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Cetuximab Therapy in Head and Neck Cancer: Immune Modulation with Interleukin-12 and Other Natural Killer Cell Activating Cytokines

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Abstract

We hypothesized that IL-12 would enhance the anti-tumor activity of the anti-HER1 antibody cetuximab against squamous cell carcinomas of the head and neck (SCCHN) by activating the FcR effector mechanisms of NK cells. All cell lines showed high expression of HER1 by flow cytometry and immunoblot analysis. NK cell lysis of cetuximab-coated SCCHN cell lines was markedly enhanced by 12 hr pre-treatment of NK cells with IL-12 ($p=0.005$ vs. cetuximab). Similar levels of lysis were noted for both HPV-positive and HPV-negative cell lines. Other NK cell-activating factors such as IL-2, IL-15 and IL-21 also enhanced antibody-dependent cell-mediated cytotoxicity (ADCC). The stimulus of IL-12 and cetuximab-coated tumor cells induced synergistic production of nanogram levels of interferon-gamma ($\text{IFN-}\gamma$; >6-fold increase over controls) ($p<0.001$). A similar effect was seen for NK cell production of the chemokines MIP-1 α , RANTES and IL-8. Phosphorylation of ERK (critical for FcR functions) was enhanced in NK cells costimulated with cetuximab and IL-12 compared to controls. Cytokine stimulation of NK cells in the presence of cetuximab-coated SCCHN cells leads to enhanced NK cell-mediated ADCC and cytokine secretion independent of tumor cell HPV-status. Cytokine administration could be a useful adjuvant in the cetuximab treatment of HER1-positive head and neck cancer.

Keywords

Cetuximab; NK cells; Interleukin-2; Interleukin-12; Interleukin-15; Interleukin-21¹

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INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide, and the global incidence of this cancer has risen significantly over the past decade. More than 650,000 new cases of head and neck cancer will be diagnosed this year alone. The main risk factors for this disease include tobacco use and alcohol consumption. However, many patients with SCCHN have no identifiable risk factors other than a sub-clinical infection with human papilloma virus (HPV-16). SCCHN is twice as common in men as in women and about one-third of these cancers occur in patients younger than 55.¹ Combined modality therapy is generally recommended for the 60% of patients who present with locally advanced disease.² The standard treatment for patients with unresectable disease is concurrent cisplatin-based chemoradiation. This approach is also employed in patients with resectable disease who desire organ preservation and as adjuvant therapy in patients with high-risk pathological findings at surgical resection. Despite this multi-modality approach, the majority of these patients will develop local and/or regional recurrences and 20%–30% will go on to develop distant metastases.³ Thus, there is a real need for new, highly active therapeutic agents that can be combined with existing treatment modalities.

The epidermal growth factor receptor (EGFR or HER1) is a member of the HER family of tyrosine kinase growth factor receptors. The majority of SCCHN (>90%) over-express HER1, which correlates with a poor prognosis and overall resistance to therapy.⁴ Cetuximab is a humanized anti-HER1 monoclonal antibody (mAb) that binds to HER1 over-expressing tumor cells and has activity when administered as a single agent to patients with HER1-overexpressing SCCHN.⁵ The direct cytotoxic effect of cetuximab on HER1-positive cancer cells markedly enhances the activity of standard therapies for SCCHN. The addition of cetuximab to platinum-based chemotherapy in patients with untreated recurrent or metastatic SCCHN led to a significant improvement in overall survival (from 7.4 to 10.1 mos) and increased the response rate from 20% to 36% ($p < 0.001$).⁶ In addition, the overall survival of patients with locoregionally advanced SCCHN improved from 29.3 to 49.0 mos ($p = 0.03$) with the addition of cetuximab to radiation therapy.⁷

IL-12 is a potent pro-inflammatory cytokine produced by innate immune cells such as dendritic cells, macrophages and neutrophils that serves as a link between innate and adaptive immunity. Normally, IL-12 appears to be important for the resolution of bacterial infections via its ability to stimulate NK cell cytokine production and enhance T cell mediated immunity. The anti-tumor effects of IL-12 depend upon its anti-angiogenic effects and ability to augment interferon-gamma (IFN- γ) production and the lytic capacity of NK cells, CD8+ T cells, and NKT cells against tumor targets.⁸

Innate immune cells (e.g., NK cells, monocytes) bear specialized receptors for the constant or “Fc” region of immunoglobulin G that permits them to bind to antibody-coated target cells and destroy them via the induction of antibody-dependent cell-mediated cytotoxicity (ADCC) and the secretion of various cytokines. Our group has shown in vitro and in murine models that co-stimulation of these effector cells with IL-12 can significantly enhance the immune response to Ab-coated tumor cells.⁹ Co-administration of therapeutic mAbs plus IL-12 is associated with co-localization of the IL-12 receptor and the activating receptor for IgG (Fc γ RIIIa) to NK cell membrane lipid rafts with subsequent enhancement of downstream MAP kinase signal transduction, cytokine secretion, and ADCC.^{10,11} These findings led to two CTEP-sponsored phase I trials which demonstrated that IL-12 markedly enhances the activity of mAb therapy.^{12,13} Importantly, the anti-tumor responses observed in these studies correlated with the induction of a robust innate immune response characterized by NK cell activation and the secretion of anti-tumor cytokines (IFN- γ , TNF-

α), anti-angiogenic factors (IP-10, MIG), and chemokines (MIP-1 α , IL-8, RANTES). These NK cell-derived factors exerted a strong chemotactic effect on both naïve and activated T cells that led to the infiltration of tumors by CD8+ T cells.¹⁴ Furthermore, we have demonstrated that the anti-tumor actions of cetuximab against HER1+ lung tumors are greatly enhanced by IL-12.¹⁵

Based on these observations, we hypothesized that cetuximab therapy of SCCHN could be significantly enhanced by co-administration of an NK cell activating cytokine such as IL-12. We also hypothesized that enhanced tumor cell killing would occur irrespective of tumor cell HPV status. The development of an effective immunotherapeutic approach to SCCHN would be an important addition to the standard therapeutic modalities of surgery, chemotherapy and radiation therapy. The present study was undertaken in order to evaluate the efficacy of NK cell stimulatory cytokines (specifically IL-12) when employed in combination with cetuximab for the treatment of HER1-overexpressing head and neck tumors.

MATERIALS AND METHODS

Cell lines

The human head and neck carcinoma cell lines Cal-27, UM-SCC-2, UM-SCC-47 and UM-SCC-74B were provided by the laboratory of Dr. Theodore Teknos (The Ohio State University) and propagated in DMEM (Life Technologies: Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 ug/mL streptomycin, and non-essential amino acids.

Cytokines

Recombinant human (rhu) IL-2 was obtained from Hoffmann-La Roche Pharmaceuticals (Nutley, NJ). rhu-IL-12 was obtained from R&D Systems (Minneapolis, MN). rhu-IL-15 was obtained from PeproTech, Inc. (Rocky Hill, NJ). rhu-IL-21 was kindly provided by ZymoGenetics (Seattle, WA).

HER1 Analysis

The expression of HER1 in human squamous cell carcinoma of the head and neck (SCCHN) cell lines was validated via immunoblot analysis and flow cytometry as previously described.¹⁰

Polymerase chain reaction (PCR) for HPV-16 expression

Purified DNA from tumor cells was extracted using the DNeasy Blood and Tissue Kit (Qiagen: Valencia, CA) and PCR was performed using HPV-16 E7 primers (forward primer, TGTGTCCTGAAGAAAAGCAAAGAC; reverse primer, AGCTGTCATTTAATTGCTCATAACAGTA). Amplifications were carried out under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 30s, followed by a final extension of 72°C for 10 min. Reaction products were analyzed on 2% agarose gels containing 0.5 ug/ml ethidium bromide. Bands were visualized by photography using the Kodak EDAS 290 under UV illumination (Kodak: Rochester, NY).

Isolation of human NK cells and monocytes

NK cells were isolated directly from fresh peripheral blood leukopacks (American Red Cross: Columbus, OH) by 30-minute incubation with RosetteSep cocktail (Stem Cell Technologies: Vancouver, BC) followed by Ficoll Hypaque (Sigma-Aldrich: St. Louis, MO)

density gradient centrifugation. For monocyte isolation, PBMCs were isolated from fresh leukopacks using Ficoll-Hypaque density gradient centrifugation. CD14⁺ monocytes were subsequently isolated from PBMCs using magnetic beads (Miltenyi Biotech: Bergisch Gladbach, Germany).¹⁰

Antibody-dependent cellular cytotoxicity (ADCC) assays

Purified human NK cells from normal human donors were plated in 96-well V-bottom plates in 10% HAB medium supplemented with IL-2 (100 IU), IL-12 (10 ng/mL), IL-15 (10 ng/mL), or IL-21 (10 ng/mL), and incubated overnight at 37°C. The following morning cetuximab or control IgG-coated (100 ug/mL) ⁵¹Cr-labeled tumor cells were added to the NK cells at various effector:target (E:T) ratios. Following a 4 hr incubation period at 37°C, supernatants were harvested for quantification of chromium release. Mean percent cell lysis was determined as previously described.⁹

***In vitro* co-culture assays**

NK cell co-culture assays were performed as previously described.¹⁶ SCCHN cell lines were cultured overnight at 37°C in the wells of a 96-well flat-bottom culture plate. The culture supernatants were aspirated the following day and the wells were treated with 100 ug/mL of cetuximab or medium alone for 1 hr at 37°C. Purified human NK cells were subsequently added to the wells (2×10⁵ cells per well) in 200 uL of 10% HAB medium supplemented with IL-2 (100 IU), IL-12 (10 ng/mL), IL-15 (10 ng/mL), or IL-21 (10 ng/mL). Control conditions consisted of NK cells plus tumor cells treated with medium alone, cetuximab alone, or cytokine alone. Cell-free culture supernatants were harvested after 48-72 hrs and analyzed for IFN- γ , MIP-1 α , RANTES and IL-8 by ELISA (R&D Systems).⁹

T-cell chemotaxis

Normal T cells were activated for 2 days with 1 ug/mL phytohemagglutinin (PHA: Sigma-Aldrich) and for 3 days with 500 pmol/L huIL-2 in RPMI 1640 complete medium supplemented with 10% FBS.¹⁴ NK cell culture supernatants (400 uL) were placed in the lower chambers of a 24-well flat bottom plate. Medium $\pm$ 1 ug/mL MIG served as control. 5-um pore size Transwell inserts (Corning Inc: Corning, NY) were placed in the wells and migration experiments were conducted by placing 2×10⁵ purified activated T cells in 100 uL of 10% HAB medium in the upper chambers of the inserts. The plates were then incubated for 4 hours at 37°C with 5% CO₂, followed by a 10 min incubation at 4°C to loosen any cells adhering to the undersides of the insert membranes.¹⁴ 10X microscopic images of the fluid in the lower chambers were obtained and the number of migrated T cells per HPF was calculated for each condition.

Analysis of ERK activation

Purified human NK cells were isolated and allowed to rest overnight in 10% HAB medium. *In vitro* co-culture assays were subsequently performed as described above. Purified human NK cells were added to the wells (2×10⁵ cells per well) in 200 uL of 10% HAB medium supplemented with IL-12 (10 ng/mL). Control conditions consisted of NK cells plus tumor cells treated with medium alone, cetuximab alone, or cytokine alone. NK cell FcR stimulation at various time points was detected using an Alexa Fluor 488-conjugated mAb to phosphorylated human ERK-1/2 and an APC-conjugated mAb to the NK cell marker CD56 (BD Biosciences: Franklin Lakes, NJ).¹⁰

Murine tumor model

Age-matched female 01B74 athymic mice from NCI Frederick (Frederick, MD) were injected with 6×10⁶ Cal-27 cells in 200 l volume s.c. in the right flank. When tumors

became palpable (Day 6), the mice were randomly allocated to treatment groups. All treatments were administered i.p. thrice weekly.¹⁵ Tumors were measured and volumes were calculated 3x/week as follows: tumor volume = $0.5 \times ((\text{large diameter}) \times (\text{small diameter})^2)$. Treatments continued until tumors were greater than 2 cm in maximum dimension or until ulceration, at which time mice were sacrificed. All protocols are approved and monitored by the Ohio State University Animal Care and Use Committee.

Statistics

Significance was determined by the Student's t test ($p < 0.05$ significant).

RESULTS

Human head and neck cancer cell lines express HER1

Immunoblot analysis was conducted to determine the level of HER1 expression in head and neck cancer cell lines (Fig. 1A). There was strong expression of HER1 in all cell lines, regardless of HPV status. Lower levels of HER1 were consistently observed in the UM-SCC-74B cell line. These results were confirmed by flow cytometry (Fig. 1B).

Evaluation of HPV status in human head and neck cancer cell lines

PCR for HPV-16 E7 was used to confirm the HPV status of the SCCHN cell lines used in this study. Expression of HPV-16 E7 transcript was observed in the UM-SCC-2 and UM-SCC-47 cell lines, but not in the UM-SCC-74B or Cal-27 cell lines (Fig. 2).

IL-12 enhances NK cell lytic activity against cetuximab-coated cells

Previous work in our laboratory has shown *in vitro* and in murine tumor models that IL-12 (an NK cell activating cytokine) leads to the activation of FcR-bearing immune cells and synergistically enhances the anti-tumor activity of mAb therapy.^{15,17} We hypothesized that the lytic activity of normal human NK cells against cetuximab-coated SCCHN cell lines would be enhanced *in vitro* following the pre-treatment of NK cells for 12 hours with IL-12. ADCC assays were performed using normal donor NK cells and various HER1+ head and neck cancer tumor targets. Human NK cells were cultured overnight in medium $\pm$ IL-12 (10 mg/mL). Target cells were labeled with ⁵¹Cr and treated with either immunoglobulin G (IgG) (control) or cetuximab before use in a standard 4 hr ADCC assay. There was statistically significant enhancement of NK cell-mediated ADCC using IL-12 in combination with cetuximab as compared to cytokine-activated NK cells alone ($p < 0.02$) (Fig. 3A and B). In the UM-SCC-47 cell line, combination therapy also reached clinical significance against Ab therapy alone ($p < 0.02$). For example, low levels of cell lysis was observed in the HPV-positive cell line UM-SCC-47 by unstimulated NK cells (20.39% at 50:1 E:T ratio). However, pre-treatment of NK cells with IL-12 significantly enhanced the level of cell lysis (57.38%). Treatment of the target cell with cetuximab led to 50.8% lysis by untreated NK cells and 86% lysis by IL-12-treated NK cells. Similar results were obtained with the Cal-27 and SCC-74B cell lines, which are HPV-negative.

Other immune stimulatory cytokines also enhance NK cell lytic activity irrespective of tumor HPV status

To determine the ability of other cytokines to stimulate NK cell FcR-mediated effector functions against Ab-coated tumor cells, human NK cells were cultured overnight in medium or medium supplemented with IL-2 (100 IU), IL-15 (10 ng/mL), or IL-21 (10 ng/mL). NK cell mediated lysis of head and neck tumor cells was significantly enhanced by the combination of cetuximab therapy plus IL-2, IL-15, or IL-21 at each effector:target ratio

examined. Enhancement of tumor cell lysis was observed for both HPV-negative (Fig. 4A, $p < 0.03$ for combination therapy vs. controls) and HPV-positive (Fig. 4B) cell lines.

IL-12 enhances production of IFN- γ by NK cells co-cultured with cetuximab-coated head and neck tumor cells

Although the cytolytic potential of NK cells is widely appreciated, the capability of NK cells to initiate and shape adaptive immune responses by providing an early source of IFN- γ has become evident.¹⁶ We have previously demonstrated that co-stimulation of NK cells via the IL-12R and Fc γ RIIIa leads to synergistic production of IFN- γ .¹⁰ We therefore hypothesized that IL-12 would enhance NK cell production of IFN- γ following exposure to cetuximab-coated head and neck tumor cells. An *in vitro* assay was performed in which purified NK cells were co-cultured with cetuximab-coated SCCHN cells. Cal-27 (HPV-negative) and UM-SCC-47 (HPV-positive) cells were coated with cetuximab and incubated with 1×10^5 human NK cells in the presence of IL-12 (1 ng/ml) for 72 hours. Control conditions consisted of IL-12 alone, cetuximab alone, and media alone. After 72 hrs, supernatants were harvested and assayed for IFN- γ content. Cetuximab in combination with cytokine therapy resulted in a statistically significant increase in IFN- γ production in both HPV-negative (Fig. 5A) and HPV-positive (Fig. 5B) cell lines as compared to controls ($p < 0.001$). These results provide evidence that co-administration of cytokines can significantly enhance NK cell IFN- γ production in response to cetuximab-coated head and neck tumor cells.

Co-stimulated NK cells secrete high levels of T cell attracting chemokines

HER1-overexpressing HPV-negative (Cal-27) and HPV-positive (UM-SCC-47) cell lines were grown to confluence in flat-bottom wells and coated with cetuximab. Each well was then supplemented with purified human NK cells in media containing IL-2, IL-12, IL-15, or IL-21. Cetuximab-coated HER1+ SCCHN tumor cells were a potent costimulus for NK cell production of the chemokines MIP-1 α , RANTES, and IL-8 in the presence of IL-2, IL-12, IL-15, and IL-21 (Fig 5C and D). The control conditions resulted in lesser NK cell production of these chemokines.

NK cell supernatants from co-stimulated NK cells induce chemotaxis of activated T cells

We have shown that NK cells costimulated with antibody-coated head and neck tumor cells and IL-2, IL-12, IL-15, or IL-21 produce large quantities of several T cell-attracting chemokines. We next sought to determine whether these NK cell-derived factors were capable of inducing T cell migration. Peripheral blood T cells were isolated from a normal donor and activated with PHA and IL-2. Activated T cells were then placed in the upper chamber of Transwell inserts (see Materials and Methods). The lower chambers contained cell culture supernatants from the various conditions of the costimulation assays that were described in Fig. 5C & 5D. Medium supplemented with MIG, a chemotactic factor known to induce T-cell migration, served as a positive control for T-cell chemotaxis.¹⁴ Culture supernatants from NK cells costimulated with cetuximab-coated tumor plus cytokine induced significantly higher levels of chemotaxis (determined by a greater # of T cells per HPF) as compared with supernatants derived from single stimulation conditions (Fig. 6).

Co-stimulation of NK cells with Ab-coated SCCHN tumor cells plus IL-12 results in enhanced p-ERK activation

Previously, our group has shown that dual stimulation of NK cells via Fc γ RIIIa and IL-12R initiates a signaling cascade that results in the activation of ERK with resultant potent IFN- γ secretion from costimulated NK cells.¹⁰ We hypothesized that the interaction of human NK cell FcR with cetuximab-coated head and neck tumor cells would maximally activate the MAP kinase pathway and result in enhanced phosphorylation of ERK within NK cells.

Human NK cells were co-cultured with cetuximab-coated head and neck tumor cells in the presence or absence of IL-12. After a 2 hr incubation period, culture supernatants were collected and cells were dual stained for CD56 and phospho-ERK. As predicted, NK cells costimulated with antibody-coated tumor cells and IL-12 demonstrated the highest level of ERK activation (54.8%) (Fig. 7). Media control (6.4%), IL-12 stimulation alone (23.9%), and Ab therapy alone (27.9%) resulted in relatively low levels of ERK activation.

Therapy with cetuximab and IL-12 in a murine tumor model

Tumors were generated in athymic mice by subcutaneous injection of Cal-27 (HPV-negative) tumor cells. Mice were subsequently treated intraperitoneally (i.p.) with PBS, IL-12 (50 ng), cetuximab (0.5 mg/kg), or the combination. There was no significant difference in tumor size at baseline prior to treatment initiation. At day 25 of treatment, there was a statistically significant difference between mice receiving IL-12 ($p=0.0497$), cetuximab ($p=0.0157$), or the combination treatment ($p=0.0083$) when compared to treatment with PBS alone (Fig. 8). Although the lowest tumor burden after 25 days of therapy was in the combination treatment group, the difference between this treatment and either IL-12 or cetuximab therapy alone was not found to be statistically significant.

DISCUSSION

There is no clear mechanism of action for most clinically employed mAbs although it is possible that immune effector cells which bear receptors for the Fc (or “constant”) region of immunoglobulin are involved. Clynes *et al.* reported that the anti-tumor effects of trastuzumab (anti-HER2/neu mAb) in a murine model of breast cancer required the expression of functional FcR by host immune effectors.¹⁸ Fan *et al.* compared the abilities of cetuximab and the F(ab)₂ fragment of cetuximab to inhibit the growth of HER1 positive xenografts in vivo.¹⁹ Although both Abs had similar effects on HER1-mediated signaling, the anti-tumor activity of the unmodified cetuximab Ab was stronger than that of the F(ab)₂ fragment. Ciardiello *et al.* generated a cetuximab-resistant colon carcinoma cell line. Although this cell line was insensitive to the anti-proliferative effects of cetuximab in vitro, a modest inhibition of tumor growth was observed when cetuximab was administered to mice bearing xenografts of the cetuximab-resistant cell line.²⁰ Recently, Ferris *et al.* showed that lysis of SCCHN cells was influenced by EGFR expression level, cetuximab concentration, and FcγR polymorphism.²¹ Interestingly, the extent of cell lysis was enhanced in the presence of immune stimulatory cytokines. Taken together, these results suggest that both FcR-dependent and FcR-independent mechanisms contribute to the anti-tumor effects of Ab therapy and imply that co-administration of immune stimulatory cytokines might enhance these effects.

Despite the efficacy of cetuximab in combination with standard therapy in the treatment of SCCHN, clinical responses are seen in only a subset (10–20%) of treated patients. Though the molecular basis of clinical resistance in these cancers is not fully understood, Ras or other downstream cell-signaling pathway mutations may play a pivotal role in conferring resistance to Ab therapy. Given the observed interaction between Abs and FcR-bearing immune cells, we propose that combination therapy with immune stimulatory cytokines and mAbs may provide a potential mechanism for overcoming antibody resistance in HER1-positive tumors. In other words, NK cells that have been activated by IL-12 seem to be able to respond to and kill Ab-coated SCCHN cells irrespective of their sensitivity to cetuximab.

The current study evaluated the efficacy of NK cell stimulatory cytokines when employed in combination with cetuximab for the treatment of HER1-overexpressing head and neck tumors. FcR stimulation of NK cells with Ab-coated targets, including immobilized IgG and mAb-coated human head and neck cancer cells modestly enhanced NK cell ADCC,

production of IFN— γ and various chemokines. Costimulation of NK cells with various immune stimulatory cytokines markedly enhanced these responses. Culture supernatants from costimulated NK cells were also able to direct the migration of activated T cells. Co-stimulation of NK cells activated the MAP kinase pathway and resulted in enhanced phosphorylation of ERK within NK cells. Our results indicate that an immunotherapeutic approach to SCCHN may be beneficial and should be investigated further as an important adjunct to the standard therapeutic modalities of surgery, chemotherapy and radiation therapy.

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Figure 1.

Expression of HER1 in SCCHN cell lines. (A) Lysates were prepared from human head and neck cancer cell lines and subjected to immunoblot analysis with antibodies directed against HER1. β -actin was the loading control. HeLa and SKBR3 served as positive and negative

controls. (B) Representative histograms for human head and neck cancer cell lines stained with anti-HER1-PE mAb and analyzed by flow cytometry.

Figure 2.

Expression of HPV-16 E7 in SCCHN cell lines. Purified DNA from the indicated cell lines was analyzed for the expression of HPV-16 E7 (224 bp) by PCR using HPV-16 E7A (forward) and HPV-16 E7B (reverse) primers. HeLa and Raji tumor cell lines served as positive and negative controls, respectively.

A

B **Figure 3.**

IL-12 enhances ADCC of NK cells against SCCHN cell lines. Purified NK cells were incubated overnight with medium $\pm$ IL-12 (10 ng/mL). The lytic activity of NK cells against cetuximab-coated HPV-positive (A) and HPV-negative (B) head and neck cancer cell lines was assessed via a standard 4 hr Cr-51 release assay. Each graph depicts results from one representative donor. At least 3 normal donors were tested per cell line. $p < 0.02$ for combination treatment vs. cytokine activated NK cells (*).

A **B** **Figure 4.**

ADCC of NK cells against SCCHN cell lines is enhanced by cytokines. Purified NK cells were incubated overnight with medium $\pm$ IL-2 (100 IU), IL-15 (10 ng/mL), or IL-21 (10 ng/mL). The lytic activity of NK cells against cetuximab-coated HPV-negative (A) and HPV-positive (B) cell lines was assessed via a 4-hr Cr-51 release assay. Each graph depicts results

from one donor. At least 3 normal donors were tested per cell line. $p < 0.03$ for combination treatment vs. control conditions (*).

B**UM-SCC-47 (HPV-positive)**

C

Figure 5. Human NK cell cytokine secretion. Cetuximab-coated SCCHN cells were cultured with purified human NK cells in media supplemented with various NK cell-activating cytokines in an *in vitro* co-culture assay. Control conditions consisted of PBS, cytokine alone, or cetuximab-coated tumor cells alone. Culture supernatants were harvested at 48 hrs and analyzed by ELISA for IFN- γ , MIP-1 α , RANTES, and IL-8 production in both HPV-negative (A and C) and HPV-positive (B and D) cell lines. Each graph depicts results from one representative donor. A minimum of 3 normal donors was tested per cell line. * = ($p < 0.001$).

Figure 6.

Supernatants from NK cells co-stimulated with immobilized antibody plus cytokine induce chemotaxis of activated T cells. Activated T cells were assayed for chemotaxis in response to culture supernatants derived from NK cells costimulated with cetuximab-coated tumor cells plus IL-2, IL-12, IL-15, or IL-21. Supernatants from control-stimulated NK cells were used as a comparison.

Figure 7.

ERK activation. Purified human NK cells were co cultured with cetuximab coated head and neck tumor cells in the presence of IL-12 for 2 hrs at 37°C. Control conditions consisted of media alone, IL-12 alone, and cetuximab-coated tumor cells alone. Following incubation, cells were collected and underwent dual flow staining using anti-CD56 and anti-phospho-ERK1/2 Abs. The percentage of cells positive for p-ERK is depicted in the figure by the addition of cells in the upper right and lower right quadrants. The flow data depicts results from one representative donor, however, the percentages represent the average from n=3 donors.

Figure 8.

The combination of IL-12 and cetuximab results in reduced tumor burden in a mouse xenograft model. The effects of thrice weekly i.p. treatments of PBS, IL-12 (50 ng), cetuximab (0.5 mg/kg) or the combination on tumor volume were assessed in athymic mice bearing Cal-27 HER1+ tumor xenografts. n=3 mice/group, bars=SE.