Anti-EGFR antibody cetuximab enhances the cytolytic activity of natural killer cells towards osteosarcoma

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Translational Relevance

In the last decades, no improvement in the prognosis of patients with advanced bone sarcomas has been achieved despite advancements in surgical techniques and intensification of chemotherapy regimens. Novel treatment modalities are needed which combine anti-tumor activity with an acceptable toxicity profile. Therefore, we have focused on NK cell-based immunotherapeutic approaches. In this study, we demonstrate that the anti-EGFR monoclonal antibody cetuximab can mediate lysis of patient-derived osteosarcoma cells by NK cells via antibody-dependent cellular cytotoxicity. In addition, NK cells from osteosarcoma patients possess normal cytolytic function when specifically directed to autologous tumor with cetuximab. Since the administration of cetuximab has already resulted in objective responses in patients with other tumors in association with only mild adverse effects, these findings provide a rationale for the implementation of cetuximab-mediated immunotherapy in the treatment of advanced osteosarcoma.
Abstract

Purpose

Osteosarcoma and Ewing’s sarcoma are the most common bone tumors in children and adolescents. Despite intensive chemotherapy, patients with advanced disease have a poor prognosis, illustrating the need for alternative therapies. Sarcoma cells are susceptible to the cytolytic activity of resting NK cells which can be improved by IL-15 stimulation. In this study, we explored whether the cytolytic function of resting NK cells can be augmented and specifically directed towards sarcoma cells by antibody-dependent cellular cytotoxicity (ADCC).

Experimental Design

EGFR expression was examined on osteosarcoma and Ewing’s sarcoma cell lines by flow cytometry and in osteosarcoma biopsy and resection specimens by immunohistochemistry. Cetuximab-mediated ADCC by NK cells from osteosarcoma patients and healthy controls was measured with 4 h $^{51}$Cr release assays.

Results

EGFR surface expression was demonstrated on chemotherapy-sensitive and -resistant osteosarcoma cells (12/12) most primary osteosarcoma cultures (4/5) and few Ewing’s sarcoma cell line (2/7). In the presence of cetuximab, the cytolytic activity of resting NK cells against all EGFR-expressing sarcoma cells was substantially increased and comparable to that of IL-15-activated NK cells. Surface EGFR expression on primary osteosarcoma cultures correlated with EGFR expression in the original tumor. The cytolytic activity of osteosarcoma patient-derived NK cells against autologous tumor cells was as efficient as of NK cells from healthy donors.

Conclusion
Our data demonstrate that the cytolytic potential of resting NK cells can be potentiated and directed towards osteosarcoma cells with cetuximab. Therefore, cetuximab-mediated immunotherapy may be considered a novel treatment modality in the management of advanced osteosarcoma.
**Introduction**

Osteosarcoma and Ewing's sarcoma most frequently arise in adolescents and young adults and represents the majority of all malignant primary bone tumors in this patient group [1-3]. The current treatment consists of a combination of systemic multi-drug chemotherapy and complete surgical resection [3-5]. In cases with localized disease, up to 70% of the patients achieve persistent remission. In contrast, patients with advanced, metastasized and recurrent disease experience a very poor prognosis, which has not improved during the last decades despite intensification of chemotherapy regimens. Therefore, novel treatment strategies with a favorable toxicity profile are warranted. In this perspective, we and others have recently reported on the potential exploitation of cellular immunotherapy against sarcomas by NK cells [6-8].

NK cells can respond to and kill cells undergoing cellular stress due to virus infection or malignant transformation. The cytotoxic activity of NK cells is regulated by the equilibrium of inhibiting and activating signals conveyed by target cells. On tumor cells, MHC class I expression (NK cell inhibitory signal) may be down-regulated to evade cytotoxic T cell recognition. Conversely, the expression of stress ligands (NK cell activating signal) may be up-regulated on tumor cells. Both of these processes may lead to increased sensitivity of tumor cells to NK cells [9;10]. In addition, the interplay with other immune cells and the pro- or anti-inflammatory microenvironment may modulate the function and activity of NK cells [10;11].

Recently, we and others have shown that sarcoma cell lines are moderately susceptible to the cytolytic potential of resting NK cells [6-8]. The cytolytic activity of NK cells can be directly potentiated by activating cytokines, such as IL-15, leading to increased lysis of sarcoma cells [6-8]. In this study we set out to explore whether the
cytolytic activity of resting NK cells can be improved and directed specifically towards sarcoma cells. Therefore, we intended using a monoclonal antibody (mAb) of the human IgG1 subtype which recognizes an antigen expressed on sarcoma cells and is able to induce antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Osteosarcoma has previously been shown to express the epidermal growth factor receptor (EGFR, erbB1/Her1) [12], which is recognized by the clinically approved, chimeric IgG1 type mAb cetuximab [13]. So far, the application of anti-EGFR mAb targeted therapy in bone sarcomas has not been reported. We focused on exploring whether the cytotoxic potential of allogeneic and autologous NK cells can be specifically directed towards sarcoma cells with cetuximab.
**Materials and Methods**

**Patient samples**

Formalin-fixed, paraffin-embedded tumor samples were obtained from one biopsy (obtained at the time of diagnosis, pre-chemotherapy) and four resections of local recurrent or metastatic tumors (post-chemotherapy) from four high-grade osteosarcoma patients (diagnosed between 2008 and 2010) by the Department of Pathology, Leiden University Medical Center. Five short-term-grown primary osteosarcoma cell cultures (between passage 2 and 13) were generated from the tumor material as previously described [6;14]. Clinicopathological details of these patients and samples are summarized in Table 1. Peripheral blood samples from these patients were collected prior to the initiation of chemotherapy after written informed consent approved by the Review Board on Medical Ethics of the Leiden University Medical Center and used for cytotoxicity experiments (Table 1). Tumor specimens were obtained and analyzed according to the ethical guidelines of the national organization of scientific societies (FEDERA, http://www.federa.org/gedragscodes-codes-conduct-en).

**Cell lines**

The following extensively characterized sarcoma cell lines were included in this study: osteosarcoma cell lines OHS, OSA (SJSA-1), SAOS-2, U2-OS, ZK-58 [15]; and the Ewing’s sarcoma cell lines A673, CADO-ES, SK-ES-1, SK-N-MC, STA-ET2.1, TC71 [15] and L1062 [14]. All sarcoma cell lines were obtained from the EuroBoNeT cell line repository (by 2007) and were confirmed for their identity by short tandem repeat DNA fingerprinting in 2011. The cell line TC71 was maintained in IMDM medium (Invitrogen, Carlsbad, CA, USA). All other cell lines were grown in...
RPIM-1640 medium (Invitrogen). Both media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 ug/ml streptomycin (all Invitrogen). All Ewing’s sarcoma cell lines were grown in 0.1% gelatin coated tissue culture flasks. The chemotherapy-resistant variant cell lines of SAOS-2 and U2-OS [16-18] were cultured in IMDM medium with 10% FCS and penicillin/streptomycin and maintained in chemotherapeutic drugs as follows: SAOS-2-DX580 and U2-OS-DX580 with 580 µg/ml of doxorubicin (DX); SAOS-2-MTX1000 and U2-OS-MTX300 with 1000 and 300 ng/ml of methotrexate (MTX), respectively; SAOS-2-CDDP6 (SAOS-2-CDDP6µg) and U2-OS-CDDP4 (U2-OS-CDDP4ug) with 6 and 4 µg/ml of cisplatin (cis-diamminedichloroplatinum, CDDP), respectively. Drug sensitivities of each cell line were calculated from the drug dose-response curves and expressed as IC50 (drug concentration resulting in 50% inhibition of cell growth after 96 hours of in vitro treatment). Fold-increases in drug resistance, quantified as the ratio between IC50 of each drug resistant variant to that of its corresponding parental cell line, were as follows: 315 for U2-OS-DX580, 328 for SAOS-2-DX580, 135 for U2-OS-MTX300, 281 for SAOS-2-MTX1ug, 63 for U2-OS-CDDP4ug, and 112 for SAOS-2-CDDP6ug. All cell lines were negative for mycoplasma infection as regularly checked by PCR. The primary osteosarcoma cultures were maintained in RPMI-1640 medium supplemented with 20% FCS and penicillin/streptomycin in gelatine-coated culture tissue culture flasks.

Cell isolations and stimulations

Peripheral blood mononuclear cells (PBMC) were isolated from osteosarcoma patients' blood samples (autologous) or buffy coats of healthy adult donors (allogeneic) (Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands)
Cetuximab enhances NK cell killing of osteosarcoma cells

by Ficoll-Hypaque density gradient centrifugation. NK cells were purified by negative
selection, depleting non-NK cells through a combination of biotin-conjugated
monoclonal anti-human antibodies and MicroBeads using the “Human NK cell
Isolation Kit” (Miltenyi Biotech, Bergisch Gladbach, Germany); NK cell purity was
>95% as determined by flow cytometry, analyzing NK cells as CD56^+, CD3^−, CD14^−
and CD19^− cells. NK cells were depleted from PBMC (NKD PBMC) by positive
selection using anti-CD56 MicroBeads (Miltenyi Biotech); NKD PBMC contained less
than 0.1% of NK cells as analyzed by flow cytometry. IL-15-activated NK cells were
obtained by incubating purified NK cells in AIM-V medium with 2 mM of glutamine
(Invitrogen) supplemented with 10% of pooled human AB serum (Sanquin,
Rotterdam, the Netherlands), penicillin/streptomycin and 10 ng/ml of IL-15
(Peprotech, Rocky Hill, NJ, USA) for two to three weeks in 24-well format tissue
culture plates without feeder cells.

To measure NK cell activation after cetuximab cross-linking, up-regulation of CD69
expression on NK cells (300,000) was measured after co-culture with STA-ET2.1
(150,000), L1062 (80,000) and OSA (75,000) cells for 48 hrs in 24-well plates in the
absence or presence of cetuximab (10 µg/ml).

**Flow cytometry**

Determination of NK cell percentages in PBMC, validation of NK cell purity and
expression of the NK cell activation marker (CD69) was analyzed phenotypically by
staining with fluorescently labeled antibodies followed by FACS. The following
antibodies were applied according to the manufacturer’s instructions: anti-CD3^FITC
(SK7), anti-CD3^PerCP-Cy5.5 (SK7), anti-CD14^PerCP-Cy5.5 (M5E2), anti-CD19^PE (4G7),
CD69^FITC (L78) (Becton Dickinson, Franklin Lakes, NJ, USA); anti-CD56^APC (N901
NKH1), anti-NKG2D<sup>PE</sup> (ON72) (IOTEST Immunotech, Marseille, France). Expression of EGFR on the surface of sarcoma cell lines and primary osteosarcoma cultures was measured using the anti-EGFR mAb cetuximab (Erbitux<sup>®</sup>, Merck KGaA, Darmstadt, Germany) followed by the Alexa-Fluor-647 goat anti-human IgG secondary antibody (A21445) (Invitrogen). The anti-CD20 mAb rituximab (MabThera<sup>®</sup>, Roche, Basel, Switzerland) was used as an IgG1 isotype-matched negative control for cetuximab. FACS measurements were performed with the FACSCalibur (BD Biosciences) and analyzed with the “BD Cell Quest ProTM” software (version 5.2.1).

**51Cr release assay**

The cytolytic activity of PBMC, NKD PBMC and purified NK cells against sarcoma cell lines and primary osteosarcoma cultures was measured in 4 h <sup>51</sup>Cr release assays. Target cells were labeled with 100 µl Na-chromate (<sup>51</sup>Cr, 3.7 MBq) for 1 hr. After washing, 2.5x10<sup>3</sup> target cells were added to the effector cells in duplicate or triplicate at the indicated effector-target (E:T) ratios and incubated in the presence or absence of cetuximab (10<sup>-7</sup> to 10 µg/ml as indicated) or the control mAb rituximab (10 µg/ml) for 4 h at 37 °C. Supernatants were collected, and the release of <sup>51</sup>Cr was measured with a beta-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and total release were obtained by incubation with medium and Triton X-100 (2.5%; Merck Chemicals, Darmstadt, Germany), respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 x (experimental release-spontaneous release / total release-spontaneous release).

**Immunohistochemistry**
Sections of 4 μm of representative tumor sections were de-paraffinized and pepsin-antigen retrieval was performed. Expression of EGFR was assessed using a mouse monoclonal anti-EGFR antibody (31G7, 1:10; Zymed, Invitrogen) followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP-linker antibody conjugate (DPVO-110HRP; Immunologic, Duiven, the Netherlands) and DAB+Substrate Chromogen System (Dako, Glostrup, Denmark) detection. The sections were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica, Solms, Germany).

Statistical analysis

Statistical analyses were performed with Graphpad Prism version 5.04 (La Jolla, CA, USA) or SPSS version 16.0 (IBM, Armonk, NJ, USA) using paired student t-tests, comparing means between groups of samples, and linear regression analysis. Error bars represent the standard error of the mean. A P-value of <0.05 was considered statistically significant. ns, not statistically significant; *, P<0.05; **, P<0.01; ***, P<0.001.
Results

EGFR is expressed on the surface of osteosarcoma cell lines

ADCC by NK cells requires antibody-binding to an antigen expressed on the tumor cell surface. Therefore, surface expression of EGFR, as detected by cetuximab, was measured on a panel of osteosarcoma (n=12) cell lines by flow cytometry. All osteosarcoma cell lines expressed EGFR on the cell surface, with the highest expression on the cell lines HOS, OHS and OSA (Figure 1). The chemoresistant variants of SAOS-2 and U2OS also expressed EGFR. Previously, EGFR has been reported undetectable in Ewing’s sarcoma cell lines (n=3) [19]. To extend these findings, surface EGFR expression was assessed on a panel of Ewing’s sarcoma cell lines (n=7). EGFR expression was not detectable on five out of seven Ewing’s sarcoma cell lines. Correspondingly, EGFR expression was not detectable in Ewing’s sarcoma biopsy and resection specimens as determined by immunohistochemistry (data not shown).

Cetuximab enhanced cytolysis of EGFR-expressing osteosarcoma cell lines by NK cells

To investigate whether cetuximab can enhance cytolysis of EGFR-expressing osteosarcoma and Ewing’s sarcoma cell lines by NK cells, resting NK cells were incubated with one EGFR⁻ and several EGFR⁺ cell lines in the presence of cetuximab or the isotype-matched, non-binding anti-CD20 control mAb in a 4 h ⁵¹Cr release assay. As compared to cytolysis in the absence of mAb, the negative control mAb did not alter killing of sarcoma cells by NK cells (data now shown). In contrast, the addition of cetuximab increased the lysis of EGFR-expressing sarcoma cells but not of EGFR-negative cell lines (Figure 2, panel A and B, i; data not shown). The lysis of
the chemotherapy-resistant variant cell lines of SAOS-2 and U2OS was equally enhanced by cetuximab. Cetuximab-enhanced lysis by resting NK cells was comparable to the lysis by IL-15-activated NK cells (Figure 2, panel A and B, ii). The addition of cetuximab to IL-15-activated NK cells hardly led to a further increase of the cytolytic activity. As an alternative parameter for NK cell activation, it was observed that the percentage of CD69-expressing NK cells increased after co-culture of NK cells with EGFR-expressing sarcoma cells in the presence of cetuximab (Figure 3, panel A).

Thus, the cytotoxic function of resting NK cells towards EGFR-expressing sarcoma cell lines as well as their activation status was substantially augmented in the presence of cetuximab.

Cetuximab-mediated lysis is independent of EGFR expression intensity

Despite different sensitivities to NK cell killing, the magnitude of cetuximab-enhanced lysis by resting NK cells was comparable among most EGFR-expressing cell lines (Figure 2, panel B, i). This increase did not correlate significantly with EGFR surface densities (Figure 3, panel B). Thus, even the minimal EGFR expression levels on some of the sarcoma cells were sufficient for the induction of ADCC.

Cetuximab-mediated lysis is dependent on NK cells

In the absence of effector cells, cetuximab did not elicit cytolytic effects on EGFR-expressing cell lines during the 4 h cytotoxicity assay (Figure 4). FcRIIIa/CD16 expression is required to elicit ADCC by NK cells. Since FcRIIIa/CD16 can also be expressed by monocytes it was studied whether cetuximab-mediated ADCC by PBMC is dependent on NK cells. In the presence of cetuximab, lysis of EGFR-
expressing sarcoma cells by PBMC was comparable to the cetuximab-enhanced
lysis by purified NK cells (Figure 4, panel A). In contrast, NK cell depletion abolished
killing by PBMC both in the absence and presence of cetuximab, indicating that in
this in vitro system cetuximab-mediated killing was strictly dependent on NK cells
present in the PBMC.

Next, the dependence of cetuximab-mediated lysis on the concentration of cetuximab
was investigated in a serial dilution experiment. The lysis induced by cetuximab was
comparable between 10 and $10^{-2}$ µg/ml of cetuximab, but it was reduced by at
least 50% at a concentration of $10^{-3}$ µg/ml (Figure 4, panel B). Lower cetuximab
concentrations failed to enhance lysis. Hence, 0.01 µg/ml of cetuximab was the
minimal concentration to substantially enhance cytolysis by NK cells.

EGFR+ primary cultures derived from EGFR+ osteosarcoma tumors are highly
susceptible to cetuximab-mediated lysis by autologous PBMC

Primary tumor cell cultures (n=5) were generated from osteosarcoma biopsy (n=1)
and resection (n=4) specimens derived from four different osteosarcoma patients
(Table 1). EGFR expression in these osteosarcoma samples was membranous, as
determined by immunohistochemistry, and correlated to the EGFR densities on the
corresponding primary cultures, as determined by flow cytometry between passages
(p) 3 and 13.

Except for osteosarcoma patient 398 in which EGFR was not detectable in the biopsy
and only weakly on the corresponding primary culture L2635 p6, all osteosarcoma
patients (369, 404 and 407) presented EGFR expression both in the original tumor
material and the respective primary cultures (L2792 p3; L2826 p9 and L3312 p13;
L2857 p7) (Figure 5, panel A and B). The cytolytic activity of NK cells from patient-
derived PBMC towards EGFR-expressing autologous osteosarcoma cultures was substantially enhanced by cetuximab (Figure 5, panel C). Notably, patient-derived NK cells lysed the osteosarcoma cultures as efficient as NK cells from healthy donors. Thus, when sarcoma cells are specifically targeted with cetuximab, their lysis by resting NK cells can be enhanced by ADCC.
Discussion

The identification of antigens specifically expressed on tumor cells has fueled the development of tumor-specific, mAb-based targeted therapies. The introduction of anti-CD20 mAb (Rituximab, MabThera®) and anti-Her2 mAb (Trastuzumab, Herceptin®) against B cell malignancies and breast cancer, respectively, has improved patient prognosis [20;21].

Surface expression of EGFR has been shown in several tumors, such as colorectal cancer, head and neck squamous cell carcinoma, and is specifically recognized by the chimeric IgG1 mAb cetuximab [13;20]. In numerous clinical phase II and III studies, the addition of cetuximab to conventional multi-drug chemotherapy or radiotherapy has led to a significant improvement of clinical response rates, progression-free survival and overall survival. Therefore, cetuximab therapy was approved for the treatment of recurrent, refractory and metastatic colorectal cancer [22-25] and head and neck squamous cell carcinoma [26;27] by the Food and Drugs Administration (FDA).

Previously, we and others have demonstrated the cytolytic potential of NK cells against sarcoma cells [6-8]. In this study we sought to explore whether this cytolytic activity can be more specifically targeted towards sarcoma cells using a sarcoma-reactive mAb with a human Fc portion that can bind to FcRIIIa/CD16 on human NK cells. As several studies have described surface EGFR expression in osteosarcoma tumors and on osteosarcoma cell lines [12;19;28-31], we explored the potential of the anti-EGFR mAb cetuximab to specifically direct NK cell-mediated killing to sarcoma cell lines.

In agreement with previous studies on other tumor types, cetuximab induced NK cell-dependent ADCC against EGFR-expressing sarcoma cells. Similar to previous
studies [32-35], we demonstrate that 0.01 µg/ml of cetuximab was the minimal concentration to induce cetuximab-mediated lysis by NK cells. These concentrations have been reported in sera of patients treated with cetuximab and in the tumor environment in a xenograft model [36;37], indicating that cetuximab-mediated ADCC could be a functional anti-cancer mechanism in vivo. While in other studies the magnitude of cetuximab-induced ADCC correlated with the level of EGFR expression [34;35;38], this correlation was not evident in osteosarcoma despite the use of highly comparable methods to assess ADCC [32;39]. In fact, minimal EGFR densities were sufficient for the cetuximab-induced lysis of sarcoma cells [32;39]. Cetuximab-induced ADCC was comparable to the maximal killing achieved by IL-15-activated NK cells. In contrast to some other models [32;34;40;41], we did not observe an additive effect of cetuximab on the lysis by cytokine (IL-15)-activated NK cells. Multiple mechanisms may account for the anti-tumor effect of cetuximab in patients. Masking of the EGFR extracellular binding site from its natural ligand EGF inhibits the activation of the receptor tyrosine kinase and downstream signaling pathways [13;42]. EGFR blockage has been shown to arrest cell cycle progression and lead to apoptosis [13]. Cetuximab can inhibit tumor angiogenesis, neo-vascularization and invasion and sensitizes tumor cells to radiation and chemotherapy-induced growth inhibition and apoptosis in vivo [13]. Finally, cetuximab may induce complement-dependent cytotoxicity or cytolytic effects by immune cells via ADCC [13;32;38;39;43]. An advantage of cetuximab-mediated ADCC is that it would be independent of the EGFR mutation status [34;38] and persistently activated EGFR signaling pathways [13;40]. The primary mode of action of cetuximab in vivo is difficult to determine. In a murine model, the anti-cancer effect of cetuximab was presumed to be mediated by NK cells
Depletion of NK cells in murine osteosarcoma xenograft models or in mice with syngeneic mesenchymal stem cell-induced osteosarcoma could address whether an anti-tumor effect of cetuximab or murine anti-EGFR mAb relies on the presence of NK cells [44-46]. In humans, the relevance of ADCC has been suggested by the finding that FcγRIIIa/CD16 polymorphism of NK cells correlated with the survival of colorectal cancer patients [47;48] as well as with the efficacy of cetuximab-mediated ADCC by NK cells in vitro [34]. Interestingly, the intratumoral NK cells have recently been associated with improved survival when colorectal cancer patients had been treated with cetuximab [49]. In this study, we used a unique combination of tumor specimens, primary tumor cultures and PBMC from osteosarcoma patients. This allowed us to establish that cetuximab treatment can improve the lysis of EGFR-expressing, autologous primary osteosarcoma cells by patient-derived NK cells via cetuximab-mediated ADCC. Cetuximab treatment is associated with relatively mild adverse effects and has been approved for clinical usage by the FDA [37;50]. Therefore, in the treatment of osteosarcoma patients, cetuximab-mediated immunotherapy could be scheduled in the presence of endogenous or adoptively transferred NK cells. As such, cetuximab may provide an interesting treatment modality for patients with chemotherapy-resistant or metastatic EGFR+ sarcomas.
Acknowledgments

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Reference List


Pahl et al., Cetuximab enhances NK cell killing of osteosarcoma cells


Pahl et al., Cetuximab enhances NK cell killing of osteosarcoma cells


Table 1: Clinicopathological details of patient material

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Figure 1: EGFR is expressed on the surface of sarcoma cell lines
Surface expression of EGFR on osteosarcoma and Ewing’s sarcoma cell lines was measured by flow cytometry using the anti-EGFR mAb cetuximab followed by the Alexa-Fluor-647 goat anti-human IgG secondary antibody. (A) Representative FACS overlay plots, detecting EGFR by cetuximab (bold solid line) and CD20 by the isotype-matched, negative control mAb rituximab (solid line) both followed by secondary antibody, and secondary antibody only (grey area). The indicated fold expression of EGFR was calculated by dividing the geometric mean fluorescence intensity of EGFR by the geometric mean fluorescence intensity of the control CD20. (B) Combined data of the fold change of EGFR expression on all tested sarcoma cell lines of multiple experiments.

Figure 2: Cetuximab-enhanced killing of sarcoma cell lines by NK cells
Lysis of EGFR+ and EGFR− sarcoma cell lines by purified, resting NK cells and IL-15-activated NK cells was measured in triplicate in a 4 h ⁵¹Cr release assay in the presence of cetuximab or the isotype-matched, negative control mAb rituximab. The specific lysis (%) of sarcoma cells in the presence of the control mAb was comparable to killing without mAb addition. (A) Representative data of the specific lysis of sarcoma cell lines (ranked by increasing EGFR density) by resting NK cells (squares) and IL-15-activated NK cells (triangles) in the presence of cetuximab (filled symbols) and the control mAb (open symbols). (B) Combined data of the specific lysis of sarcoma cells by resting NK cells (i, no pattern) and IL-15-activated NK cells (ii, horizontal pattern) calculated at a 10:1 E:T ratio. Data represent three
independent experiments done in triplicate, showing lysis in the presence of the control mAb (open bars) and the respective extra lysis induced by the presence of cetuximab (filled bars, +/- SEM). Significance of cetuximab-induced lysis was calculated by comparing whether the total lysis in the presence of cetuximab (filled bar plus open bar) was statistically different to the lysis in the presence of the control antibody (open bar).

**Figure 3: Cetuximab-induced activation of NK cells**

(A) EGFR-expressing (OSA and STA-ET2.1) and EGFR-negative cell lines (L1062) were co-cultured with purified NK cells in the presence of cetuximab for 48 hours and CD69 expression on NK cells was assessed. The percentage of CD69 positive NK cells is indicated.

(B) The extra lysis induced by cetuximab, as calculated by subtracting the lysis the control mAb from the lysis by cetuximab, was correlated with EGFR expression levels on the cell lines. Lysis of EGFR⁺ sarcoma cell lines (n=13) by resting NK cells at a 10:1 E:T ratio was measured in the presence of cetuximab or the negative control mAb (Figure 2). The extra lysis was calculated by subtracting the specific lysis in the presence of the control mAb from the total lysis in the presence of cetuximab, and plotted against the log of the fold change of EGFR expression (Figure 1). The regression coefficient (r²) between extra lysis and EGFR expression was 0.14 (p=0.21) as calculated by linear regression analysis.

**Figure 4: Cetuximab-mediated killing is dependent on NK cells**

(A) The specific lysis of the osteosarcoma cell line OSA (i) and the Ewing’s sarcoma cell line STA-ET2.1 (ii) in the presence of cetuximab or the control mAb by resting NK
cells was compared to the lysis by total PBMC, NKD PBMC and medium (absence of effector cells). Killing by PBMC was analyzed at a 150:1 E:T ratio. Killing by NKD PBMC was analyzed at a 150:1 E:T ratio corrected for the absence of NK cells in accordance to the percentage of NK cells in the corresponding PBMC. Killing by purified NK cells was extrapolated to a 150:1 E:T ratio of PBMC in accordance with the percentage of NK cells in the corresponding PBMC. Combined data of two experiments. (B) The extra lysis of the two EGFR+ cell lines OSA and STA-ET2.1 by resting NK cells was measured in the presence of increasing concentrations of cetuximab, from $10^{-7}$ to 10 µg/ml, at a 10:1 E:T ratio. Combined data of three independent experiments.

Figure 5: EGFR+ primary osteosarcoma cultures, derived from EGFR+ osteosarcoma specimens, are highly susceptible to cetuximab-mediated lysis by autologous PBMC

(A) EGFR expression in biopsies and resection specimens from osteosarcoma patients was detected using a mouse monoclonal anti-EGFR antibody followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP-linker antibody conjugate. Brown-colored EGFR staining patterns and counterstaining with Mayer’s hematoxylin are displayed at a 40-fold magnification for the respective tumor origin and patient. (B) Primary osteosarcoma cultures were generated from EGFR+ and EGFR- biopsies and resections of osteosarcoma patients as indicated. EGFR expression on the primary osteosarcoma cultures was measured as described in figure 1 at the indicated passage numbers. (C) Killing of primary osteosarcoma cultures by autologous PBMC, derived from the same osteosarcoma patient, and allogeneic PBMC (one representative result from two healthy donors) was analyzed in duplicate
in the presence of cetuximab or the control mAb at the indicated E:T ratios. p,
passage number; prim, primary.
Surface expression of EGFR on sarcoma cell lines

A

L1062
SAOS-2
STA-ET2.1
OSA

fold expression:  1
10
39
71

B

Figure 1
Figure 2

Cetuximab-enhanced killing of sarcoma cells by NK cells

A

Killing of L1062
Killing of SAOS-2
Killing of STA-ET2.1
Killing of OSA

EGFR -
EGFR +
EGFR +
EGFR +

% of specific release

0.3 0.5 1 2 4 8 16 32 64

0 20 40 60 80

NK:L1062
NK:SAOS-2
NK:STA-ET2.1
NK:OSA

- ctrl.
- Cetuximab
- IL15 + ctrl.
- IL15 + Cetuximab

B, i

% specific lysis at E:T 10:1

HOS OSA ZK-58 SAOS-2 S-DX S-MTX S-CDDP U2-OS U-DX U-MTX U-CDDP A673 L1062 STA-ET2.1

ns * ns ns ns ns ns

B, ii

% specific lysis at E:T 10:1

HOS OSA ZK-58 SAOS-2 S-DX S-MTX S-CDDP U2-OS U-DX U-MTX U-CDDP A673 L1062 STA-ET2.1

ns ns ns ns ns ns ns

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Figure 3

A Percentage of CD69+ NK cells after cetuximab cross-linking

B Correlation of Cetuximab-induced extra lysis with EGFR expression

\[ r^2 = 0.14 \]
\[ p = 0.21 \]
Figure 4

A  Cetuximab-mediated killing by PBMC, NKD PBMC and NK cells

i, OSA

ii, STA-ET2.1

% specific lysis at E:T 150:1

no effectors  PBMC  NKD PBMC  NK cells

B  Cetuximab concentration-mediated increase of killing by NK cells

% extra lysis by Cetuximab at 10:1

Cetuximab concentration [µg/ml]

STA-ET2.1  OSA
A  EGFR expression in osteosarcoma tissue

369  local relapse  
398  biopsy  
404  prim. resection  
404  local relapse  
407  prim. resection

B  EGFR expression on primary osteosarcoma cultures

369 L2792 p3
398 L2635 p6
404 L2826 p9
404 L3312 p13
407 L2857 p7

fold expression:  27 1.9 62 35 5.2

C  Cetuximab-mediated killing of primary osteosarcoma cultures by autologous NK cells

Lysis of 369 L2792 p3
Lysis of 398 L2635 p6
Lysis of 404 L2826 p9
Lysis of 404 L3312 p13
Lysis of 407 L2857 p7

allogeneic PBMC + ctrl.
allogeneic PBMC + Cetuximab
autologous PBMC + ctrl.
autologous PBMC + Cetuximab

Figure 5
Anti-EGFR antibody cetuximab enhances the cytolytic activity of natural killer cells towards osteosarcoma


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