). We have confirmed here that p-NK cell activity, notably IFN-γ pro-
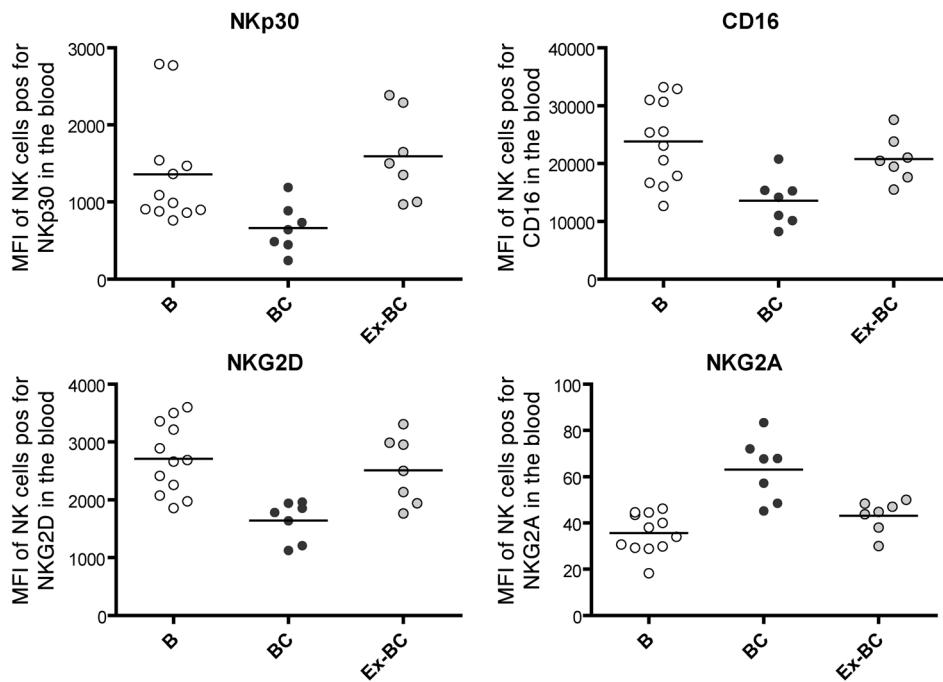
Figure 8
Reversibility of p-NK cell phenotype in patients in remission. The phenotypes of NKp30, CD16, NKG2D, and NKG2A receptors were restored in former BC patients compared with matched (age, group, and TNM classification) BC patients at diagnosis. BC (*n* = 7); B (*n* = 12); Ex-BC (*n* = 7). Data are represented as mean ± SEM.

duction, is strongly impaired in invasive breast tumors (39, 40), especially when the stage of disease is advanced. In situ, the immune infiltrate has long been neglected, but we now know that it is not the quantity but the quality of the immune infiltrate, mostly composed of cytotoxic cells, that can predict tumor outcome of several cancers, such as early-stage colorectal cancer, gastrointestinal tumor, pulmonary adenocarcinoma, and, more recently, BC (41–43).

Our data show that at least 4 major mechanisms are associated with an escape from NK cell antitumor immunity in invasive breast tumors. First, we showed that breast tumor cells alter NK cell functions through the modulation of their surface receptors, a mechanism observed in several other malignancies (28, 29, 44–47). These alterations are associated with invasive characteristics and a poor prognosis. Among invasive BCs, the cases in which NK cell infiltrates still displayed high expression of NCRs, NKG2D, and DNAM-1 receptors, and so which could still be considered as activated cells, have a good prognostic index. NK cells were our primary population of interest considering that invasive tumors preferentially develop in an NK-deficient mouse background (6, 7). However, some of the affected receptors, such as DNAM-1, NKG2D, and NKG2A, are also expressed by specific populations of T cells. We found that these receptors are also altered on tumor-infiltrating T cells, but we did not detect any association with the acquisition of invasive characteristics (data not shown). Data obtained on large cohorts have demonstrated a beneficial effect on BC patients' survival of the presence in the tumor of cytotoxic T cells at diagnosis, suggesting that these cells also play a major role in breast tumor outcome. It would thus be interesting to look at the functional behavior and/or regulation of other antitumor immune cells. By inducing the downregulation of activating receptors and the upregulation of inhibitory receptors on lymphocytes, the tumor might become "invisible" to antitumor immunity.

Second, we looked at the immunogenicity of breast tumors and found that both the protein and mRNA profiles of NK cells' ligands are different from the profiles seen in healthy mammary tissues. This is also a phenomenon frequently observed in several tumors (28, 48, 49). The heterogeneity of the profiles suggested that the phenotype of tumor cells at diagnosis is the result of a more or less successful immuno-editing (28, 50).

Third, the alterations of Ti-NK cells, and their reversibility in BC patients in long-term remission, suggested that the tumor induces its own tolerance from NK cell antitumor immunity (51). We tried to identify how the tumor might influence the expression of activating and inhibitory ligands. Based on previously published data, we showed that the alterations of NK cell receptors could be induced by the breast tumor microenvironment. TGF-β1 was an obvious candidate because it is a powerful inhibitor (52–54) elevated in advanced breast tumors (55). Accordingly, blocking TGF-β1 in mice suppresses the occurrence of metastases and restores NK cell activity (56). Furthermore, TGF-β1 is also one of the key factors promoting epithelial-to-mesenchymal transition (EMT), a major mechanism active at the invasive front of solid tumors and responsible for disease progression, metastatic potential, and chemotherapeutic resistance (57–60). Mesenchymal stem cells, which support cancer growth in situ or at the site of BC metastasis, can protect BC cells from immune recognition directly by producing TGF-β1 or indirectly by increasing the recruitment of Tregs (61, 62). This is in line with the correlations we observed between the in situ levels of TGF-β1 or Treg infiltrate and NK cells' impaired phenotype and functions (refs. 31, 63, 64; Figure 7 and Supplemental Figure 6). However, because TGF-β1 only partially explained this effect, we did not limit our study to TGF-β1 and Tregs as

potential inhibitors of NK cell functions. We found that not only PGE$_2$ (65, 66), sMICA (67), LGALS3 (68), and ADAM17 (69), but also IDO1 (data not shown and ref. 70) contributed to the immunosuppressive environment. All these molecules have previously been implicated in tumor progression and impairment of NK cell phenotypes and functions. In the MMTV/Neu mouse model, which spontaneously develops invasive mammary tumors (71), we observed alterations of NK cell phenotypes and functions concordant with those found in humans (Supplemental Figure 7). It would be interesting to determine whether the administration of blocking antibodies against the various actors of the tumor microenvironment previously mentioned, alone or in combination, could delay or accelerate invasive tumors and/or metastasis occurrence in this model.

Finally, we looked at how the microenvironment influences final maturation of NK cells in tissues. A limited amount of information on other tissue-infiltrating NK cell phenotypes is available (72, 73). NK cells usually show tissue-specific patterns of expression and disturbance of the cytotoxic CD56^{Dim}CD16$^+$KIR$^{+/-}$ and helper/precursor CD56^{Bright}CD16$^-$KIR$^-$ balance (74, 75). Notably, human NK cells collected from nonreactive LNs are CD16$^-$KIR$^-$, whereas NK cells derived from reactive/efferent LN and blood express CD56^{Dim}CD16$^+$KIR$^+$ (76). The current belief is that immature CD56^{Bright}NK cells acquire these molecules in the LN during inflammation and then circulate as KIR$^+$CD16$^+$NK cells. CD56^{Bright}CD16$^-$KIR$^-$ and CD56^{Dim}CD16$^+$KIR$^{+/-}$ NK cells might thus correspond to sequential steps of differentiation, like the CD56^{Bright}CD16^{low}KIR$^{+/-}$ found in breast tumors (77, 78). Together, these observations support the hypothesis that secondary lymphoid organs, or any microenvironment, can be the site not only of human NK cell final maturation but also of self-tolerance acquisition during immune reaction. Several recent studies showing that NK cell developmental programming is not entirely fixed and that mature NK cells can be reeducated by their environment support this hypothesis (79–81). We showed that BC Ti-NK cells expressed activating molecules (CD56^{Bright}, CD25, CD69, NKp44) like mature activated NK cells, but the levels of all other markers were instead characteristic of immature and nonfunctional NK cells (82). The most intriguing feature was the disappearance of the KIR, otherwise present on Mt-NK cells, confirming that Ti-NK cells could not exert cytotoxic functions like noneducated cells (83). The education process strongly depends on the engagement of activating and inhibitory receptors (84), but ligands were extremely heterogeneous in the different breast tumors, whereas KIR loss was homogeneous. We suggest that a strong inhibitory environment can reorient or reverse the transcriptional program of NK cell maturation toward a nonreactive self-tolerant profile.

In conclusion, our study highlights the role of NK cells in the control of invasive breast tumors, suggesting that restoring NK cells' antitumor efficiency (with adoptive transfer of preactivated or alloreactive NK cells) after surgery might help clear residual tumor cells as demonstrated in other malignancies (85–87). Most importantly, we showed that invasive breast tumors induce self tolerance in NK cells, resulting in attenuated malignant cell immunogenicity and the creation of a multifaceted immunosuppressive microenvironment that blunts NK cells' cytotoxicity and prevents their final maturation process. NK cell subversion is thus another active mechanism, in addition to the other breast tumor–derived mechanisms such as increased Treg infiltrates, dendritic cell modulation, and inhibitor secretion, that contributes to breast tumor progression. Finally,

these results are particularly interesting in the context of BC dormancy and immune evasion and highlight the importance of devising future therapies able to enhance NK cell cytotoxicity efficiently to further prevent invasive breast tumor recurrence (88).

Methods

Patients. Benign breast tumor and BC patients 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 or therapeutic act, before administration of any treatment related to tumor progression. Fresh samples were extemporaneously treated, before the determination of the diagnosis by the surgeon. After analysis of morphological tumor characteristics by pathologists, patients were retrospectively classified into 5 groups, as follows (Table 1): patients with a benign tumor (the B group), patients with an in situ cancer (the Tis group), patients with invasive localized cancer (pT1N0 an pT2N0, referred to as the LOC group), patients with invasive locally advanced cancer (pT2N1-2 to pT4, referred as the LA group), and patients whose initial breast tumor had given rise to metastases in distant organ(s) (referred to as the M group). We also included a control group of former BC patients, followed in our institution, who had undergone surgery at least 5 years ago and had not relapsed since. NPI was used to establish the precise prognostic value in invasive tumors, based on 3 factors: tumor grade, number of LNs involved, and size of the tumor.

NK cell phenotype by flow cytometry. 100 µl of fresh whole blood or 0.7×10^6 Ti-cells were incubated with the appropriated antibodies (Supplemental Table 1) on a rocking platform for 30 minutes. Red blood cells were lysed with OptiLyse B (Beckman Coulter). Samples were extemporaneously analyzed on a BD FACS Canto (BD Biosciences). Before and after reading patient samples, fluorescence intensities of the FACS Canto were standardized over time with PMT 7-Color Setup Beads (BD Biosciences) to compensate for fluorescence intensity variability. The gating strategy consisted of the elimination of the doublets based on the FCS-A/FCS-H parameters, followed by the removal of dead cells. NK cell population was selected based on the following phenotype: CD45^{pos}CD3^{neg}CD56^{pos}. Treg-cells were selected on the following expression: CD45^{pos}CD3^{pos}CD4^{pos}CD25^{hi}CD127^{lo}.

NK cell isolation from peripheral blood samples. NK cells were negatively isolated with the NK cell StemSep System (StemCell Technology) according to the manufacturer's instructions. The purity and viability of sorted cells were established and always greater than 94%.

NK cells were incubated in RPMI, 10% FCS, complemented with suboptimal concentrations of IL-2 (100 U/ml; Proleukine, Chiron) and IL-15 (5 µg/ml; R&D Systems) overnight.

Isolation of epithelial tumor cells from mammary tissue. The minced mammary tumor was incubated with 1× collagenase/hyaluronidase for 16 hours at 37°C on a rotary shaker, according to the manufacturer's instructions (StemCell Technology). Briefly, the liquefied fat layer and supernatant were discarded after centrifugation. The pellet, enriched in epithelial cells, was resuspended in prewarmed trypsin-EDTA, and then washed. Cells were resuspended in prewarmed dispase (5 mg/ml) complement with DNase I (1 mg/ml). After additional washes, cells were counted and resuspended in PBS, 2% BSA, for fluorescent staining if viability exceeded 75%.

Isolation of lymphocyte infiltrates in mammary tissue. A section made within the core of the malignant area was selected by the pathologist and extemporaneously cleared of pieces of fat, then weighed; an equal volume (w/v) of medium (RPMI 1640) was added to the tumor. The tumor was mechanically disrupted; then the supernatant was harvested and centrifuged at high speed to remove residual cellular elements. This supernatant, later referred to in the text as "supernatant of dissociation," was aliquoted and

frozen at –20°C until further use. The mean volume of the tumor supernatant of dissociation was 0.640 ± 0.420 ml. After mechanical disruption and removal of the supernatant of dissociation, the tumor was digested for 1 hour under agitation with collagenase Ia (1 mg/ml) and DNase I (50×10^3 units/ml; Sigma-Aldrich). Cell suspension was then used for flow cytometry staining or NK cell isolation if viability was greater than 80%.

Cytotoxic activity analysis. NK cells were tested for cytotoxic activity against the leukemic HLA-I[neg] K562 cell line (direct cytotoxicity), the FcγR-positive P815 mastocytoma murine cell line (redirected cytotoxicity). or the erbB-2–overexpressing BC cell line SK-BR-3 (used to measure ADCC potential of NK cells in the presence of trastuzumab, the therapeutic mAb targeting erbB-2). These cytotoxic tests were all done in 4-hour assays. The measured parameters were degranulation (CD107a and CD107b) and cytokine production (IFN-γ and TNF-α) by NK cells or the percentage of absolute number of dead targets, referred to as the cell death index (CDI). The respective effector/target (E/T) ratios are indicated in the figure legends.

In redirected experiments, NK cells were preincubated (20 minutes at 37°C) with saturating amounts of purified antibodies (IgG1, anti-CD16, anti-NKp46, anti-NKG2D, anti-NKG2A, anti-KIR, anti-NKp30, and anti-DNAM-1 mAb), then used in cytotoxic assays.

In ADCC experiments, SK-BR-3 cells were preincubated (30 minutes at 4°C) with increasing amounts of purified trastuzumab, then used in cytotoxic assays.

For flow cytometric experiments measuring the degranulation and cytokine production of NK cells and CD107a and CD107b mAb plus GolgiStop, 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 CD56 and permeabilized with Cytofix/Cytoperm (BD Biosciences). Intracellular antibodies were next added (IFN-γ and TNF-α) before cell analysis.

For cytotoxic assays measuring the CDI (percentage of dead targets compared with targets not exposed to NK cells), experiments were done in True-Count Beads Tubes (BD Biosciences) to precisely quantify the number of NK cell–mediated dead cells. Dead cells were visualized with the Live/Dead red reagent according to the manufacturer's instructions (Invitrogen).

For the study of alterations induced by breast supernatants, purified NK cells were cultured with or without healthy or tumor supernatants (dilution 1:2) before staining 48 hours later or used in CD107 assays 40 hours later (followed by 4 hours of CD107 assay).

Finally, 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 anti–TGF-β1 (20 μg/ml).

Immunohistochemistry. Immunohistochemical staining was performed on 5-μm cryostat sections fixed in 4% paraformaldehyde. After neutralization of the endogenous peroxidase and saturation, sections were layered with the anti-CD56 mAb (Dako) or isotype-matched control mAb, revealed with the Vectastain ABC Kit (Dako), and counterstained with Gill hematoxylin (Merck). Although CD56 is not strictly specific for NK cells, the percentage of CD56^{+}CD3^{+} cells determined by flow cytometry staining was negligible. Therefore, we estimated that CD56 staining gave a reasonable representation of NK cell infiltrates within the tumor.

ELISA. ELISAs were performed according to the manufacturer's instructions. sMICA, TGF-β1, LGALS3, and ADAM17 were purchased from R&D Systems, while the PGE$_2$ ELISA kit was purchased from Cayman Chemical (Interchim).

Microarray experiments. U133+2 pan-genomic Affymetrix data from malignant and healthy mammary tissues were downloaded from the public GEO data sets (GSE21653).

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 from 5 months of age and, once mammary gland nodules were detected, tumor diameter was measured twice weekly with calipers. 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. At the end of the protocol, mice were sacrificed and breast tumors and organs were collected.

Statistics. All statistical analyses were done with StatView software. Differences between breast tumor patients' groups were evaluated using 2-tailed nonparametric unpaired Mann-Whitney U test. The comparisons between NK cell markers in paired tumor and peripheral blood samples were done with nonparametric Wilcoxon tests. Finally, all correlations between MFIs of NK cell markers expressed on Ti-NK and p-NK were evaluated with nonparametric Spearman correlation tests. The equality of the population medians among the different groups was triggered with the nonparametric ANOVA (Kruskal-Wallis) for each marker. In all figures with histograms, data are represented using the mean ± SEM. $P < 0.05$ was considered as significant.

Human study approval. This study was approved by 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 written informed consent for research use.

Mouse study approval. All animal procedures were performed in accordance with protocols approved by the local Committee for Animal Experimentation (CAE) of Marseille, France.

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