PRECLINICAL STUDY

IL-15 and IL-2 increase Cetuximab-mediated cellular cytotoxicity against triple negative breast cancer cell lines expressing EGFR

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Abstract Triple negative breast cancer (TNBC) patients are not likely to benefit from anti-estrogen or anti-HER2 therapy and this phenotype is associated with a more aggressive clinical course and worse clinical outcome. Taking into account the limited treatment possibilities in TNBC, the aim of the present work was to study a potential therapy based on Cetuximab-mediated immune activity by natural killer (NK) cells. We performed in vitro studies on human breast cancer (BC) cell lines, IIB-BR-G, and the in vivo metastatic variant IIB-BR-G MT. The immunohisto-chemical analysis showed a TNBC phenotype with high but different levels of EGFR expression on each cell line, measured by flow cytometry. DNA sequencing showed that both cell lines have a mutated K-RAS status, 38 G > A at codon 13. Consequently, Cetuximab did not inhibit cellular proliferation or induce apoptosis. We investigated if Cetuximab could trigger immune mechanisms, and we determined that both cell lines treated with 1 µg/ml Cetuximab were susceptible to antibody dependent cellular cytotoxicity (ADCC), mediated by peripheral blood mononuclear cells (PBMC). At 50:1 effector:target ratio, lytic activity was 34 ± 2% against IIB-BR-G and 27 ± 6% against IIB-BR-G MT cells. PBMC pretreatment with IL-2 allowed reaching 65 ± 3% of Cetuximab-mediated ADCC against IIB-BR-G and 63 ± 6.5% against IIB-BR-G MT. Furthermore, IL-15 pretreatment increased the ADCC up to 71 ± 3% in IIB-BR-G and 79 ± 3.5% in IIB-BR-G MT. We suggest that NK cells are the effectors present in PBMC since they were able to induce ADCC at lower effector:target ratios. Besides, IL-2- and mainly IL-15-induced upregulation of NK activating receptors CD16 and NKG2D and enhanced IFN-γ production. EGFR-expressing TNBC could be killed by Cetuximab-mediated ADCC at clinically achievable concentrations. IL-15 could advantageously replace IL-2 in most of its immunologic activities, stimulating the ability to produce IFN-γ, and paralleling the up-regulation of activating receptors.

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Introduction

Approximately, 10–15% of breast cancers (BCs) are known to be "triple-negative (TN) receptor," not expressing estrogen receptor (ER) or progesterone receptor (PR) and not exhibiting overexpression and/or gene amplification of HER2-neu. TNBC comprises approximately 85% of all basal-type tumors. "triple-negative receptor" is a term based on established clinical immunohistochemical and fluorescence in situ hybridization assays for ER, PR, and HER2,

whereas "basal-like" is a molecular phenotype defined by using cDNA microarrays [1–3]. TNBC is associated with a more aggressive clinical course and worse clinical outcome than non-TNBC [4]. As patients with TNBC are not likely to benefit from anti-estrogen or anti-HER2 therapy, first-line treatment usually consists of conventional cytotoxic chemotherapy. Although TNBCs are quite sensitive to taxane- and anthracycline-containing regimens in the preoperative setting, they are nevertheless associated with poor long-term outcomes [5]. Epidermal growth factor receptor (EGFR) is frequently overexpressed in TNBC. EGFR regulates cancer cell proliferation, apoptosis, and tumor-induced neoangiogenesis, and has been validated as a relevant therapeutic target in several human cancers [6]. The anti-EGFR chimeric monoclonal antibody (mAb) Cetuximab has been approved by the FDA for the treatment of patients with metastatic colorectal cancer (mCRC). Although EGFR is expressed in approximately 85% of patients with mCRC, the clinical efficacy of treatment with anti-EGFR mAbs is limited to a subset of patients [7, 8]. For instance, activating mutations within K-RAS constitutively activates the RAS-signaling pathway and can predict resistance to anti-EGFR mAbs [8, 9]. These genetic alterations in K-RAS are found in approximately 35–40% of patients with mCRC and are almost exclusively detected in codons 12 and 13 of exon 2 [7]. Therefore, development of new therapeutic strategies for K-RAS mutated tumors is highly desirable. It has been suggested that immune mechanisms could contribute to the antitumor activity of Cetuximab [10]. In particular, Cetuximab, alone or in combination with other Abs, may elicit immunological responses such as antibody dependent cellular cytotoxicity (ADCC) or complement activation [11–14]. In previous reports, we and others proved that natural killer (NK) cells produce Cetuximab-mediated ADCC of mCRC and that this activity is not affected by the K-RAS mutational status [15, 16]. This effect is expected because NK cells recognize the surface-bound Abs and are able to kill tumor cells independently of the EGFR pathway activation. ADCC is triggered by an interaction between Ab-coated target cells and $Fc\gamma RIII$ (CD16) on NK cells, which initiates a sequence of cellular events culminating in the release of cytotoxic, granzyme-containing granules, and $INF-\gamma$ secretion [17–19]. Nevertheless, it remains to be answered why in mCRC patients with K-RAS mutated status Cetuximab-mediated ADCC does not induce clinical remissions. One of the possibilities is the low proportion of $CD3^-CD56^+$ (NK cells) infiltrating CRC tumors [20] and the low functional capacity of these cells observed in cancer patients [21–23]. Therefore, if we could enhance the activity of NK cells, the efficacy of treatment with Cetuximab could be increased. As reported previously, IL-2 and IL-12 play an important role in NK cell biology [24–26]. However, in recent years, IL-15 has been shown to be the major NK

cell homeostatic cytokine displaying effects on NK-cell differentiation, peripheral expansion and survival [27, 28]. Besides, mature human circulating NK cells treated with IL-15 upregulate the expression of their receptors, and this upregulation is associated with a concomitant increase of NK cell activity [29].

Given the limited possibilities of treatment in TNBC, in the present work, we studied in vitro whether EGFR expressing K-RAS mutated TNBC cell lines could be target of Cetuximab. Our hypothesis was that if Cetuximab-mediated ADCC may be achieved, the resistance to mAb due to mutations in K-RAS may be overcome. We found that (i) Cetuximab-mediated ADCC takes place at clinically achievable concentrations, (ii) cytokines IL-15 and IL-2 stimulated the lytic capacity of peripheral blood mononuclear cells (PBMC) against Cetuximab-coated tumor cell lines, and (iii) purified NK cells strongly mediated cytotoxicity. Moreover, these cytokines increased NK ability to produce IFN-γ, paralleling the upregulation of activating receptors.

In other studies, EGFR-expressing BCs have been treated with Cetuximab in vitro [30], in mouse models [31] and in patients [32] based on the direct activity of the mAb on the EGFR signaling pathway. Our study, instead, was focused on the involvement of Cetuximab-mediated immune response rather than on the blockade of intracellular signaling as mechanisms of antitumor activity.

Methods

Cell lines

IIB-BR-G BC cell line has been established from a primary infiltrating ductal carcinoma [33]. IIB-BR-G MT is a spontaneous metastatic variant generated in vivo in nude mice [34]. Both cell lines were grown at 37°C in a humid atmosphere containing 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS, Natocor), 2 mM L-glutamine, 3.5 mg/ml sodium carbonate, 2 mg/ml insulin, and 4.5 mg/ml glucose (BRG medium).

K-RAS mutations

Human K-RAS codon 12 and 13 mutations were assessed by sequencing. Genomic DNA was subjected to PCR amplification with the following set of intronic primers: sense 5′-TACAGTTCATTACGATACACG-3′, antisense 5′-ATA TGCATATTAAAACAAGATT-3′. The PCR products were sequenced using the same set of primers by Genetic Analyzer 3130 (Applied Biosystems).

Isolation of human cells and treatments

PBMC were obtained from peripheral blood of healthy human donors by Ficoll (Sigma)-Hypaque (Justesa Imagen Argentina SA) gradient. All subjects signed an informed consent approved by the Institutional Review Board of the Instituto Alexander Fleming. Cyotokine treatment consisted of overnight incubation of cells in RPMI medium 10% FCS plus 100 IU/ml IL-2 or 10 ng/ml IL-15 (Prepro Tech Inc).

NK purification and treatment

NK cells were isolated from PBMC by negative selection using NK cell Isolation Kit II and MACS columns (Miltenyi Biotec) following the manufacturer's protocol. Experiments were performed when purity of NK cells was more than 95% as determined by flow cytometry. Cytokine pretreatments were the same as described for PBMC.

Antibodies

For immunohistochemistry (IHC), the following mAbs were used: anti-human ER, clon 1D5; anti-human PR, clon 1294; anti-human HER-2 receptor clon c-erbB2-Oncoprotein; anti-human EGF receptor clon 2-18C9; and anti-cytokeratins 5/6, clon B5/16 B4 (Dako Cytomation). For flow cytometry: Cetuximab (Erbitux®, Merck), anti-human-FITC and human IgG (Dako Cytomation), and anti-CD20 mAb (Rituximab, MabThera/Rituxan, Roche). For Immunophenotyping of NK receptors: CD16-PE, NKG2D-PE, IgG$_1$PE, CD56-APC, and CD3-FITC (BD Pharmingen).

Immunohistochemistry

For IHC evaluation, each cell line was fixed in 4% formaldehyde and embedded into a paraffin block. IHC stains pretreatments consisted of sample dehydration in graded alcohols, enzyme digestion, or other heat mediated retrieval methods. Sections were stained using either Envision labeled polymer-HRP K4003 (Dako) or ABC system (Vectastain Universal Elite ABC kit, PK-6200 Vector Lab) and counterstained with hematoxylin.

Immunofluorescence analysis by FACS

Direct and indirect immunofluorescences were performed on BC cell lines, PBMC or NK cells using specific mAbs and isotype-matched mAbs. Antibodies were incubated for 45 min at 4°C and samples were analyzed with FACSCalibur flow cytometer using the CellQuest software (BD Biosciences). Intensity was calculated as follows: Mean intensity level (MIL) = % positive cells × mean fluorescence intensity (MFI).

MTT cell proliferation assay

Cell lines were incubated in BRG Medium with Cetuximab (concentrations ranging from 10^0 to 2×10^2 μg/ml) or control anti-CD20 mAb Rituximab, in a 96-well flat-bottomed plate in quadruplicate. After 96 h at 37°C, 20 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma) were added to each well and incubation was carried out for 2 h. Colorimetric evaluation was performed with a plate reader spectrophotometer at 570 nm (Microplate Reader 550 BioRad). The inhibition of proliferation was calculated as percentage of cell growth induced by Cetuximab in comparison with that developed without mAb.

Apoptosis

To evaluate apoptosis, each cell line was incubated in the presence of Cetuximab (100 and 200 μg/ml BRG Medium); Doxorubicin (1 μg/ml; Delta Farma) was used as a positive control. After incubation for 96 h at 37°C, apoptosis was measured by flow cytometry using the Annexin V–FITC Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's recommendations. The populations of apoptotic cells (Annexin-V positive/propidium iodide negative) and late apoptotic cells (Annexin-V positive/propidium iodide positive) were calculated as a percentage of total cells.

Antibody-dependent cell-mediated cytotoxicity assay

IIB-BR-G and IIB-BR-G MT were used as target (T) and labeled with Calcein-acetyoxymethyl (Calcein-AM) (Molecular Probes, Invitrogen Life Technology). Effector cells (E) were PBMC or NK where indicated. The cytotoxicity assay was performed using different E:T ratios ranging from 25:1 to 100:1 for PBMC or 10:1 to 40:1 for NK, in triplicate, with indicated concentrations of Cetuximab or control mAb (Rituximab). Three replicate wells for spontaneous (only target cells in RPMI-medium with 10% FCS) and maximum release (only target cells in medium plus 1% Triton X-100) were also measured. After incubation at 37°C in 5% CO_2 for 4 h, supernatants were analyzed by fluorimetry to measure cell death (Calcein release), and the percentage of specific lysis was calculated as follows: (experimental fluorescence – spontaneous fluorescence)/(maximum fluorescence – spontaneous fluorescence) × 100.

Measurement of IFN-γ release

Purified NK (pretreated or not with cytokines) were plated at a 15:1 E:T ratio in RPMI 10% FCS in 24-well plates in the presence of the tumor cell lines coated or not with 1 μg/ml Cetuximab, and incubated at 37°C. Controls consisted of NK cells or tumor cells alone. Supernatants were collected after 24 h and IFN-γ concentration was measured in triplicates by ELISA according to the manufacturer's instructions (OptEIA kits, BD PharMingen), using a calibration curve plotted in log–log regression analysis (Cembal 2.2 software).

Statistical analysis

Data were analyzed by ANOVA. A p-value <0.05 was considered as statistically significant.

Results

TNBC cell lines IIB-BR-G and IIB-BR-G MT presented a mutation in K-RAS

Hematoxylin and eosin staining of IIB-BR-G and IIB-BR-G MT showed high-grade poorly differentiated cell morphology. We then analyzed the expression patterns of the routine clinical panel of markers for primary BC classification. We performed the IHC using the same Abs, the same experimental conditions, and the same scoring systems that are used for clinical specimens, for both cell lines and positive controls. We determined that IIB-BR-G and IIB-BR-G MT were negative for ER, PR, and HER-2 (Fig. 1a). Since TNBC frequently expresses other basal markers, we further analyzed them for EGFR and cytokeratins 5/6 (CK 5/6) (Fig. 1a). In both cell lines, EGFR showed a strong membrane pattern while CK 5/6 were not expressed. To determine K-RAS mutational status, we amplified by PCR a 338 bp fragment that was subsequently sequenced. Both cell lines carried the same point mutation 38 G > A at codon 13 (Fig. 1b).

Cetuximab had no effect on the inhibition of proliferation or induction of apoptosis in IIB-BR-G or IIB-BR-G MT cells

To investigate Cetuximab direct action on EGFR tyrosine kinase functions, we first studied the anti-proliferative activity of Cetuximab against IIB-BR-G and IIB-BR-G MT cells. A MTT assay was performed incubating each cell line with different concentrations of Cetuximab (up to 200 μg/ml) and control mAb (Rituximab at 200 μg/ml). For both cell lines, a mild anti-proliferative effect of Cetuximab was observed only between 100 and 200 μg/ml. At 200 μg/ml of Cetuximab, inhibition of proliferation was only 18% and 26% for IIB-BR-G and IIB-BR-G MT, respectively, and was not significant compared to untreated controls (Fig. 2a). Control mAb did not have any effect on IIB-BR-G and IIB-BR-G MT (data not shown). Colorectal cancer Caco-2 cells EGFR-positive and K-RAS wild type were used as positive controls showing 90% of proliferative inhibition at concentrations higher than 10 μg/ml (Supplementary 1). To examine apoptosis-inducing activity of Cetuximab, we next performed an Annexin–propidium iodide assay in both BC cell lines using the maximal concentration of Cetuximab tested in the viability assays (200 μg/ml) (Fig. 2b and Table 1). No significant levels of apoptosis were induced by Cetuximab after 96 h treatment. This result was consistent with the high levels of viability previously observed even after doses of Cetuximab treatment as high as 200 μg/ml. As a positive control of apoptotic process, Doxorubicin was used at 1 μg/ml that lead to an 80.6 ± 8.0% and 76.7 ± 6.8% of apoptotic cells for IIB-BR-G and IIB-BR-G MT, respectively.

### Cetuximab increased lysis in TNBC cell lines with mutated K-RAS

We performed a flow cytometric analysis of EGFR expression in both cell lines (Fig. 3a, *left*). EGFR expression was measured both as percentage of positive cells and as mean intensity level. We then calculated the MIL for each cell line: $MIL_{IIB-BR-G} = 7781.512 \pm 53.39$ and $MIL_{IIB-BR-G\ MT} = 4623.346 \pm 21.62$. The relative comparison of MIL revealed that in IIB-BR-G, EGFR expression was approximately twofold higher than in IIB-BR-G MT. We then measured ADCC activity by Calcein release assay. We first performed a dose–response curve to obtain the concentration of Cetuximab for optimal PBMC lytic activity (Fig. 3a, *right*). For both cell lines, PBMC showed optimal lytic activity at 1 μg/ml of Cetuximab, though the different concentrations of the mAb range did not present any significant differences, except at 100 μg/ml where the percentage of lysis decreased probably due to in vitro saturation of the system. Taking into account these results, 1 μg/ml was the selected dose for subsequent experiments with different E:T ratios (Fig. 3b). Even though there was variability in the EGFR expression levels between IIB-BR-G and IIB-BR-G MT, both cell lines were susceptible to ADCC and we observed that cell lysis was significantly enhanced by E:T increment.

Given that in peripheral blood, the main cells that express FcRγIII are NK cells, and to examine more precisely the therapeutic effect of Cetuximab achieved by the NK FcR-dependent mechanism, we evaluated purified NK from PBMC on ADCC activity. We observed that both

Fig. 1 Immunohistochemical and molecular characterization of TNBC cell lines, IIB-BR-G and IIB-BR-G MT. **a** Determination by IHC of BC receptors. *Upper panel*: IIB-BR-G and IIB-BR-G MT BC cell lines resulted negative for ER, PR, and HER-2, and known clinical specimens were used as positive controls. *Lower panel*: Immunostaining of two relevant markers of TNBC (EGFR and CK 5/6). Bar = 5 μm, magnification ×1,000. **b** Determination of K-RAS mutations at codon 12 and 13. IIB-BR-G and IIB-BR-G MT were homozygous for the same 38 G > A point mutation of K-RAS at codon 13. *arrows* indicate point mutations
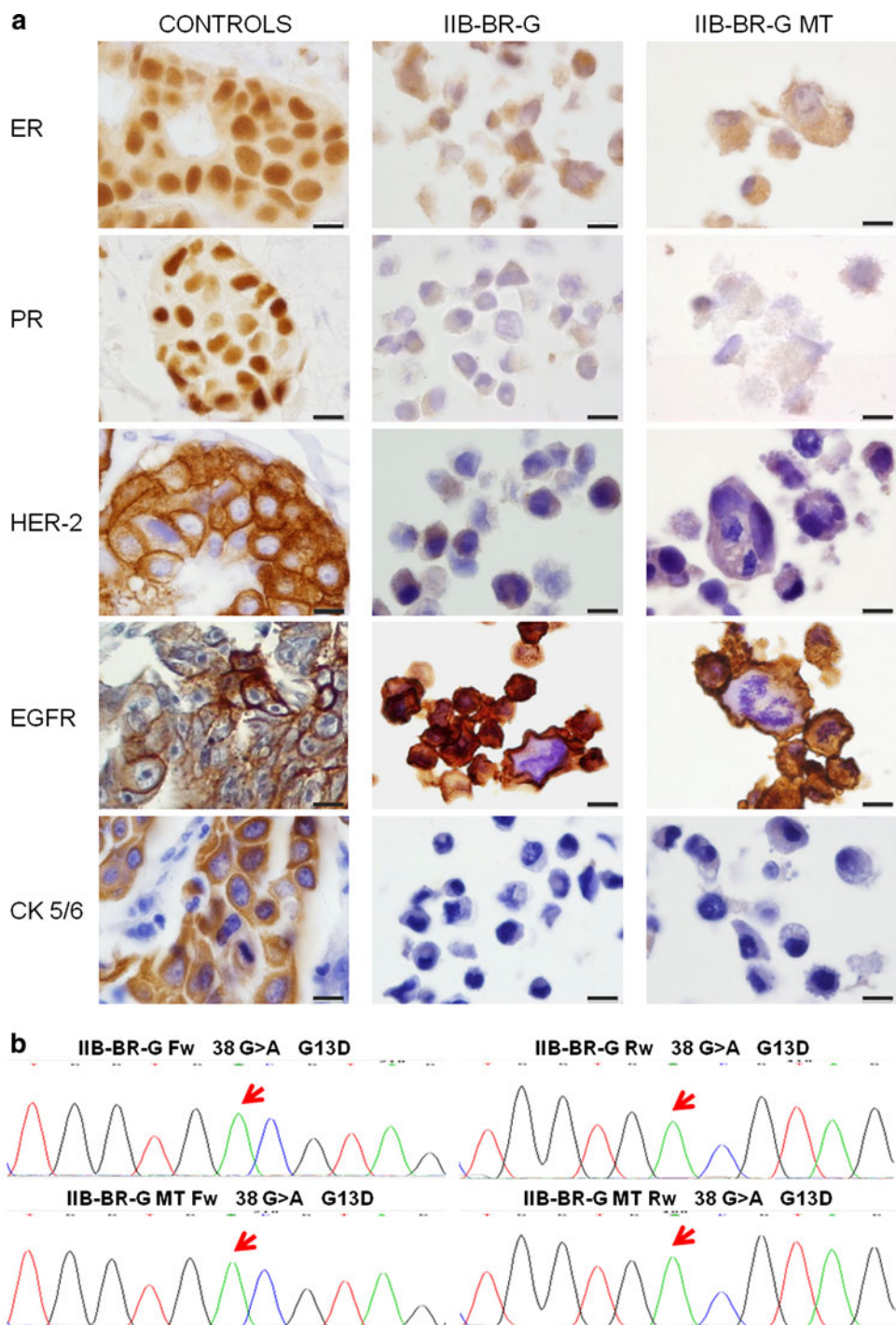
Cetuximab-coated cell lines were highly susceptible targets of NK-mediated ADCC (Fig. 3c). NK cells represented between 5 and 15% of PBMC in our healthy donor samples. Hence, the ADCC reached by 10:1 NK:T ($55 \pm 5\%$ and $42 \pm 9\%$ for IIB-BR-G and IIB-BR-G MT, respectively; Fig. 3c), was comparable to ADCC by 100:1 PBMC:T ($50 \pm 2\%$ and $29 \pm 3\%$ for IIB-BR-G and IIB-BR-G MT, respectively; Fig. 3b). The 20:1 NK:T reached a maximal ADCC; higher ratios did not increment lytic activity, probably due to an excess of effector cells.

IL-2 and IL-15 increased PBMC cytotoxicity against IIB-BR-G and IIB-BR-G MT cell lines

With the purpose of testing immune modulators to enhance ADCC, we first evaluated the addition of IL-2 to

Fig. 2 Blockade of intracellular signaling of tumor cells in antitumor activity of Cetuximab for TNBC. **a** Growth inhibitory effect of Cetuximab on TNBC-K-RAS mutated cell lines. Dose–response curve of Cetuximab effect on TNBC cell lines proliferation. Results are expressed as percentage of the untreated (*control*) values. Data show mean ± SEM values of six independent experiments. **b** Apoptosis-inducing activities of Cetuximab for TNBC cell lines. Each cell line (2×10^5 cells) was incubated in medium alone ±Cetuximab (200 µg/ml) or a positive apoptosis control performed with 1 µg/ml Doxorubicin. After incubation for 96 h, apoptosis was measured by staining with FITC-conjugated Annexin-V and propidium iodide (PI) and analyzed by FACS. The populations of early apoptotic cells (Annexin-V positive/PI negative) and late apoptotic cells (PI positive) were calculated as a percentage of total cells. Dot plots show a representative data of four independent experiments
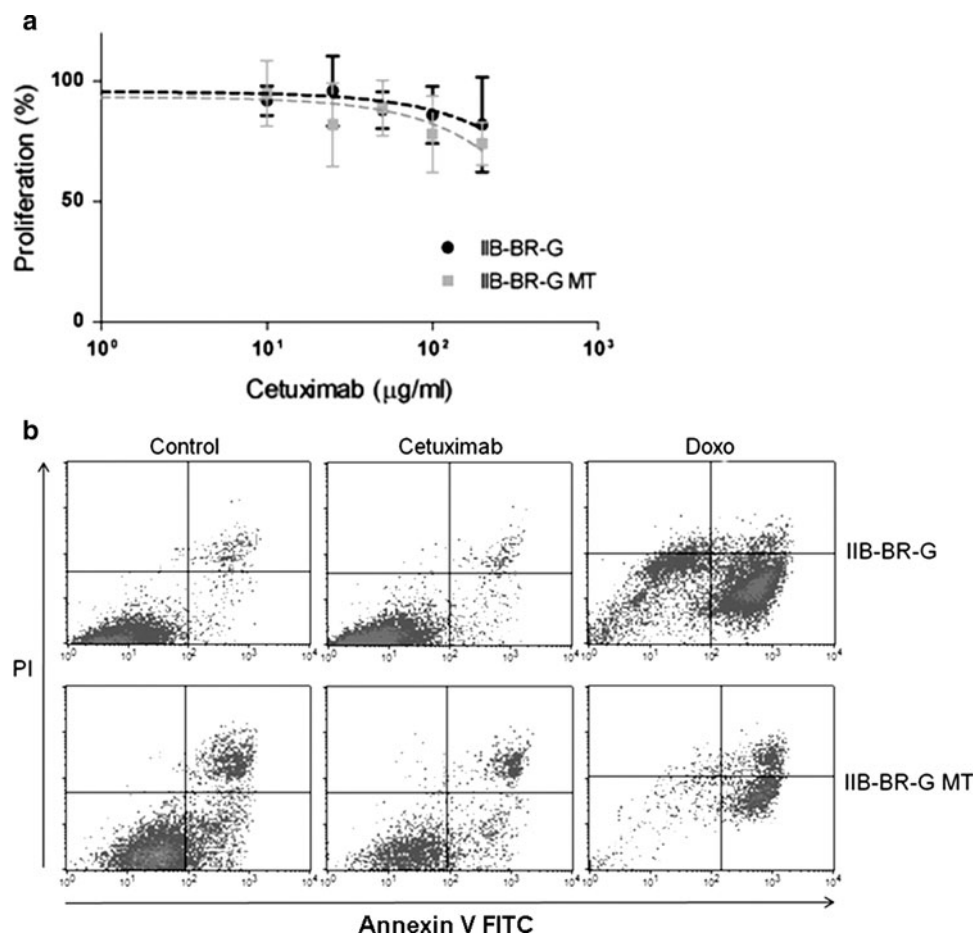

Table 1 Cetuximab effect on apoptosis induction on IIB-BR-G and IIB-BRG MT cell lines

Treatment	Cell death	
	IIB-BR-G	IIB-BR-G MT
Control	12.0 ± 5.6%	18.7 ± 9.4%
Cetuximab 200 µg/ml	6.4 ± 2.2%	18.4 ± 11.3%
Doxorubicin 1 µg/ml	80.6 ± 8.0%***	76.7 ± 6.8%***

Each cell line was incubated in three different conditions: medium alone (control), medium with Cetuximab (200 µg/ml), or medium with Doxorubicin (1 µg/ml) (positive apoptotic control). Percentage of cell death was calculated including three quadrants (the populations of early apoptotic cell (Annexin-V positive/PI negative), late apoptotic cells (PI positive), and necrotic cells a percent of total cells were evaluated. Data show mean ± SD values of four independent experiments. Percentual values of Annexin V positive cells at 96 h treatment measured by flow cytometry. *P* values were calculated by Dunett's multiple comparisson test ***$p < 0.001$

Cetuximab, because the induction of an activated phenotype of effector cells is well demonstrated for this cytokine. Cetuximab-mediated ADCC was enhanced by the pretreatment of PBMC with IL-2, while basal cytotoxic activity of IL-2 pretreatment showed less effect (Fig. 4a). We sought next to determine if IL-15 could influence the lytic capacity

of PBMC as IL-2 does. Thus, we performed Calcein release assays using both tumor cell lines and PBMC pre-treated or not with either IL-2 or IL-15. Interestingly, the PBMC pre-activation of with IL-15 caused a statistically significant enhancement (35%) of ADCC against Cetuximab-coated tumor cell lines, over the IL-2 effect, at an E:T ratio of 20:1 (Fig. 4b). Even in the absence of Cetuximab, IL-15 pre-treated PBMC did increase basal cytotoxicity.

IL-15 increased the expression of activating NK cell receptors and promoted INF-γ secretion in presence of Cetuximab

To further investigate the molecular mechanisms accompanying the increase in ADCC induced by IL-15 and IL-2, we determined first the phenotypic profile of the NK subpopulations before and after treatment with the cytokines. We found that IL-15-induced upregulation of activating NK receptors, mainly CD16 and NKG2D (Fig. 5a), with an increase observed, both in the percentage of cells expressing the receptor and the MFI, without substantial modification of the inhibitory receptors (data not shown). We were also interested in determining if IL-15 could enhance IFN-γ
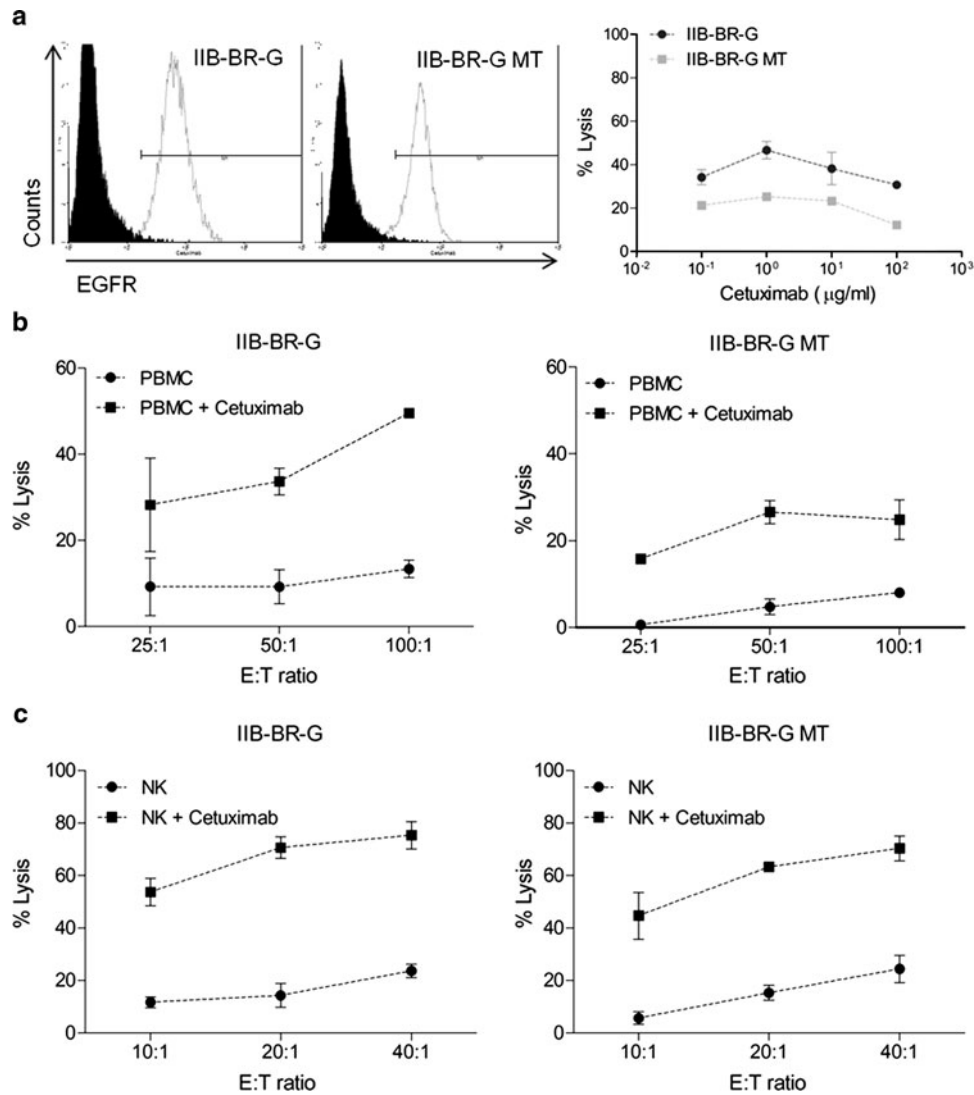
Fig. 3 Cetuximab mediated ADCC against TNBC cell lines. **a** Concentration-dependent curve of Cetuximab-mediated ADCC activity (*left*). PBMC ADCC from healthy donors against IIB-BR-G and IIB-BR-G MT cell lines were evaluated by Calcein-release assay at E:T ratio of 25:1. Data showed mean ± SEM values of three independent experiments. EGFR expression on TNBC cell lines (**a** *right*). Histograms show EGFR membrane expression (*gray empty peaks*) and isotype control (*black fill peaks*) in each TNBC cancer cell line analyzed by FACS. **b** Cetuximab-mediated PBMC ADCC activity against TNBC cell lines at different E:T ratios. IIB-BR-G and IIB-

BR-G MT cell lines were analyzed for ADCC using PBMC from healthy donors in the presence of 1 µg/ml Cetuximab at different E:T ratios. Data show mean ± SEM values of five independent experiments. **c** Cetuximab-mediated ADCC activity of NK cells. Effectors cells were tested for cytotoxicity against Cetuximab-treated TNBC cell lines. CD56$^+$CD3$^-$ cells from healthy donors were purified by negative selection (purity >95%). ADCC was performed against Cetuximab-treated BC cell lines at different E:T ratios in the presence of 1 µg/ml Cetuximab data show mean ± SEM of three independent experiments

production, as IL-2 does, in response to Cetuximab-coated tumor cells. We performed cocultures with purified NK, pretreated or not with these cytokines, and Cetuximab-coated IIB-BR-G cells (Fig. 5b). The overall levels of IFN-γ production that were observed varied among donors. Nevertheless, for all donors tested, NK cells in the presence of Cetuximab-coated tumor cells alone secreted modest amounts of INF-γ and costimulation with IL-2 or IL-15 substantially enhanced IFN-γ production. In all cases, IL-15 resulted in two- to five-fold higher levels of IFN-γ

production than IL-2, being the most potent stimulus. Of note, IL-15 was capable of inducing basal IFN-γ release in the absence of Cetuximab and tumor cells.

Discussion

IIB-BR-G cell line and its metastatic variant IIB-BR-G MT were previously established in our laboratory from a patient's primary breast tumor [33, 34]. IIB-BR-G cells are

Fig. 4 IL-2 and IL-15
increased PBMC cytotoxicity
against IIB-BR-G and IIB-BR-
G MT cell lines. **a** Increased
PBMC cytotoxicity after IL-2
treatment. PBMC from healthy
donors were incubated
overnight in medium alone or in
medium supplemented with
100 UI/ml IL-2. Each point
denotes mean ± SEM values of
three independent experiments.
b Effects of IL-2 or IL-15
stimulation on PBMC
cytotoxicity. PBMC were
analyzed for basal lytic activity
or Cetuximab-mediated ADCC
against TNBC cell lines,
comparing different
pretreatments with IL-2
(100 IU/ml) or IL-15 (10 ng/ml)
and the control (medium alone).
Figure shows a representative
data of three independent
experiments. *$p < 0.05$,
**$p < 0.01$, and ***$p < 0.001$
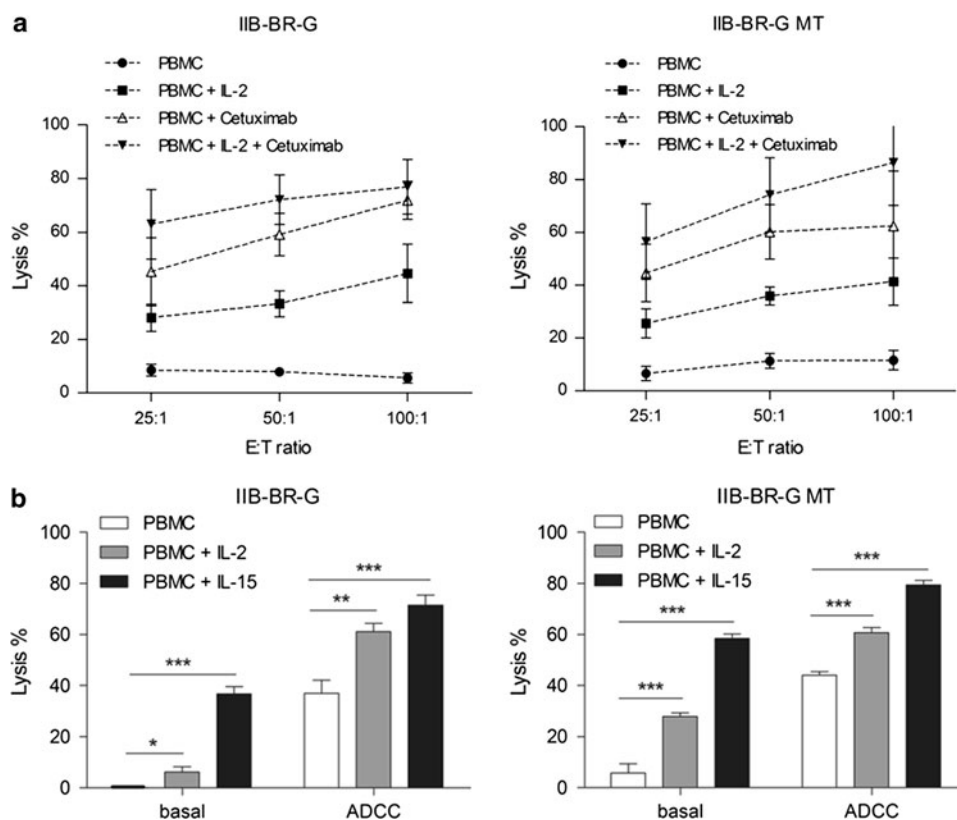
able to grow in nude mice as subcutaneous tumors that resemble the original human tumor, whereas IIB-BR-G-MT cells have adapted to grow as subcutaneous xenografts that spontaneously give rise to metastasis in a high proportion in nude mice. We determined by IHC that both cell lines are *TN* (negative for ER, PR, and HER-2), both have strong, but *different EGFR expression levels*, and carry the same point mutation 38 G > A at codon 13 within K-RAS gene. Hence, TN *K-RAS-mutated* cell lines offer an interesting model to assay therapeutic strategies mediated by Cetuximab. First, we determined that Cetuximab-mediated antiproliferative and apoptosis-inducing activities on TNBC cell lines were relatively small or had no effect, even when the highest achievable plasmatic doses were used. In this sense, failure to respond to Cetuximab was associated with mutations in K-RAS that lead to constitutive mitogen-activated protein kinase signaling [35]. Nevertheless, Cetuximab should still be able to bind to tumor cell with mutations of the EGFR kinase domain or with K-RAS mutations, because the extracellular portion of EGFR is unaffected by these alterations [36]. Hence, we focused on the ability of the mAb to initiate tumor-specific immune responses. As a new finding in the present study, we clearly showed that EGFR-expressing TNBC cell lines could be killed in vitro, by Cetuximab-mediated ADCC, in spite of having mutated K-RAS, manifesting the independence of the immune mechanism triggered by Cetuximab

from tyrosine kinase signaling pathways. Based on previous reports, the average plasma concentration of Cetuximab in cancer patients is estimated to be within the range of 56 and 100 µg/ml under current clinical dosing regimens [37]. In our study, the maximum specific lysis of target cells was observed at 1 µg/ml Cetuximab, suggesting that Cetuximab-mediated ADCC activity could have an important antitumor effect. Even when metastatic cell line IIB-BR-G MT displayed lower EGFR expression level, PBMC and/or NK cells were capable to kill them in presence of Cetuximab. Improved understanding of the cytokines involved in modulating effector cells of the innate immune system, together with deeper knowledge of the mechanisms of NK cell recognition and killing of target cells, has provided a basis for the rational investigation of immunoregulatory cytokine combinations in the treatment of specific cancers. Cytokines such as IL-2 [38] and interferons [39] have been shown, both in mouse models and clinical trials, to enhance ADCC by stimulating or expanding in vivo populations of NK cells, macrophages, and monocytes. In our work, we have shown that Cetuximab-mediated ADCC activity against BC cell lines is enhanced in response to IL-2, in concordance to previous reports which showed that NK cells are the major effectors of ADCC activity, which is augmented by IL-2 [40, 41]. On the other hand, IL-15 is a four-α helix, short chain, type I T-cell growth factor that shares some structural and

Fig. 5 IL-15 effects on the expression of activating NK cell receptors and INF-γ secretion in presence of Cetuximab. **a** Flow cytometry histograms (*top*) illustrate the levels of expression of CD16 and NKG2D in NK after culture for 24 h with medium alone (*gray full peaks*), IL-2 (*black lines*), or IL-15 (*gray lines*). Isotype controls are indicated as *black fill peaks*. **b** NK cell IFN-γ production in response to Cetuximab-coated tumor cells. Purified NK cells from healthy donors were cocultured with Cetuximab-coated IIB-BR-G tumor cell line in presence of IL-2 (100 IU/ml) or IL-15 (10 ng/ml). IFN-γ secretion was measured after 24 h. Data show mean $\pm$ SEM values of three independent experiments. ***$p < 0.001$
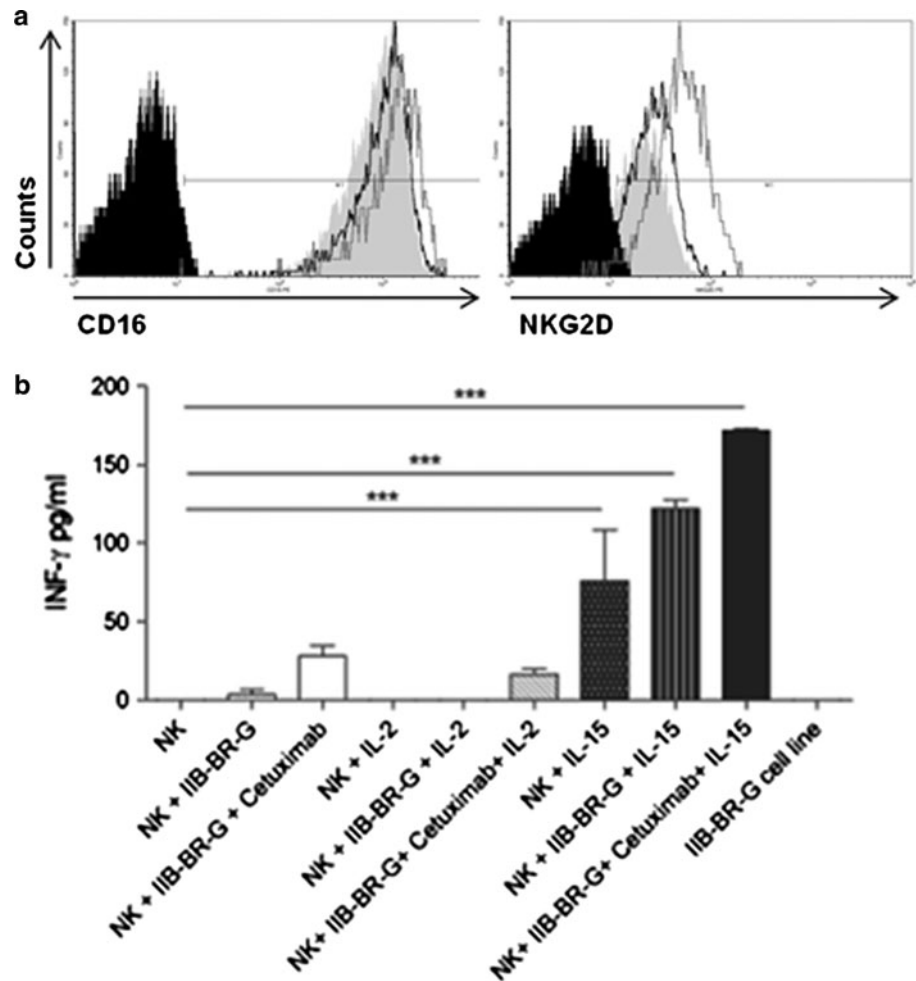
biological similarities with IL-2 [42, 43] and which can replace IL-2 in most of its activities in the immune system, including induction of T-cell proliferation, stimulation of NK cell growth, and IFN-γ production. Studies using knockout mice have revealed that IL-15 is the major physiologic growth factor responsible for NK cell differentiation and for survival and cytolytic activity of mature NK cells, through the enhancement of the expression of NKG2D receptor at 72 h [44–46]. Even in shorter preincubation times, we found that NK cells from healthy donors upregulated the activating receptor expression (mainly, CD16 and NKG2D) in the presence of exogenous IL-15 or IL-2, along with a significant threefold and twofold increase, respectively, in Cetuximab-mediated ADCC by PBMC against TNBC cell lines. FcγRs provide the key link between therapeutic mAbs and the cellular immune system, and they enable mAbs to induce adaptive immune responses. The magnitude and quality of the innate immune responses induced by ADCC are likely to influence the ensuing adaptive immune response. We observed that IFNγ production increased up to fivefold when NK cells were

previously stimulated with IL-15 rather than with IL-2 in the presence of Cetuximab-coated tumor cells. IFN-γ produced by NK cells could also exert direct antiproliferative effects against tumor cells and promote T-cell infiltration of tumor deposits [47].

This in vitro study suggests that ADCC, independently of the blockade of EGFR intracellular signaling, could be a useful mechanism for antitumor activity of Cetuximab in TNBC. The combination of Cetuximab with modulators of the immune response as IL-15 or IL-2 may be considered an attractive therapeutic approach to enhance the clinical efficacy of Cetuximab in TNBC.

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