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Altered Expression of Natural Cytotoxicity Receptors and NKG2D on Peripheral Blood NK Cell Subsets in Breast Cancer Patients[1]

Nayeli Goreti Nieto-Velázquez[*,‡], Yessica Dorin Torres-Ramos[†], José Luis Muñoz-Sánchez[‡], Lorena Espinosa-Godoy[*], Susana Gómez-Cortés[*], José Moreno[*] and Mario Adán Moreno-Eutimio[*]

[*]Immunity and Inflammation Research Unit, Hospital Juárez de México, Ministry of Health, Mexico City, Mexico; [†]Immunobiochemistry Department, National Institute of Perinatology, Isidro Espinosa de los Reyes, Ministry of Health, Mexico City, Mexico; [‡]Cellular Regulation Laboratory, Biochemistry Department, National School of Biological Sciences, IPN, Mexico City, Mexico

Abstract

Human natural killer (NK) cells are considered professional cytotoxic cells that are integrated into the effector branch of innate immunity during antiviral and antitumoral responses. The purpose of this study was to examine the peripheral distribution and expression of NK cell activation receptors from the fresh peripheral blood mononuclear cells of 30 breast cancer patients prior to any form of treatment (including surgery, chemotherapy, and radiotherapy), 10 benign breast pathology patients, and 24 control individuals. $CD3^{-}CD56^{dim}CD16^{bright}$ NK cells ($CD56^{dim}$ NK) and $CD3^{-}CD56^{bright}CD16^{dim/-}$ NK cells ($CD56^{bright}$ NK) were identified using flow cytometry. The circulating counts of $CD56^{dim}$ and $CD56^{bright}$ NK cells were not significantly different between the groups evaluated, nor were the counts of other leukocyte subsets between the breast cancer patients and benign breast pathology patients. However, in $CD56^{dim}$ NK cells, NKp44 expression was higher in breast cancer patients ($P = .0302$), whereas NKp30 ($P = .0005$), NKp46 ($P = .0298$), and NKG2D ($P = .0005$) expression was lower with respect to healthy donors. In $CD56^{bright}$ NK cells, NKp30 ($P = .0007$), NKp46 ($P = .0012$), and NKG2D ($P = .0069$) expression was lower in breast cancer patients compared with control group. Only NKG2D in $CD56^{bright}$ NK cells ($P = .0208$) and $CD56^{dim}$ NK cells ($P = .0439$) showed difference between benign breast pathology and breast cancer patients. Collectively, the current study showed phenotypic alterations in activation receptors on $CD56^{dim}$ and $CD56^{bright}$ NK cells, suggesting that breast cancer patients have decreased NK cell cytotoxicity.

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Introduction

Breast cancer is a highly heterogeneous disease presenting a broad range of molecular and clinical characteristics and is the most diagnosed malignancy in women worldwide [1]. There is strong evidence that the innate and adaptive immune response plays a role in tumor growth and progression. An effective immune response may lead to recognition of tumor cells, resulting in their eradication. However, due to their genetically unstable nature, tumor cells may arise with properties that enable them to escape from the immune system [2,3].

Address all correspondence to: Mario Adán Moreno-Eutimio, PhD, Hospital Juárez de México, Av. Instituto Politécnico Nacional 5160, Colonia: Magdalena de las Salinas, CP: 07760, Delegación: Gustavo A. Madero, México City, Mexico.
E-mail: marioadan@inmunoquimica.com

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In breast cancer, more favorable clinical outcomes have been associated with the presence of higher numbers of tumor-infiltrating lymphocytes since 1922 [4]. Early studies identified tumor-infiltrating lymphocytes in breast cancer as a lymphocyte population comprising mainly cytotoxic T cells, together with varying proportions of helper T cells and B cells, and rare natural killer (NK) cells [5,6].

NK cells are important components of the innate immune system and play a central role in the defense against viral infections, as well as in tumor surveillance [7]. NK cells are also associated with the adaptive immune response through the production of cytokines. In humans, NK cells are usually defined as $CD3^{-}CD56^{+}$[8] and can be further subdivided based on CD56 expression. $CD56^{dim}CD16^{bright}$ ($CD56^{dim}$ NK) cells and $CD56^{bright}CD16^{dim/-}$ ($CD56^{bright}$ NK) cells differ in terms of phenotype, effector function, and tissue localization.

$CD56^{dim}$ NK cells constitute the majority (90%) of peripheral blood NK cells and express high levels of the low-affinity Fcγ receptor CD16, through which they can exert antibody-dependent cell-mediated cytotoxicity [9,10]. Engagement of CD16 is sufficient to induce interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) secretion, in addition to chemokine secretion. NK cell function is controlled by the integration of signals from various activation and inhibitory receptors, which bind to components of pathogens and tumoral antigens [11–13]. The most potent activation receptors of NK cells are the antibody-dependent cell-mediated cytotoxicity–mediating molecule CD16 and natural killer group 2D (NKG2D) [10–13]. Moreover, NK cells mediate "natural cytotoxicity" via a set of activating natural cytotoxicity receptors (e.g., NKp30, NKp44, and NKp46), which recognize their ligands in tumor or virus-infected cells [9,10,14].

In contrast, $CD56^{bright}$ NK cells are poorly cytotoxic and are major cytokine producers that respond to cytokines such as IL-12, IL-18, or IL-15. Although $CD56^{bright}$ NK cells constitute the minority of peripheral blood NK cells, they represent the large majority of NK cells in secondary lymphoid organs [9,10]. It remains unclear whether the $CD56^{bright}$ NK subsets are precursors of $CD56^{dim}$ NK cells or whether the $CD56^{bright}$ population represents an activated or differentiated $CD56^{dim}$ NK cell subtype.

Breast tumors act systemically to sustain cancer progression, affecting the physiological processes in the host and triggering responses in the peripheral blood cells [15]. The peripheral blood cells monitor the body's physiological status and modify their immunophenotype in response to pathological changes [16–19]. Due to its easy access, peripheral blood constitutes an interesting source to measure functional competence of immune cell subsets. Immune cell dysfunctions were found in peripheral blood from breast cancer patients detected through whole blood multiparametric flow cytometry assay [20]. Hence, the aim of this study was to evaluate the frequency of $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells in the peripheral blood of women with breast cancer, women with benign breast pathology, and healthy controls.

Materials and Methods

Patients and Healthy Donors

A group of 64 women was studied. Study participants were enrolled at the Department of Oncology of the Hospital Juárez de México. All women were informed of the goal of the study and provided informed consent. The breast cancer group comprised women matching the disease's diagnostic criteria after physical exams; mammograms; ultrasounds; blood chemistry studies; biopsies; and, in some cases, magnetic resonance imaging. The noncancerous (benign) breast tumor group comprised women matching the diagnostic criteria of fibroadenoma after physical exams, mammograms, ultrasounds, blood chemistry studies, and biopsies. The exclusion criteria for the studied groups included concurrent medical problems that may cause disordered inflammatory responses, such as diabetes and autoimmune diseases. Twenty-four blood samples from healthy women were also collected.

Blood Sample Collection

Four milliliters of peripheral blood were collected into EDTA tubes (Becton Dickinson and Company, Franklin Lakes, NJ) from the antecubital vein of each subject. All blood samples were processed within 2 hours after sampling. Peripheral blood mononuclear cells (PBMCs) were isolated using the density centrifugation technique (Ficoll-Paque PLUS; Amersham Biosciences, Uppsala, Sweden) and then immediately utilized. Total circulating counts for leukocytes, lymphocytes, monocytes, and neutrophils were determined based on 2 ml of EDTA-treated blood using a Siemens high-volume hematology analyzer with an ADVIA 2120i System with Autoslide (Siemens AG, Munich, Germany).

Flow Cytometry Analyses

To identify NK cell subsets, PBMCs were stained for surface antigens with a phycoerythrin-conjugated mAb specific for NKp44 (BD Pharmingen, San Jose, CA; clone p44-8), phycoerythrin-conjugated mAb specific for NKp46 (BD Pharmingen; clone 9E2/NKp46), Alexa Fluor 647–conjugated mAb specific for NKp30 (BD Pharmingen; clone p30-15), Allophycocyanin (APC)-conjugated mAb specific for NKG2D (BD Pharmingen; clone 1D11), allophycocyanin-Hilite 7 (APC-H7)–conjugated mAb specific for CD3 (BD Pharmingen; clone SK3), violet 450 (V450)–conjugated mAb specific for CD56 (BD Horizon; clone B159), and violet 500 (V500)–conjugated mAb specific for CD16 (BD Horizon; clone 3G8) in accordance with the manufacturer's instructions. Briefly, 1×10^6 PBMCs were stained with fluorochrome-conjugated mAbs specific for cell surface antigen markers for 20 minutes in the dark at 4°C. After initial staining, the cells were washed twice using phosphate-buffered saline at pH 7.4, followed by surface marker fixation. The negative control samples were incubated with isotype-matched antibodies. After incubation, the cells were resuspended in 200 μl of phosphate-buffered saline for subsequent flow cytometry analysis using a FACS Verse flow cytometer (BD Biosciences, San Jose, CA). The resultant data were analyzed using FlowJo software V10.0.8 (Tree Star, San Carlos, CA).

Lymphocytes were defined and separately gated on the basis of forward light scatter and side light scatter for further analysis. Furthermore, the proportions of the major subsets of cells stained by antibodies were determined using gating of individual areas based on the first-gated lymphocytes. NK cells were divided into two subsets on the basis of CD56 surface density. $CD3^{-}CD56^{dim}CD16^{bright}$ NK cell ($CD56^{dim}$ NK) and $CD3^{-}CD56^{bright}CD16^{dim/-}$ NK cell ($CD56^{bright}$ NK) subsets were distinguished using gated flow cytometric analyses (Figure 1). Absolute cell counts were derived by multiplying the percentage of a given cell subset by the total lymphocyte concentration found in the peripheral blood. The results were expressed as the percentage of cells in a gated $CD3^{-}$ region.

Ethics Statement

The Hospital Juarez of Mexico Scientific Research Committee (composed of Scientific, Ethics, and Bio-security Committees) approved the project (project number: HJM 2321/14B), and the protocols that

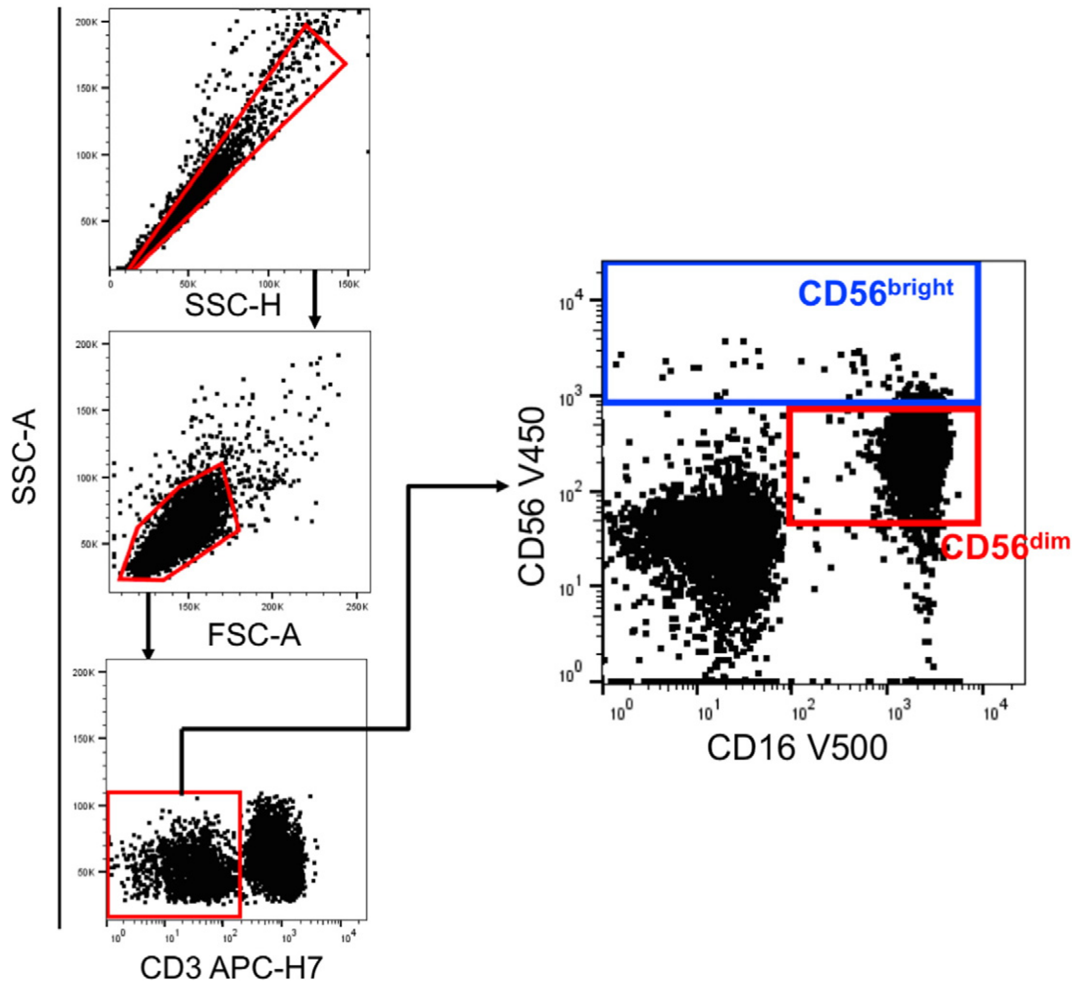

Figure 1. Gating strategies for flow cytometry analysis of CD56^{dim} NK and CD56^{bright} NK cells. Representative gating strategy of peripheral blood breast cancer patient. PBMCs were isolated using the density centrifugation technique. To identify NK cell subsets, PBMCs were stained for surface antigens with APC-H7–conjugated mAb specific for CD3, V450-conjugated mAb specific for CD56, and V500-conjugated mAb specific for CD16. Dot plot CD56 V450 versus CD16 V500 graph, lower box: CD56^{dim} NK cell subset (CD3^{-} CD16^{+} CD56^{dim}), upper box: CD56^{bright} NK cell subset (CD3^{-} CD16$^{+/-}$ CD56^{bright}).

were used conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All enrolled individuals provided written informed consent.

Statistics

The distribution of the data was tested for normality using the Kolmogorov-Smirnov test. Student's *t* test was used for continuous variables that were normally distributed, and the nonparametric Mann-Whitney *U* test was used to compare cell percentages between groups. *P* values <.05 were considered significant. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).

Results

Thirty breast cancer patients ranging from 27 to 84 years of age and 10 noncancerous breast tumor patients ranging from 24 to 69 years of age were analyzed between April 2014 and March 2016, as were 24

Table 1. Sociodemographic Characteristics of the Study Groups

Group	Healthy Donors (n = 24)	Noncancerous Breast Tumor (n = 10)	Breast Cancer (n = 30)	P Value
Age, mean ± SD	49.6 ± 13.4	44.8 ± 12.4	55.3 ± 13.5	P = .062^aP = .069^b
First menstruation age, mean ± SD	12.5 ± 1.4	12.4 ± 0.8	12.8 ± 1.6	P = .618^aP = .577^b
Family history of breast cancer	12.5% (n = 3)	10% (n = 1)	16.7% (n = 5)	P = .72^aP = 1.0^b
Use of oral contraceptives	29.2% (n = 7)	60% (n = 6)	46.7% (n = 14)	P = .263^aP = .716^b
First pregnancy age, mean ± SD	20.9 ± 4.7	16.5 ± 1.2	23.2 ± 3.2	P = .099^aP < .0001^b
Number of pregnancies, mean ± SD	3.1 ± 1.4	2.3 ± 1.3	3.5 ± 1.6	P = .015^aP = .039^b
Breastfeeding	95.8% (n = 23)	100% (n = 10)	100% (n = 30)	P = 1.0^aP = 1.0^b
Obesity	25% (n = 6)	30% (n = 3)	56.7% (n = 17)	P = .027^aP = .273^b
Alcoholism	20.8% (n = 5)	20% (n = 2)	6.7% (n = 2)	P = .231^aP = .256^b
Smokers	12.5% (n = 3)	20% (n = 2)	23.3% (n = 7)	P = .483^aP = 1.0^b

Notes: Sociodemographic data were obtained by personal questionnaires. *P* value is from the Mann-Whitney *U* test for comparisons of means and from Fisher's exact test for comparisons of the categorical variables between breast cancer versus healthy donors (a) and breast cancer versus noncancerous breast tumor (b).

Table 2. Breast Cancer Tumor Characteristics

Characteristics	
Tumor size (cm), mean ± SD	4.43 ± 2.02
Tumor status	
T1	16.6% (*n* = 5)
T2	36.6% (*n* = 11)
T3/4	46.6% (*n* = 14)
Stage of breast cancer	
Stage IIA	23% (*n* = 7)
Stage IIB	27% (*n* = 8)
Stage IIIA	27% (*n* = 8)
Stage IIIB	23% (*n* = 7)
Estrogen receptors	
Positive	60% (*n* = 18)
Negative	40% (*n* = 12)
Progesterone receptors	
Positive	50% (*n* = 15)
Negative	50% (*n* = 15)
HER2/Neu	
Positive	53% (*n* = 16)
Negative	47% (*n* = 14)

Note: The presence of estrogen receptors, progesterone receptor, and HER2/Neu was detected by immunohistochemistry.

control group blood samples from healthy subjects ranging from 33 to 86 years of age. No significant differences were found in age, first menstruation age, family history of breast cancer, use of oral contraceptives, maternal lactancy, consumption of alcoholic beverages, and tobacco use among the groups studied. The group of breast cancer patients showed higher first pregnancy age and number of pregnancies, as well as greater presence of obesity (Table 1).

The main characteristics of the 4970 eligible cases included in the analysis are summarized in Table 1.

In this study, there were 30 patients with histologically confirmed breast cancer; the mean age at diagnosis was 55.3 ± 13.5 years. The tumor size ranged from 2 to 10 cm in diameter with an average (mean) of 4.43 ± 2.02 cm. Test for hormone receptor expression revealed that 18 patients were ER positive, 15 patients were PR positive, and 16 patients were HER2/Neu positive (Table 2).

There were no significant differences in the absolute numbers or percentages of white blood cells, CD4+ T cells, CD8+ T cells, B cells, NK cells, monocytes, eosinophils, neutrophils, and basophils (Tables 3 and 4).

Frequencies of Peripheral NK Cell Subsets

The group of breast cancer patients showed 90.98% (78.0-98.9) of $CD56^{dim}$ NK cells, whereas the healthy-donors group has 91.96% (86.3-97.1) of $CD56^{dim}$ NK cells in PBMC; there was no significant difference P = .6259. In the noncancerous group, the percentage of $CD56^{dim}$ NK cells was 93.95% (91.4-96.1); compared with the breast cancer patients, there was no significant difference (P = .3329) (Figure 2*A*).

With respect to the percentage of $CD56^{bright}$ NK cells in PBMC, the breast cancer patients showed 9.02% (1.1-2.2) and the healthy-donors group had 8.04% (2.9-13.6); these differences were not significant (P = .7277). The noncancerous group showed 6.05% (3.9-8.6) of $CD56^{bright}$ NK cells in PBMC; compared with healthy donors and breast cancer patients, there were no significant differences (P = .084 and P = .3329, respectively) (Figure 2*B*).

Expression of NK Cell Activating Markers

To determine whether breast cancer modulates the expression of the main NK cell activating receptors involved in tumor cell killing, we evaluated the presence of NKG2D, NKp46, Nkp44, and NKp30 on peripheral blood $CD56^{dim}$ NK and $CD56^{bright}$ NK cells in breast cancer patients, noncancerous breast tumor patients, and healthy donors women. Our results showed that on $CD56^{dim}$ NK cells, NKp44 expression determined by mean fluorescence intensity by flow cytometry was significantly higher in women with breast cancer than in healthy donors (P = .0302), although no differences were found between the healthy control group and noncancerous breast tumor group.

Interestingly, no differences were found in NKp44 expression on $CD56^{bright}$ cells between any of the groups studied (Figure 3*A*).

We found that breast cancer patients showed decreased NKG2D expression in both NK cell subsets compared with both the healthy control group ($CD56^{dim}$ NK, P = .0001; $CD56^{bright}$, P = .0001) and the noncancerous breast tumor group ($CD56^{dim}$ NK, P = .0005; $CD56^{bright}$, P = .0069). Furthermore, in both NK cell subsets, noncancerous breast tumor patients showed decreased NKG2D expression compared with the control group ($CD56^{dim}$ NK, P = .0005; $CD56^{bright}$, P = .0069) and increased NKG2D expression compared with breast cancer patients ($CD56^{dim}$ NK, P = .0439; $CD56^{bright}$, P = .0208) (Figure 3*B*).

Finally, our analysis indicated decreased expression of NKp30 on NK cell subsets in breast cancer patients ($CD56^{dim}$ NK, P = .0001; $CD56^{bright}$, P = .0001) and noncancerous breast tumor patients ($CD56^{dim}$ NK, P = .0005; $CD56^{bright}$, P = .0007) compared with healthy controls group (Figure 3*C*). Furthermore, the expression of NKp46 on NK cell subsets showed the same pattern (breast cancer versus control: $CD56^{dim}$ NK, P = .0001; $CD56^{bright}$, P = .0001; noncancerous versus control: $CD56^{dim}$ NK, P = .0298; $CD56^{bright}$, P = .0120) (Figure 3*D*).

Discussion

There is ample evidence that NK immune surveillance is of crucial importance for solid tumors [21]. Detecting subsets of immune cells may be one beneficial way to understand immune function, which may assist in clinical diagnoses of diseases and provide evidence for disease pathogenesis, course, and prognosis [22]. We implemented this study using peripheral blood in an attempt to assess immune function variation by measuring NK cell subsets. An important strength of our study was the use of breast cancer patient blood samples that had been taken prior to any form of treatment, including surgery, chemotherapy, and radiotherapy. This allowed us to examine differences in host immune status without the confounding influence of treatment effects.

Our results did not show any significant changes in NK cell numbers and frequency in peripheral blood of untreated breast cancer

Table 3. Absolute Numbers and Percentages of White Blood Cells of Breast Cancer and Noncancerous Breast Tumor Patients

Group	Noncancerous Breast Tumor (*n* = 10)		Breast Cancer (*n* = 30)		*P* Value
	($\times 10^3/\mu l$)	(%)	($\times 10^3/\mu l$)	(%)	
Lymphocytes	2.3 ± 0.7	28.2 ± 5.2	2.2 ± 0.6	28.5 ± 7.8	P = .684^aP = .912^b
Monocytes	0.4 ± 0.1	4.9 ± 1.1	0.4 ± 0.1	5.4 ± 1.1	P = .416^aP = .100^b
Eosinophils	0.2 ± 0.1	2.0 ± 0.9	0.2 ± 0.1	2.1 ± 1.4	P = .648^aP = .753^b
Neutrophils	5.2 ± 1.3	62 ± 5.4	4.6 ± 1.4	59.5 ± 10.5	P = .229^aP = .390^b
Basophils	0.04 ± 0.02	0.5 ± 0.3	0.05 ± 0.03	0.7 ± 0.4	P = .161^aP = .144^b

Notes: Data were obtained by Siemens high-volume hematology analyzer with an ADVIA 2120i System. Data are presented as absolute number (×103/μl) and percentages (%) of peripheral blood leukocytes. *P* value is from the Mann-Whitney *U* test for comparisons of means of absolute number (a) and percentage (b) of cells between breast cancer versus noncancerous breast tumor.

Table 4. Absolute Numbers and Percentages of T Cells, B Cells, and NK Cells of the Study Groups

Group	Healthy Donors (n = 24)		Noncancerous Breast Tumor (n = 10)		Breast Cancer (n = 30)		P Value
	(×10³/μl)	(%)	(×10³/μl)	(%)	(×10³/μl)	(%)	
T cells (CD3+/CD45+)	1.2 ± 0.4	19.2 ± 1.7	1.6 ± 0.5	19.5 ± 3.6	1.5 ± 0.4	19.8 ± 5.5	P = .0045[a] P = .8027[b] P = .2883[c] P = .7311[d]
CD8+ T cells	0.4 ± 0.1	30.6 ± 4.9	0.56 ± 0.16	35.2 ± 3.2	0.57 ± 0.17	37.4 ± 2.9	P < .0001[a] P = .6846[b] P < .0001[c] P = .0676[d]
CD4+ T cells	0.6 ± 0.2	53.3 ± 4.9	0.89 ± 0.27	54.8 ± 5.1	0.82 ± 0.24	54.2 ± 5.1	P = .0021[a] P = .5953[b] P = .8074[c] P = .3903[d]
CD19+ B cells (CD3–/CD45+)	0.3 ± 0.1	5.2 ± 1.6	0.4 ± 0.13	4.8 ± 0.9	0.37 ± 0.14	4.9 ± 1.8	P = .1635[a] P = .5844[b] P = .4805[c] P = .9378[d]
CD16+CD56+ total NK cell (CD3-CD19/CD45+)	0.2 ± 0.1	3.4 ± 1.3	0.27 ± 0.1	3.3 ± 0.8	0.24 ± 0.09	3.2 ± 1.1	P = .1337[a] P = .3902[b] P = .5714[c] P = .6732[d]

Notes: T cells, B cells, and NK cells are presented as absolute number (×10³/μl) and percentages (%) of peripheral blood leukocytes. CD8+ T cells and CD4+ T cells are presented as percentages (%) of T cells. P value is from the Mann-Whitney U test for comparisons of means of absolute number (a) and percentage (c) of cells between breast cancer versus healthy donors and absolute number (b) and percentage (d) of cells between noncancerous breast tumor versus healthy donors.

patients, which is in agreement with a previous study that did not find alteration in NK cell numbers in breast cancer based on expression of CD56 and CD16 [23]. However, another group showed an increased percentage of $CD3^-CD56^+$ cells in peripheral blood of breast cancer patients [24,25]. These discrepancies could be attributed to the fact that NK cells were characterized with only two markers (CD3 and CD56) and patients were receiving adjuvant tamoxifen therapy at the time of testing, which has been suggested to have immunomodulatory effects [26].

It was reported that malignant breast tissues had less $CD56^{dim}CD16^+$ cells than healthy mammary tissues; furthermore, breast tissues had increased infiltration of $CD56^{bright}$CD16 NK cells [25,27,28], and this would indicate that tumor-infiltrating NK cells had poor cytotoxic capacity. However, this phenomenon is not reflected by the frequencies NK cell subsets in peripheral blood, suggesting that altered NK cell subset numbers depended on the tumor microenvironment. But we found differences in the expression of natural cytotoxicity receptors and NKG2D on $CD56^{dim}$ NK and $CD56^{bright}$ cells; it could be indicate that NK cells gain immunoregulatory properties which may partly explain the low cytotoxic functions of NK cells in breast cancer reported earlier [23,28,29].

NKp44 expression is restricted to activated NK cells capable of initiating an immediate cytotoxic response [30]. NKp44 is implicated in the recognition and killing of numerous types of cancer, including neuroblastoma; choriocarcinoma; pancreatic adenocarcinoma; lung adenocarcinoma; colon, cervix, and hepatocellular carcinoma; and prostate and breast carcinoma [31–33]. Our result showed upregulated levels of NKP44 on $CD56^{dimm}$ NK cells, which would indicate a good outcome in killing tumor cells, but tumors may also downregulate NKp44 surface expression by shedding soluble MHC class I chain-related molecules or by releasing indoleamine 2,3-dioxygenase and prostaglandin E2 [34,35]. Additionally, tumor cells may induce expression of exosomal proliferating cell nuclear antigen when physically contacted by NKp44 expressing NK cells to inhibit NK cell effector function [36].

Interestingly, the cytoplasmic tail of NKp44 contains a tyrosine motif resembling an ITIM; contrary to initial reports, this motif is functional and inhibits the release of IFN-γ [30,36,37]. NKp44 surface expression is dependent on its association with the ITAM containing DAP12 accessory protein, which results in the release of cytotoxic agents, TNF, and IFN-γ [37]. We highlight the importance of further studies for unraveling the precise signaling of NKp33 in breast cancer patients.

High expression of NKG2D ligands has been found in various types of tumors, such as ovarian cancer, colorectal cancer, and breast cancer [38–40]. Expression of NKG2D ligands may induce an immune response by binding to the NKG2D receptor, which is present in NK cells [41]. The downregulation or complete knockout

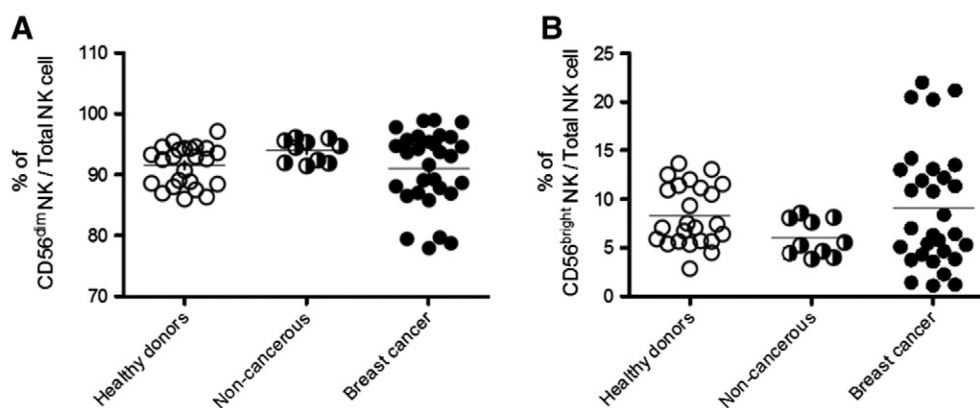

Figure 2. Frequencies of $CD56^{dim}$ ($CD3^-$ $CD16^+$ $CD56^{dim}$) NK and $CD56^{bright}$ ($CD3^-$ $CD16^{+/-}$ $CD56^{bright}$) NK cells. Percentage of each subset from total NK cells of 24 healthy donors, 10 noncancerous patients, and 30 breast cancer patients. PBMCs were stained for surface antigens with APC-H7–conjugated mAb specific for CD3, V450-conjugated mAb specific for CD56, and V500-conjugated mAb specific for CD16. There were no significant differences between the groups.

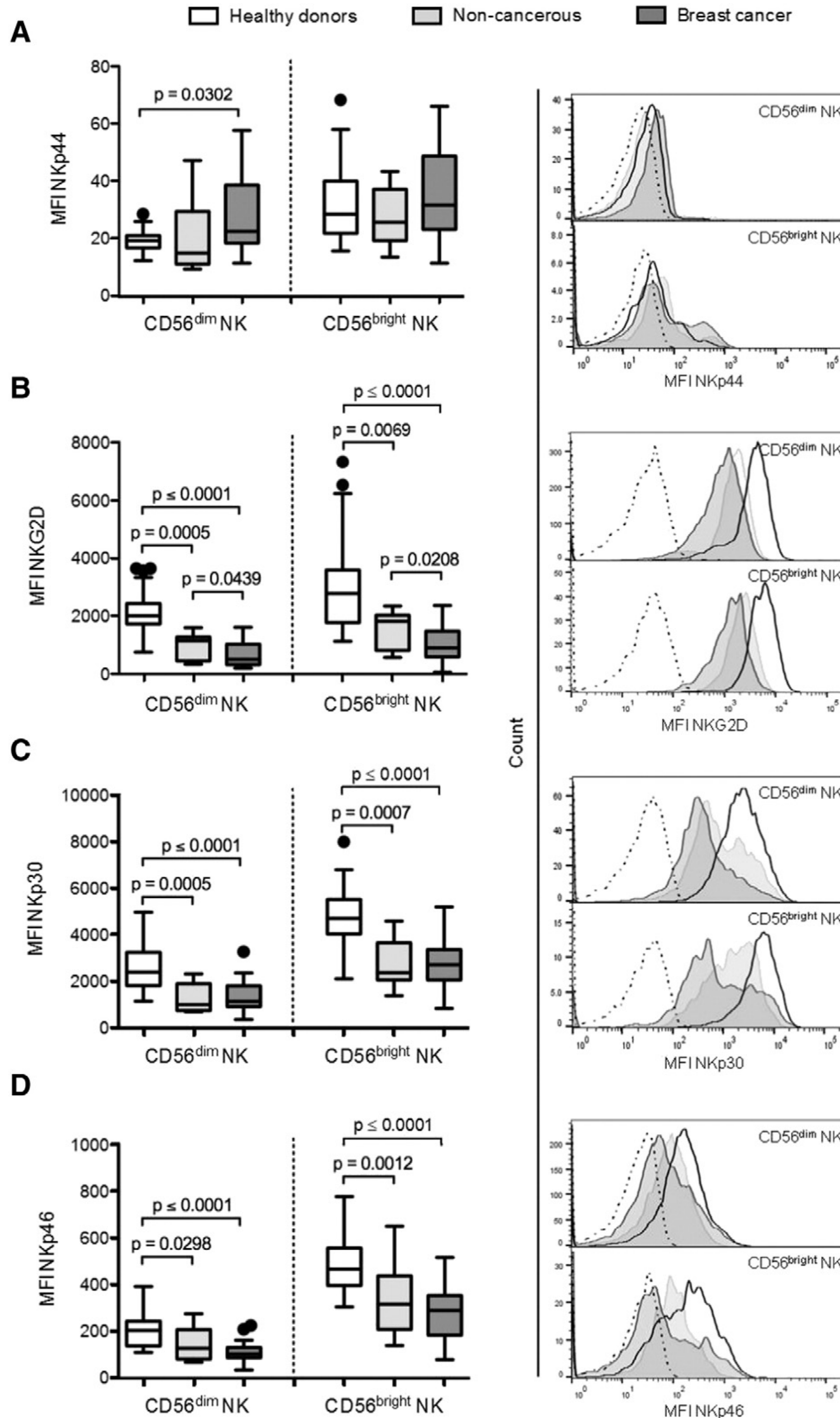

Figure 3. Phenotype analysis of activation receptors on peripheral CD56^{dim} (CD3^{-} CD16^{+} CD56^{dim}) NK and CD56^{bright} (CD3^{-} CD16$^{+/-}$ CD56^{bright}) NK cells. Box plots show expression of cytotoxicity activation markers of 24 healthy donors, 10 noncancerous patients, and 30 breast cancer patients. PBMCs were stained with mAb. Each box plot has a representative histogram of the activation receptor on CD56^{dim} and CD56^{bright} NK cells; the threshold between negative and positive was defined by the fluorescence minus one (FMO) method (dotted line). (A) MFI of NKp44, (B) MFI of C-type lectin receptor NKG2D, (C) MFI of NKp30, and (D) MFI of NKp46 on CD56^{dim} NK cell and CD56^{bright} NK cell. Analysis was performed using the Mann-Whitney *U* test. *P* values of significant differences between the groups were written.

of NKG2D in mice resulted in an impaired immune response against tumor cells, higher expression levels of NKG2D ligands, and an increased incidence of certain tumors [42,43]. We found that breast cancer patients showed decreased expression of NKG2D in both NK cell subsets, which may indicate a possible evasion mechanism for tumor cells to prevent NK lysis (Figure 3*A*).

In contrast to the knowledge on the regulation of NKG2D and NKp40 in tumor immunology, the understanding of the natural cytotoxicity receptors NKp46 and NKp30 and their ligands remains limited. A recent study revealed that neuroblastoma tumor cell–derived factors caused downregulation of NKp30 in a TGF-β1–dependent manner [44] and that inhibitory NKp30 splice variants were identified that affect the prognosis of gastrointestinal sarcoma [45]. Our results showed that breast cancer patients and patients with benign tumors had decreased NKp30 and Nkp46 expression on peripheral $CD56^{dim}$ NK and $CD56^{bright}$ NK cells, which suggests that malignant cells bypass NK surveillance by downregulating these receptors. Future studies will be necessary to dissect the effects of expression of the immunosuppressive NKp30C isoform and the activating isoforms NKp30A/B in breast cancer patients.

Conclusions

The results of this study indicate that breast cancer patients have phenotypically altered activation receptors on $CD56^{dim}$ and $CD56^{bright}$ NK cells, suggesting that these patients have decreased NK cell cytotoxicity. Furthermore, this study provides further evidence that NK cells play an important role in breast cancer.

Conflict of Interest

The authors have declared that no competing interests exist.

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