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Overexpression of CD85j in TNBC patients inhibits Cetuximab-mediated NK-cell ADCC but can be restored with CD85j functional blockade

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Abbreviations: TNBC: Triple negative breast cancer, ADCC: Antibody-dependent cell-mediated cytotoxicity, Ab: antibody; EGFR: Epidermal Growth Factor Receptor; NK: Natural Killer.

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Abstract

Clinical studies suggest that triple negative breast cancer (TNBC) patients with epidermal growth factor receptor (EGFR)-expressing tumors could benefit from therapy with Cetuximab, which targets EGFR. NK cells are the primary effectors of antibody (Ab)-dependent cell-mediated cytotoxicity (ADCC) and thus play a role in Ab-based therapies. We have previously described diminished levels of Cetuximab-mediated ADCC in vitro in patients with advanced breast cancer. Here, we investigated the potential causes of this NK-cell functional deficiency. We characterized NK-cell activating/inhibitory receptors in the peripheral blood of breast cancer patients and found CD85j inhibitory receptor overexpression. The capacity of NK cells to perform Cetuximab-triggered ADCC against TNBC cells correlated inversely with CD85j expression, even in the presence of the stimulatory cytokines IL-2 or IL-15. Hence, patients expressing high levels of CD85j had an impaired ability to lyse TNBC cells in the presence of Cetuximab. We also found that CD85j overexpression was associated with HLA-I and soluble HLA-G expression by tumors. A CD85j functional blockade with a CD85j antagonist Ab restored ADCC levels in breast cancer patients and reverted this negative effect. Our data suggest that strategies which overcome the hurdles of immune activation could improve Cetuximab clinical efficacy.

Introduction

NK cells are a major component of the innate immune system and can limit the growth and dissemination of several types of tumors [1]. In cancer immune surveillance, NK cells can exert direct cellular cytotoxicity on tumor cells without prior sensitization and secrete immunostimulatory cytokines such as IFN-γ, which can control both local tumor growth and metastasis [2, 3]. Moreover, NK cells act as effectors in antibody (Ab)-dependent cell- mediated cytotoxicity (ADCC) [4]. Accordingly, it has been determined that NK-cell activity can play a significant role in the efficacy of several therapeutic mAbs used in cancer therapy [5-9].

NK-cell activities are regulated by inhibitory and activating signals following engagement of cell membrane receptors with their cognate ligands on target cells [1]. Their main ligands are MHC class I (MHC-I) molecules; NK cells preferentially lyse cells that express few or no MHC-I molecules and therefore fail to bind inhibitory receptors [10]. Furthermore, to complete NK-cell activation the activating receptors must still recognize their specific ligands on target cells [11]. Killer Immunoglobulin-like Receptors (KIRs) [12] and Leukocyte Immunoglobulin-like Receptors (LIRs) are type I transmembrane proteins of the immunoglobulin-like receptor superfamily [13]. LIR-1, the most broadly expressed LIR family member, is an inhibitory receptor that binds multiple MHC-I molecules, both classical (HLA-A, -B and -C) and non-classical (HLA-E, -F and -G) [12-16]. The inhibitory receptor LIR-1 (also known as ILT-2 and CD85j) is the only LIR expressed on NK cells [17]. Upon ligand binding, CD85j is able to inhibit NK-cell cytotoxicity [16, 18-20], as well as IFN-γ production [21]. Despite the fact that NK-cell inhibitory receptor engagement with target cells is considered a primary event in the formation of the inhibitory synapse and the subsequent prevention of cell lysis [22], CD85j receptor involvement in cancer has been poorly addressed. In lung cancer, CD85j expression was drastically reduced on intratumoral NK cells compared to blood NK cells [23]. In contrast, CD85j was up-regulated on peripheral blood (PB) NK cells from breast cancer patients with advanced disease compared to cells from healthy donors (HD) or *in situ* carcinoma patients [24].

Triple-negative breast cancer (TNBC) accounts for approximately 15 to 20 % of breast cancer cases. These tumors are defined by a diagnosis of exclusion; they lack both estrogen (ER) and progesterone receptors (PR), and do not have the overexpression of ErbB-2 (HER-2). TNBC occurs more frequently in younger patients and tends to be aggressive [25], exhibiting worse prognosis and more frequent relapse with distant metastasis than luminal subtypes [26]. There is a dire need for new, effective, target-specific therapies. TNBCs frequently express epidermal growth factor receptor (EGFR-HER-1) [27, 28]. EGFR has a diagnostic and prognostic role in basal-like TNBC and has been validated as a relevant therapeutic target in several human cancers [29].

Cetuximab is a chimeric mAb that binds EGFR, and some reports suggest that it is effective in TNBC Despite strong preclinical data, its clinical efficacy is still a matter of discussion. Two phase II clinical trials reported that Cetuximab, in combination with chemotherapeutic agents, induced responses in 20% of metastatic TNBC patients, encouraging further investigation [30, 31].

Previously, we investigated the ability of NK cells from breast cancer patients to perform Cetuximab-mediated ADCC [32]. We observed that TNBC cell lysis was significantly diminished in patients with advanced disease. In the present study, we analyzed the causes of ADCC impairment in breast cancer patients and unraveled how the overexpression of the inhibitory receptor CD85j dampens breast cancer NK-cell effectiveness against breast cancer cells. Moreover, we evaluated NK-cell functional restoration using a CD85j blocking Ab. This study provides the first functional evaluation of CD85j in breast cancer, not only by describing a tumor escape mechanism, but also highlighting a potential new therapeutic strategy.

Results

CD85j inhibitory receptor is over-expressed in breast cancer PBLs

In previously published work we describe a significant reduction in the efficiency of Cetuximab-mediated ADCC in PB NK cells from breast cancer patients with advanced disease than HD [32]. In this work we performed an exhaustive analysis of 13 NK-cell receptors on PB breast cancer NK cells to characterize the phenotypic alterations involved in that impairment (Supporting Information Fig. S1). There was a significantly higher proportion of $CD3^{-}CD56^{+}$ NK cells in breast cancer PBL as compared to HD (HD mean 8.4 ± 3.6%; breast cancer patients mean 12.2 ± 6.3%; Supporting Information Fig. S1B). The comparative analysis of NK-cell receptor expression revealed that most receptors were similarly expressed in HD and breast cancer patients. However, there was higher dispersion of the expression levels in breast cancer patients and a loss of the normal distribution typically observed for HD receptors. The dispersion of expression levels for breast cancer patients tended to overexpress inhibitory receptors, such as KIRs CD158a/h, CD158b and NKG2A, and to underexpress activating receptors, such as NKp30, CD161 and DNAM-1 (Supporting Information Fig. S1 C). Surprisingly, the receptor that was most significantly altered in breast cancer NK cells was the inhibitory receptor CD85j (HD mean 21.7%; breast cancer patients mean 39.5% $P<0.0001$) (Fig. 1 A). Moreover, this receptor was also overexpressed in breast cancer patient's T cell ($CD3^{+}CD56^{-}$) and NKT-like cell ($CD3^{+}CD56^{+}$) populations ($P<0.001$ and $P<0.01$), respectively (Supporting Information Fig. S2A). CD85j analysis showed a positive linear association of expression with disease progression ($P=0.01$), although we found overexpression in early stages (Fig. 1 B). Interestingly, we observed that CD85j was overexpressed in patients with tumors of different tumor subtypes (Fig. 1 C).

Taking into account previous reports relating augmentation of CD85j expression with aging, we decided to evaluate the relationship between CD85j expression and age in our breast cancer patients. We observed a significant positive correlation between the percentage of NK cells expressing CD85j and breast cancer patient age (Supporting Information Fig. S2B). However, when dividing breast cancer patients and HD by age, we observed significantly higher CD85j mean values in breast cancer patients than in HD of same age group, even in the younger group ranging from 20-39 years old (Supporting Information Fig. S2C). Meanwhile, the 40-59 HD cohort and the younger HD cohort had similar CD85j expression levels. This indicates that increased CD85j expression in breast cancer patients is an effect of both age and disease status.

CD85j overexpression impairs NK-cell functionality against TNBC cells

To analyze the potential link between Cetuximab-mediated ADCC decrease and CD85j overexpression we performed a correlation analysis of NK-cell receptor expression and *in vitro* basal lysis or ADCC levels against TNBC cells. As expected, Cetuximab-triggered ADCC, but not basal lysis, was positively correlated with the percentage of $CD16^+$ NK cells in HD. This correlation was lost in the breast cancer patients (Fig. 2 A, upper panel), and no correlation with CD16 MFI was found (Supporting Information Fig. S3A). This suggests that receptors other than CD16 may modulate ADCC levels. In this respect, there was a negative correlation between ADCC levels and the percentage of $CD85j^+$ NK cells in breast cancer patients, but not in HD. No correlation was found between $CD85j^+$ NK cells and basal lysis (Fig. 2 A, lower panel). We were able to rule out a concomitant decrease in CD16 expression as the cause of decreased ADCC in patients with a high percentage of $CD85j^+$ NK cells because $CD16^+$ NK cells remained at high levels even in patients with high levels of $CD85j^+$ NK cells (Supporting Information Fig. S3B). To better examine the effect of CD85j expression on ADCC levels, we divided the patients into groups of low and high $CD85j^+$ NK cells, using the percentage of $CD85j^+$ NK-cell mean value plus 2 SD of HD as a cutoff (Fig. 1 A). The $CD85j^{high}$ breast cancer patient group, but not the $CD85j^{low}$ group, showed significantly lower ADCC levels as compared with HD control group ($p < 0.05$) (Fig. 2 B).

As previously described, stimulation with IL-2 and IL-15 increases PB NK-cell lytic activity against TNBC cells [32, 33]. However, the negative correlation between CD85j overexpression and ADCC levels was observed even after cytokine stimulation (Fig. 2 C).

HLA-I tumor expression correlates with CD85j overexpression in breast cancer

The expression of a singular receptor can only have a significant functional impact when it coexists with its cognate ligand. CD85j functions as a broad-specificity receptor recognizing both classical and non-classical HLA-I molecules. We studied HLA-I molecule expression in 66 breast cancer tumors by IHC on a Tissue Microarray (TMA). Microscopic quantification was successful in 91% (60 cases) for HLA-ABC, 92% (61 cases) for HLA-E, and 89% (59 cases) for HLA-G; results are summarized in Table 2. As internal controls, we observed that normal cells, such as vascular endothelium, fibroblasts or lymphocytes were positively labeled for HLA-ABC and occasionally HLA-E (Supporting Information Fig. S4). HLA-I expression was not associated with the different breast cancer subtypes (Fig. 3 A). We also performed an association analysis between tumor HLA-I expression or loss and $CD85j^{low}$ or $CD85j^{high}$ in PB breast cancer NK cells. Interestingly, there was an association between these variables ($P = 0.021$); no patient whose tumor had completely lost HLA-I expression (classical and non-classical) over-expressed CD85j in PB NK cells. Separate analysis by HLA type revealed that this association was mainly between HLA-ABC and CD85j expression ($P = 0.014$), while HLA-E and HLA-G expression on tumors were not associated with CD85j expression on PB NK cells (Fig. 3 B).

Measurement of plasmatic sHLA-G in breast cancer patients

It has been reported that the HLA-G soluble isoforms (sHLA-G) HLA-G1 and HLA-G5 can promote the overexpression of their receptors [34]. We measured plasmatic sHLA-G levels in HD and breast cancer patients and analyzed their relationship to CD85j overexpression. No significant differences in plasmatic sHLA-G levels were found between HD and breast cancer patients (mean 4.8 ng/ml and 8.8 ng/ml, respectively) (Fig. 3 C). Only 5 out of the 47 patients studied had plasmatic sHLA-G values higher than the HD mean + 2 SD. Interestingly, all of these patients presented CD85j^{high} NK cells, showing a significant relationship with elevated plasmatic sHLA-G values (P= 0.015, Fig. 3 D). This suggests that high systemic concentrations of sHLA-G may be one of the possible mechanisms by which CD85j overexpression is produced. However, most CD85j^{high} patients also presented low plasmatic sHLA-G levels, indicating that other mechanisms must also be involved in the overexpression of this receptor in breast cancer NK cells.

CD85j blockade increases lysis and restores deficient Cetuximab-triggered NK-cell mediated ADCC

To test whether CD85j expression could impact NK cytotoxic activity against TNBC cells, we used the previously described HP-F1 mAb for its ability to specifically recognize and functionally block CD85j [16]. As control of blockade specificity, we performed the same assay using K562 cells, which do not express HLA-I molecules and do express other NK-cell ligands (Supporting Information Fig. S5).
The CD85j receptor blockade increased NK-cell basal lysis levels against IIB-BR-G cells for breast cancer patients (Fig. 4 A, right panel), as well as ADCC levels for both HD and breast cancer patients (Fig. 4 B). It did not affect lytic activity against HLA-I-negative K562 cells (Fig. 4 C), showing that the blockade is specific to cellular targets expressing the receptor ligand. We also found a positive correlation between the expression of CD85j and ADCC increase after HP-F1 blockade (P<0.01), which is indicative of increased inhibition at higher levels of CD85j expression on NK cells (Fig. 4 D).

Discussion

NK cells play a central role in the eradication of transformed cells [35]. In Ab-based immunotherapy, NK cells cooperate with Ab-mediated antiproliferative activity by lysing opsonized tumor cells [7, 36, 37]. We have previously described PB NK cell´s ability to perform Cetuximab-triggered ADCC against TNBC cells *in vitro*. The efficiency of killing opsonized cells was almost 70%, even against K-RAS mutated target cells [33]. Clinical trials evaluating Cetuximab in breast cancer are in advanced phases [30, 31], suggesting that NK-cell activity can have clinical implications in breast cancer treatment. Although there were many altered and dysfunctional features in intratumoral breast cancer NK cells [24, 32], PB-NK cells should be evaluated as a therapeutic approach for TNBC in the adjuvant setting, since this population could be responsible for preventing metastatic spread [38]. Adjuvant therapies are administered post-surgery when patient's primary tumor has been excised. NK cells are recognized as the main ADCC effector cells in the PB, as well as in Cetuximab-mediated lysis [33]. In breast cancer patients the therapeutic application of Trastuzumab is widely accepted in HER-2+ tumors. In this context, patients who responded to Trastuzumab with complete or partial remissions demonstrated greater capacity to mediate ADCC *in vitro* in response to Ab than those patients whose tumors did not respond to therapy [8, 9].

Unfortunately, NK cells from cancer patients display altered receptor signatures that prevent them from developing their full range of potential activities [24, 39-43]. In particular, we observed a heterogeneous pattern of Cetuximab-mediated cytotoxicity by NK cells from breast cancer patients. When we classified patients by clinical AJCC stage, we observed a decrease in Cetuximab-mediated ADCC levels in patients with advanced disease (data not shown). These results indicate that the loss of breast cancer NK-cell functional activity against breast cancer tumor cells increased over the course of the disease, which is coincident with previous reports describing decreased NK-cell functionality in breast cancer PB [24, 44, 45]. Although CD16 receptor expression in breast cancer patients was comparable to HD in all stages, we found that the correlation between CD16 expression and ADCC levels observed in HD was lost in breast cancer patients. CD16 is the main receptor implicated in ADCC triggering [46, 47] and the quantity of $CD16^{+}$ NK cells is reported to be a major predictor for ADCC activity in Trastuzumab treated breast cancer patients [9]. With this understanding, the loss of correlation between CD16 and Cetuximab-mediated ADCC found in breast cancer patients was unexpected. However, response quality and intensity is the result of NK-cell receptor expression balance [48] and this would be especially important for patients with many phenotypic alterations.

Looking for phenotypic traits that could be responsible for this impairment, we found that CD85j overexpression was the most striking NK-cell receptor alteration of the 13 activating /inhibitory receptors analyzed. In fact, our study demonstrated that CD85j inhibitory receptor overexpression correlated negatively with Cetuximab-mediated ADCC in these patients. Likewise, when we stratified patients according to low or high CD85j expression levels, we observed that the $CD85j^{high}$ patient group presented diminished Cetuximab-mediated ADCC against HLA^{+} IIB-BR-G cells. Furthermore, CD85j expression increased with disease progression. In the search for tumor-associated causes of CD85j augmentation in breast cancer patients, we studied classical and non-classical HLA-I molecules in patient´s biopsies. We found an association between HLA-I and $CD85j^{high}$ expression, which supports the notion that CD85j overexpression assumes functional relevance when tumor cells express its ligands. The major mechanism that determines this upregulation of CD85j protein is still unknown. Patients with high serum sHLA-G also presented $CD85j^{high}$, a mechanism previously reported as a modulator of CD85j expression [34]. However, high sHLA-G was observed in a minority of breast cancer patients. On the other hand, cross-sectional studies showed that expression of CD85j in CD8 T cells increased with age and, while already apparent in the 40-59 cohort, was clearly accelerated in older people [49]. Furthermore, the proportion of $CD85j^{+}$ NK cells gradually increase with age in healthy adults [50]. However, our findings in breast cancer patients from 20-39 yrs, whose CD85j values were higher than HD, suggest that a cancer-related pathological factor may also be involved in CD85j overexpression. Consequently, other factors impacting the regulation of CD85j expression should be the focus of future research.

Based on our previous work to overcome NK-cell dysfunction in breast cancer patients [32], we tested whether pre-activating NK cells with IL-2 or IL-15 cytokines improved TNBC basal lysis or ADCC. As expected, pre-activation of NK cells with IL-2 or IL-15 increased their lytic activity; however the negative correlation between CD85j overexpression and ADCC levels persisted even after stimulation with cytokines. CD85j is one of many inhibitory receptors on NK cells that can detect MHC class I on other cells [15]. CD85j binds to the HLA-I conserved region α3, which imparts the versatility to recognize classical and non-classical HLA molecules. Binding between CD85j and its HLA-I ligands leads to the inhibition of NK-cell effector function [16, 51, 52]. Since overexpression of the inhibitory receptor CD85j is a possible mechanism of immune suppression in breast cancer that would diminish NK-cell activity against tumor cells, we blockaded CD85j in order to increase the anti-tumor activity of NK cells in breast cancer patients. NK-cell specific blockade with HP-F1 mAb increased both basal lysis and ADCC levels against the TNBC cell line HLA-I^{+} IIB-BR-G; we also found greater lysis derepression and improved ADCC levels when NK cells expressed high levels of the CD85j receptor. As expected, this functional enhancement did not take place with the HLA^{-} K562 cell line, since CD85j does not play any role in K562 target cell recognition.

We focused our study on TNBC because Cetuximab is a promising treatment for this group. However, HER-2+ breast cancer patients also overexpressed CD85j in PB-NK cells. Hence, CD85j could also impact Trastuzumab-mediated ADCC, since approximately 50% of HER-2+ tumors express HLA-I [53]. Although evaluation of the impact of CD85j against HER-2+ targets was out of the scope of the present study, we believe this hypothesis should be tested in a near future.

In conclusion, we detected phenotypic and functional alterations in breast cancer PB NK cells. As tumor cells develop strategies to overcome their elimination by the innate immune system, it is possible to develop counter-strategies to restore these functions. This could be achieved through direct enhancement of anti-tumor immune functions, as we proposed with the use of IL-2 or IL-15 stimulatory cytokines [32, 33], or through inhibition of negative immune regulator molecules, as we demonstrate here with the CD85j receptor blockade. Considering the biological variability and immune evasion mechanisms observed in breast cancer, an efficient therapeutic strategy could certainly rely on combinatorial approaches to successfully fight this complex disease.

Materials and Methods

Patients

105 breast cancer patients treated at the Servicio de Patología mamaria, Instituto Alexander Fleming (Ciudad Autónoma de Buenos Aires, Argentina) were recruited at diagnosis between April 2011 and July 2014. PB was sampled during surgery and before administration of any treatment. Patients were classified into 5 clinical stages following The American Joint Committee on Cancer (AJCC) staging classification and into 3 groups according to morphological tumor characteristics and hormonal and HER-2 receptor expression (Table 1). We also included a control group of healthy donors (HD) from our institution (n = 36; age mean: 34 years; range: 19-56 years). All subjects gave written, informed consent approved by the Institutional Review Board of the Instituto Alexander Fleming.

Cell lines

The K562 cell line, derived from human leukemia (ATCC # CCL-243), was grown at 37°C in a humid atmosphere containing 5% CO_2 in RPMI supplemented with 10% heat-inactivated FCS (Natocor, Córdoba, Argentina). The TNBC cell line IIB-BR-G was established from a primary infiltrating ductal breast cancer [54] and was cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 3.5 mg/ml sodium carbonate, 2 mg/ml insulin, and 4.5 mg/ml glucose.

Flow cytometry

For NK-cell phenotyping, 2.0 $X10^5$ PBMC were incubated with the appropriated Abs: FITC-conjugated anti-CD3, allophycocyanin-conjugated anti-CD56 and anti-CD69, PE-conjugated anti-NKp30, anti-NKp44, anti-NKp46, anti-CD16, anti DNAM-1, anti-CD94, anti-CD161, anti-CD56, anti-CD158a/h, anti-CD158b, anti-CD107a and anti-CD85j (BD PharMingen) or PE-conjugated anti-NKG2A (R&D Systems), for 30 min at 4°C. The NK-cell population was selected based on $CD3^-CD56^+$-phenotype of gated lymphocytes according to forward-side scatter. For NK-cell ligand analysis, tumor cell lines were incubated with FITC-conjugated anti-HLA-ABC (BD PharMingen), or PE-conjugated anti-HLA-G, anti-HLA-E (Abcam), anti-CD112 and ant-MICA/B (BD PharMingen), anti-CD155 (BioLegend) or respective isotype controls. All samples were acquired on a BD FACS Calibur using Cellquest Pro software (BD Biosciences) and analyzed with FlowJo 7.6.2 software (Tree Star, Inc.).

Cytotoxicity assays

TNBC IIB-BR-G or K562 cells were used as targets and labeled with Calcein-acetyoxymethyl (Calcein-AM) (Molecular Probes, Invitrogen Life Technology) as previously reported [55]. For ADCC experiments, IIB-BR-G cells were preincubated (30 min at room temperature –RT-) with 1 μg/ml Cetuximab (Erbitux, Merck) or rituximab (Rituxan, Roche) as irrelevant chimeric IgG_1. The effector cells were PBMC, fixing E:T ratios with NK cells. Cytotoxicity assays were performed in triplicate using different E:T ratios ranging from 5:1 to 1.25:1 (NK:IIB-BR-G or NK:K562). Three replicate wells were also measured for spontaneous (only target cells in RPMI medium with 10% FCS) and maximum release (only target cells in medium plus 1% Triton X-100). After incubation at 37 °C in 5% CO_2 for 4h, supernatants were analyzed using a microplate fluorescence reader (BTX880, Beckman Coulter) to measure cell death (Calcein release). The percentage of specific lysis was calculated as follows: (experimental fluorescence – spontaneous fluorescence)/ (maximum fluorescence – spontaneous fluorescence) X 100.

CD85j functional blockade

The HP-F1 monoclonal antibody (kindly provided by Dr. López-Botet, *Institut Hospital del Mar d'Investigacions Mèdiques*, España), which has the ability to specifically block CD85j function, was used in functional assays [16]. HP-F1 mAb or the corresponding mouse IgG1 control, were added at the beginning of cytotoxic assays.

ELISA for soluble HLA-G (sHLA-G)

Serum levels of sHLA-G (isoforms 1 and 5) were measured by ELISA [56] as previously described. MEM-G/9 (Abcam) was used as capture Ab, and biotinylated anti-beta-2—microglobulin (Abcam) was used for detection. Streptavidin-peroxidase (Abcam) was used for color development, using a TMB substrate reagent set (BD biosciences). sHLA-G concentration were calculated based on a calibration curve, using r-sHLAG (kindly provided by Dr. Daniel E. Geraghty, *Fred Hutchinson Cancer Research Center,* USA). DO was measured using a microplate reader (BioRad, USA)

Tissue microarray (TMA) and Immunohistochemistry (IHC)

The TMA construction of paraffin embedded tissues was performed using a previously described adapted *homemade* technique [57]. Each 3 mm spot contained a tumor area selected by a pathologist. IHC staining pretreatments consisted of sample dehydration in graded alcohols, enzyme digestion, or other heat-mediated retrieval methods. HLA-I, classic and non-classic molecule expression in human tumor spots were assessed using mAbs anti HLA-ABC (clone EMR8-5), HLA-E (clone MEM-E/02) and HLA-G (clone 4H84) following manufacturer's specifications (Abcam). In brief, after incubation with the primary mAb to human HLAs, sections were stained using ABC system (Vectastain Universal Elite ABC kit, PK-6200 Vector Lab), counterstained with hematoxylin and mounted. All results were interpreted using light microscopy (Olympus BX40). Using a double blind system to evaluate percentage and intensity of positive cells pictures were captured with a coupled camera (Olympus Digital Camera DP72) and acquired with DP2-BSW software (Olympus).

Statistics

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software). Comparisons between samples (breast cancer and HD) were performed using Mann-Whitney tests and non-parametric one-way ANOVA with Dunns post-tests for multiple comparison analysis. Wilcoxon matched-pair test was used when comparing two matched groups. In all figures, data are represented using the mean ± SEM. Reported *p* values are two-tailed and $p<0.05$ was considered significant.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

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Figure 1. CD85j expression in PB NK cells from breast cancer patients.

(A-C) The expression of CD85j was determined by flow cytometry in various NK-cell populations. NK-cell population was selected by $CD3^{-}CD56^{+}$ phenotype of gated lymphocytes by to FSC/SSC. (A) Percent CD85j expression in PB NK cells from HD (n= 29) and breast cancer patients (n= 89) is shown. The gray line shows the cutoff for normal values (mean + 2SD of values from HD). Values below and above the cutoff are considered as $CD85j^{low}$ and $CD85j^{high}$, respectively. Black bars represent the mean for each group. ***P <0.001 (Mann-Whitney test). (B) As for (A), with breast cancer patients divided into different clinical stages: *in situ*, stages I, II, III and IV (n= 4, 46, 29, 9 and 2 respectively). The gray line represents the significant positive linear trend between CD85j expression levels and disease progression, P= 0.007 (ANOVA test for linear trend). Mean values for each stage are shown with black bars, *P< 0.05, ***P< 0.001 (Dunn's Multiple Comparison Test). (C) As for (A), with breast cancer patients classified according to tumor subtype: HR (n= 69), HER-2+ (n=8), TN (n=7). Black bars represent the mean for each group, *P<0.05, **P< 0.01, ***P< 0.001 (Dunn's Multiple Comparison Test).

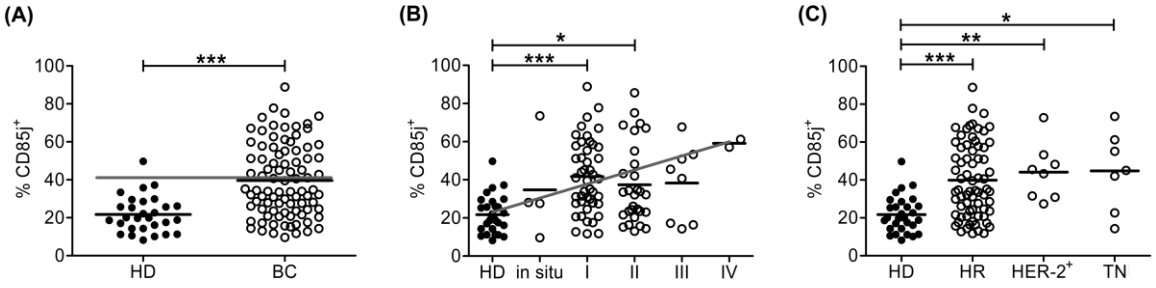

Figure 2. Correlation between PB NK-cell cytotoxic ability and NK-cell receptor expression. (A-C) Cytotoxic activity of NK cells against IIB-BR-G cells was measured by calcein release assay. IIB-BR-G cells were pre-incubated with Cetuximab or rituximab as irrelevant chimeric $IgG_{1.}$ (A) Basal lysis levels (gray dots) or Cetuximab-triggered ADCC (black squares) of PB NK cells from HDs and breast cancer patients against IIB-BR-G cells were correlated with CD16 (top, 2.5:1 NK:T ratio) and CD85j (bottom, 5:1 NK:T ratio) expression. Spearman r correlation coefficients and P values of significance are shown. (B) ADCC mediated by PB NK cells from HD (n= 14) and breast cancer patients divided into CD85j normal expression ($CD85j^{low}$, n=10) and CD85j overexpression ($CD85j^{high}$, n=11), as described in Figure 2A. NK:T ratio 5:1. Black bars represent mean values for each group, *P<0.05 (Dunnett's Multiple Comparison Test). (C) Correlation between CD85j expression in breast cancer patients and ADCC against IIB-BR-G cells by cytokine-activated NK cells. PBMC were pre-incubated overnight with IL-2 (empty gray dots), IL-15 (black diamonds). Lysis values correspond to a NK:T ratio of 5:1. Spearman r correlation coefficients and P values of significance are shown.

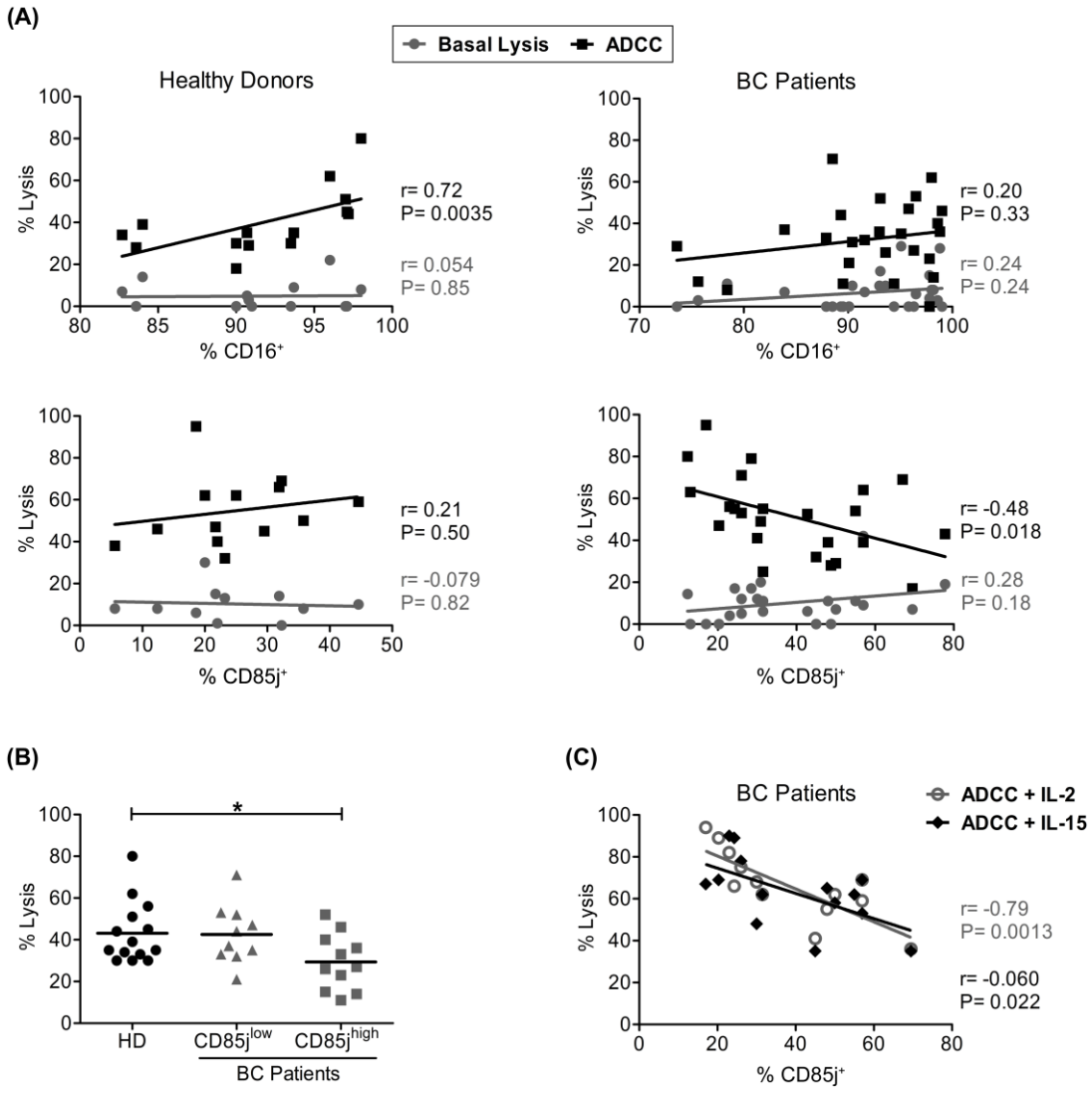

Figure 3. CD85j expression on PB breast cancer NK cells relative to HLA-I expression in tumors. HLA-I expression in breast cancer tumors was assessed by IHC on tissue microarrays.
(A) Frequency distribution of HLA-ABC (left), HLA-E (middle) and HLA-G (right) expression among different breast cancer tumor subtypes. P values are shown, Chi-square test. (B) Frequency distribution of low CD85j expression (CD85j^{low}) and overexpression (CD85j^{high}) on NK cells relative to the expression of HLA-I classical and non-classical molecules in breast cancer tumors. P values are shown, Chi-square test. (C) Plasmatic HLA-G levels in HD (n=24) and breast cancer patients (n=47) were measured by ELISA. Mean ± SEM are shown, P>0.05 (Mann-Whitney test). The gray line indicates the cutoff for normal values (mean + 2SD of values from HD). Values below and above the cutoff are considered HLA-Gs^{low} and HLA-Gs^{high}, respectively. (D) Frequency distribution of low CD85j expression (CD85j^{low}) and overexpression (CD85j^{high}) on NK cells relative to the expression of plasmatic HLA-Gs levels: HLA-Gs^{low} and HLA-Gs^{high}. P value is shown, Chi-square test.

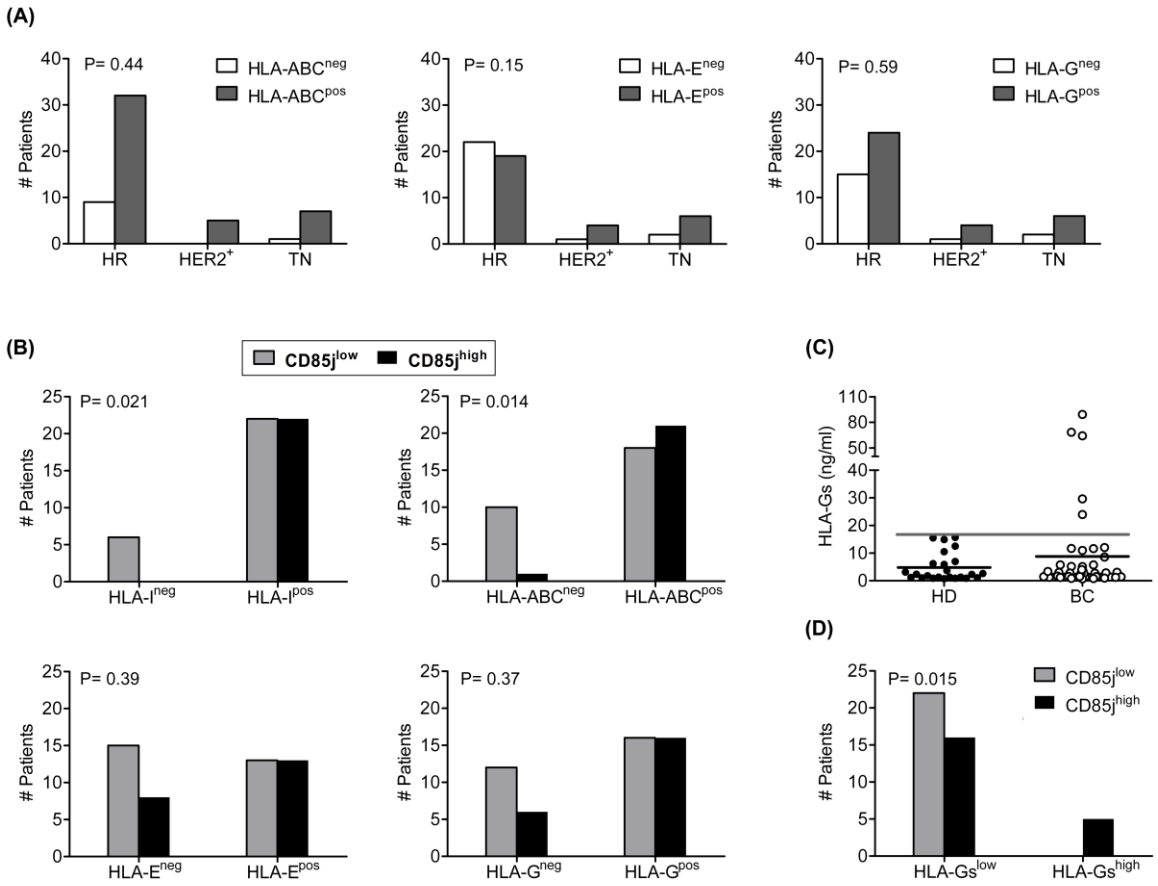

Figure 4. CD85j functional blockade restores Cetuximab-mediated NK-cell ADCC.
(A, B) IIB-BR-G cells were incubated with NK cells from HD (left, n=5) and breast cancer patients (right, n=5) in the presence of CD85j-neutralizing mAb HP-F1 or isotype control, at a 2.5:1 NK:T ratio. (A) Basal lysis and (B) ADCC against IIB-BR-G cells by NK cells was determined by calcein release assay. (C) Basal lysis against HLA-$I^{-/-}$ K562 cells by NK cells from HD (left, n=3) and breast cancer patients (right, n=3) in the presence of CD85j-neutralizing mAb HP-F1 or isotype control, at a 2.5:1 NK:T ratio. *P<0.05, **P<0.01 (Paired t-test). (D) Correlation between CD85j expression levels and the improved NK-cell ADCC against TNBC cells in the presence of CD85j-neutralizing mAb HP-F1. Percent lysis enhancement was calculated as (ADCC with HP-F1 – ADCC)/ ADCC X100. Symbols represent individual values obtained from HD (filled black dots, n=5) and breast cancer patients (empty dots, n=5). Spearman r correlation coefficients and P values are shown.

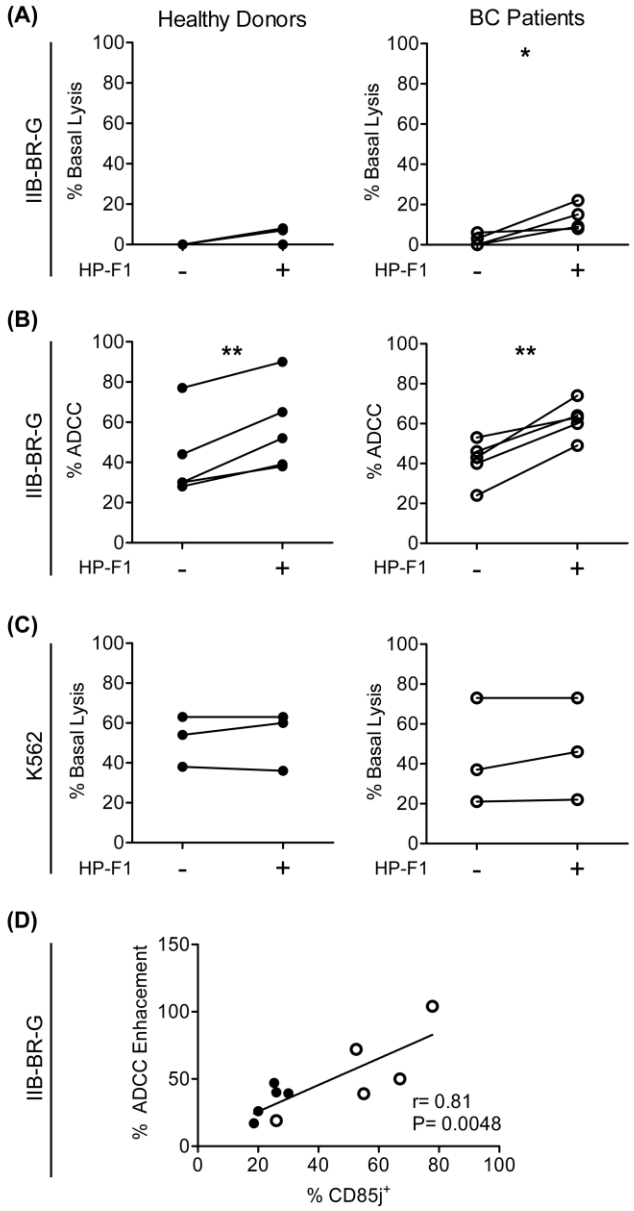

Table 1. Clinical characteristics of breast cancer patients involved in the study

Stages		**In Situ**	**I**	**II**	**III**	**IV**
n		5 5%	54 51%	35 33%	9 9%	2 2%
Mean age (years)		51	58	51	57	60
Grade	1	1	14	7	1	0
	2	0	35	14	5	1
	3	4	5	12	3	1
	ND[a)]	-	-	2	-	-
IHC expression	HR+	3	51	30	7	1
	HER-2+	ND	4	3	2	0
	HR[b)]- HER-2-	ND	1	4	1	1
Histological type	CDIS[c)]	2	43	26	6	1
	Ductal	0	4	7	3	0
	Lobulillar	0	4	1	0	1
	Ductolobulillar	3	0	0	0	0
	Others	0	3	1	0	0
%Ki67	High >13	-	36	16	4	2
	Low<13	-	10	11	4	0
	ND	5	8	8	1	-

[a)] ND - not determined

[b)] HR - hormone receptor

[c)] CDIS - in situ carcinoma

Table 2. Breast tumor expression of HLA-I molecules

HLA-I molecule	**% of tumors**		
HLA-I loss	12.9		
HLA-ABC expression	74.6		
HLA-E expression	54.0		
HLA-G expression	65.1		
HLA-I coexpression	**HLA-E**	**HLA-G**	**HLA-EG**
HLA-ABC Loss	0	3.2	9.5
HLA-ABC expression	9.5	17.5	34.9