

Anti-EGFR Targeted Monoclonal Antibody Isotype Influences Antitumor Cellular Immunity in Head and Neck Cancer Patients

Sumita Trivedi¹, Raghvendra M. Srivastava¹, Fernando Concha-Benavente², Soldano Ferrone³, Tatiana M. Garcia-Bates⁴, Jing Li⁵, and Robert L. Ferris^{1,6,7}

Abstract

Purpose: EGF receptor (EGFR) is highly overexpressed on several cancers and two targeted anti-EGFR antibodies which differ by isotype are FDA-approved for clinical use. Cetuximab (IgG1 isotype) inhibits downstream signaling of EGFR and activates antitumor, cellular immune mechanisms. As panitumumab (IgG2 isotype) may inhibit downstream EGFR signaling similar to cetuximab, it might also induce adaptive immunity.

Experimental Design: We measured *in vitro* activation of cellular components of the innate and adaptive immune systems. We also studied the *in vivo* activation of components of the adaptive immune system in patient specimens from two recent clinical trials using cetuximab or panitumumab.

Results: Both monoclonal antibodies (mAb) primarily activate natural killer (NK) cells, although cetuximab is significantly more potent than panitumumab. Cetuximab-activated neutrophils mediate antibody-dependent cellular cytotoxicity (ADCC)

against head and neck squamous cell carcinomas (HNSCC) tumor cells, and interestingly, this effect was FcγRIIIa- and FcγRIIIa genotype-dependent. Panitumumab may activate monocytes through CD32 (FcγRIIa); however, monocytes activated by either mAb are not able to mediate ADCC. Cetuximab enhanced dendritic cell (DC) maturation to a greater extent than panitumumab, which was associated with improved tumor antigen cross-presentation by cetuximab compared with panitumumab. This correlated with increased EGFR-specific cytotoxic CD8⁺ T cells in patients treated with cetuximab compared with those treated with panitumumab.

Conclusions: Although panitumumab effectively inhibits EGFR signaling to a similar extent as cetuximab, it is less effective at triggering antitumor, cellular immune mechanisms which may be crucial for effective therapy of HNSCC. *Clin Cancer Res*; 22(21): 5229–37. ©2016 AACR.

Introduction

The EGF receptor (EGFR) remains an important therapeutic target in many solid tumors, including head and neck squamous cell carcinomas (HNSCC; refs. 1–4). In 2006, the anti-EGFR, murine–human chimeric, IgG1 monoclonal antibody (mAb), cetuximab, was approved as combination or single-agent therapy for HNSCC. The same year, the anti-EGFR targeted, fully human, IgG2 mAb, panitumumab, was approved for use in EGFR-expressing metastatic colorectal carcinoma. As HNSCC has one of the highest prevalence of EGFR overexpression of all solid tumors

(80%–90%; ref. 5), panitumumab has been investigated as a second therapeutic anti-EGFR mAb. Recent studies looking at the addition of panitumumab to standard chemoradiation therapy for HNSCC indicate no improvement to locoregional control or overall survival, (6, 7) whereas others suggest it may modestly improve progression-free survival (8). However, few studies have investigated the differential biology underlying clinical outcomes of patients with HNSCC treated with either cetuximab or panitumumab.

We previously demonstrated that both monoclonal antibodies (mAb) bind to EGFR and inhibit its subsequent phosphorylation and signaling (9). Aside from inhibiting downstream signaling of EGFR, cetuximab is known to enhance antitumor immunity (10–12). Although the biologic features of IgG1 and IgG2 antibodies are known to differ, little comparative clinical data from treated cancer patients exist, and previous reports indicate that both cetuximab- and panitumumab-treated peripheral blood mononuclear cells (PBMC) were capable of mediating antibody-dependent cell cytotoxicity (ADCC; refs. 9, 13). On the basis of these findings, we sought to identify and compare which cell type(s) are activated by cetuximab or panitumumab and to compare the extent to which they mediate cellular immunity. To determine whether these effects resulted in enhanced adaptive immune responses, we analyzed lymphocytes from patients on two recent clinical trials, of cetuximab or panitumumab. We measured activation of natural killer (NK), neutrophils, dendritic cells (DC) and EGFR-specific cytotoxic CD8⁺ T lymphocytes (CTL). Taken together, our results suggest that although panitumumab effectively inhibits EGFR signaling to a similar extent as

¹Department of Otolaryngology, University of Pittsburgh, Pittsburgh, Pennsylvania. ²Department of Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania. ³Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. ⁴Department of Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania. ⁵Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Beijing, China. ⁶University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania. ⁷Cancer Immunology Program, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Robert L. Ferris, Hillman Cancer Center Research Pavilion, 5117 Centre Avenue, Room 2.26b, Pittsburgh, PA 15213-1863. Phone: 412-623-0327; Fax: 412-623-4840; E-mail: ferril@upmc.edu

doi: 10.1158/1078-0432.CCR-15-2971

©2016 American Association for Cancer Research.

Translational Relevance

EGF receptor (EGFR) overexpression in head and neck cancers provides the basis to develop targeted monoclonal antibody-based therapy. Two antibodies, differing by isotype, cetuximab and panitumumab, have been approved for the clinic. Aside from inhibiting downstream EGFR signaling, cetuximab (IgG1) has been shown to enhance antitumor immunity. Panitumumab (IgG2) is known to bind EGFR and inhibit its phosphorylation to a similar extent as cetuximab. However, its effect on antitumor immunity has not been confirmed. Here, we identify and compare the cell types that are activated by cetuximab or panitumumab and the extent to which they mediate cellular immunity. We measured *in vitro* activation of cellular components of the innate and adaptive immune systems and *in vivo* activation from patients on two recent clinical trials using cetuximab or panitumumab. We conclude that panitumumab is less effective at mediating antitumor cellular immunity. This finding may explain its lower clinical activity and guide therapeutic decisions.

cetuximab, it is much less effective at mediating antitumor, cellular immune mechanisms; this finding may explain its lower clinical activity in HNSCC.

Materials and Methods

Patient blood samples and lymphocyte isolation from PBMC

Following approval by the University of Pittsburgh (Pittsburgh, PA) Institutional Review Board (IRB #99-06), written informed consent was obtained for all patients. Peripheral venous blood samples were obtained from healthy donors from the Western Pennsylvania Blood Bank and patients with HNSCC on 2 separate clinical trials. Patients with HNSCC with stage III/IVA disease receiving chemoradiotherapy combined with cetuximab (400 mg/m² followed by 250 mg/m² weekly) on a prospective phase II clinical trial (UPCI # 08-013, NCT01218048) and patients with HNSCC with stage III/IV disease treated with chemoradiotherapy in combination with panitumumab (2.5 mg/kg; UPCI 06-120, NCT00798655; Supplementary Fig. S1). Cetuximab-treated patients also received 3 to 4 doses of preoperative treatment. Peripheral venous blood samples were drawn immediately before and again after chemoradiotherapy in combination with either cetuximab or panitumumab. Lymphocytes were purified by Ficoll-Paque PLUS centrifugation (Amersham Biosciences) and either used in experiments on the same day or stored frozen at -80°C until further use. CD56⁺ and CD14⁺ cells were purified from lymphocytes using immunomagnetic positive selection, EasySep kits (Stem cell technologies). Neutrophils were isolated by immunomagnetic negative selection from whole blood using EasySep kits (Stem cell technologies). The percentage purity for CD14⁺ cells was approximately 93%, the percentage purity for CD56⁺ cells was approximately 92%, and the percentage purity for neutrophils was approximately 94% as measured by flow cytometry (Supplementary Fig. S2).

Dendritic cell induction and maturation

Fresh PBMCs were obtained from the Western Pennsylvania Blood Bank and lymphocytes were purified by Ficoll-Paque PLUS

centrifugation. Monocytes were isolated from PBMCs by plastic adherence for 2 hours at 37°C. Plastic adherent cells were incubated at 37°C using Iscove modified Dulbecco medium (IMDM), supplemented with 10% FCS, granulocyte macrophage colony-stimulating factor (GM-CSF; 1,000 IU/mL), and IL4 (1,000 IU/mL). On day 3 of the culture, GM-CSF and IL-4 were replenished to a final concentration of 2,000 and 1,000 IU/mL, respectively. Day 8 monocyte-derived, mature (CD11c⁺) DCs were harvested with trypsin EDTA.

Reagents and antibodies

Recombinant EGF, Zombie Aqua Fixable Viability Kit, Alexa Fluor 488-EGFR, PerCP-Cy 5.5-CD3, PerCP-Cy 5.5-CD32, and PE-Cy5-CD86 flow antibodies were purchased from Biolegend. Cetuximab and panitumumab were purchased from the manufacturers, Bristol-Myers Squibb, and Amgen, respectively. FITC-CD69 and PE-Texas Red-CD8 flow antibody were purchased from Life Technologies. PE-CD107a, PE-Cy5-CD137, PE-Cy7-CD16, APC-CD56, PE-CD32, APC-H7-HLA-A2, and FITC-CD80 flow antibodies were purchased from BD Biosciences. 12B6 antibody was produced by Dr. Ferrone (Harvard Medical School, Boston, MA) and has been previously validated (14). Phycoerythrin (PE)-labeled HLA-A*0201-EGFR853-861 tetramer was provided by the NIH tetramer core facility and used for identification of EGFR-specific CTL (15). PE-labeled HLA-A*02:01 HIV-tetramer was purchased from MBL International.

Flow cytometry

Flow cytometry for EGFR-specific CTL was performed as follows. The PE-labeled HLA-A*0201-EGFR853-861 tetramer was obtained from the Tetramer Facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Specificity was confirmed by the lack of staining of HLA-A2⁻ PBMC obtained from normal donors and a nonspecific PE-HLA-A*02:01HIV-Tetramer. PBMC from patients on both trials were typed for HLA-A2⁺ status. Seven of 16 patients (43%) on the panitumumab trial and 15 of the 32 patients (46%) on the cetuximab trial were found to be HLA-A2⁺. HLA-A2⁺ PBMCs were harvested and washed once in PBS and then resuspended in 100 μL of PBS. Fluorophore-conjugated tetramer was added at a 1:300 dilution incubated for 30 minutes at 25°C and washed twice by sequential centrifugation at 1,400 rpm with FACS buffer. Then, flow cytometry for cell surface proteins was performed as follows. Cells were harvested and washed once in PBS and then resuspended in 100 μL of FACS buffer. Fluorophore-conjugated antibodies were added at 1:100 dilution, incubated for 30 minutes at 4°C, washed twice by sequential centrifugation at 1,400 rpm with FACS buffer and resuspended in 2% paraformaldehyde (PFA) solution until analyzed in the flow cytometer. Isotype control antibody staining was added for each condition and each mAb. Each experiment was repeated at least 3 times and mean and SEM were calculated and plotted using GraphPad PRISM software version 6. Statistical analysis of the data included ANOVA (two-tailed) with Tukey test when more than 2 different group means were compared or the Student *t* test when 2 means were compared.

Tumor cell lines

JHU-029 (HLA-A2⁻, EGFR^{hi}, and MAGE-3⁺) was a kind gift of Dr. James Rocco (The Ohio State University, Columbus, OH). PCI-15B (HLA-A2⁻, EGFR^{hi}, and MAGE-3⁻) cells were isolated and cultured from a patient at the University of Pittsburgh

through the explant/culture method, authenticated, and validated as unique using STR profiling and HLA genotyping every 6 months (16, 17). EGFR expression is higher in JHU-029 cells than in PCI 15B cells (Supplementary Fig. S3). All cell lines were routinely tested and found to be free of mycoplasma. Cells were grown/passaged in 10% FBS-DMEM (Mediatech), supplemented with 2% L-glutamine and 1% penicillin/streptomycin (Invitrogen Corp.) at 37°C in a 5% CO₂ atmosphere at 95% humidity.

Cytotoxicity assays

⁵¹Cr release assay was used to determine cytotoxicity. Target cells were incubated in 100 μL of media with 25 μCi of Na²⁵¹CrO₄ (Perkin Elmer) for 60 minutes at 37°C and resuspended in RPMI-1640 medium supplemented with 25 mmol/L HEPES. Cells were thoroughly washed in PBS and plated at various effector:target (E:T) ratios in 96-well plates. Cetuximab, panitumumab, human IgG1 control, or human IgG2 control (10 ng/mL) was incubated with effector cells added at the specified E:T ratios. Plates were incubated at 37°C for 4 hours in a 5% CO₂ atmosphere. Controls for spontaneous (cells only) and maximal lysis

(cells treated with 1% Triton-X) and specific mAb control (human IgG1 or IgG2 isotype) were included. Each reaction was done in triplicate and repeated 3 times. The supernatants were collected and analyzed with a Perkin Elmer 96-well plate gamma counter. Results were normalized with the formula $\text{lysis} = (\text{experimental lysis} - \text{spontaneous lysis}) / (\text{maximal lysis} - \text{spontaneous lysis}) \times 100$ and results were plotted on a graph.

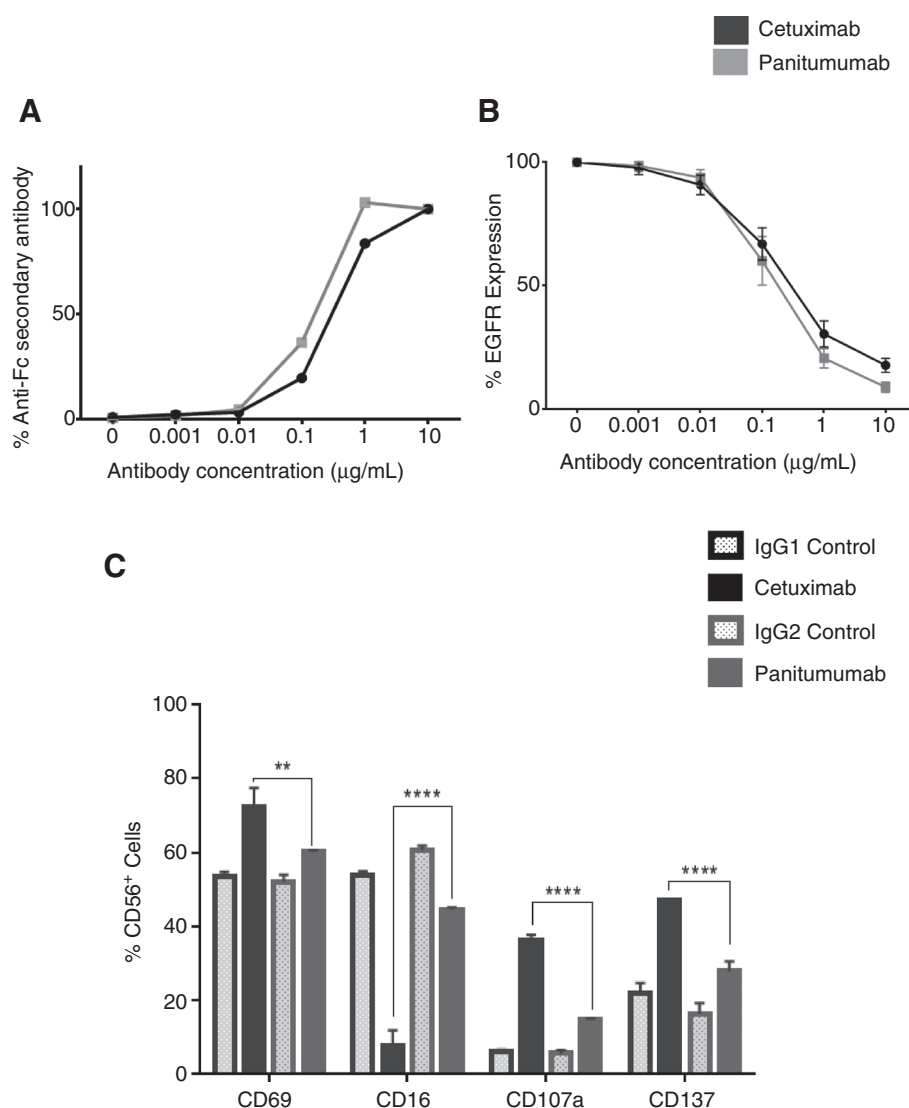
Results

Cetuximab and panitumumab bind to EGFR similarly

Previous studies indicated that panitumumab, a fully human mAb, may bind to the EGFR with greater affinity than the mouse-human chimeric mAb cetuximab (dissociation constant, $K_d = 0.12$ vs. 0.31 nmol/L, respectively; ref. 18). To directly compare the binding of cetuximab and panitumumab to EGFR on HNSCC cells, we incubated EGFR high JHU-029 HNSCC cells with either mAb, at increasing concentrations (0.001–10 μg/mL) for 30 minutes at 4°C and then stained these cells with either fluorescein isothiocyanate (FITC)-labeled Fc-specific mAb (Fig. 1A) or commercial anti-EGFR antibody (Fig. 1B) and then analyzed these

Figure 1.

Cetuximab and panitumumab bind EGF receptor similarly, but cetuximab activates PBMCs to a greater extent than panitumumab. **A** and **B**, binding of cetuximab (IgG1)- and panitumumab (IgG2)-specific monoclonal antibodies (mAbs) to JHU-029 HNSCC cells. Cells were treated with increasing concentrations of either cetuximab or panitumumab (0.001–10 μg/mL) for 30 minutes at 4°C and then stained with either FITC-labeled Fc-specific Ab or EGFR Ab and analyzed by flow cytometry. Graphs show the percentage of FITC-positive cells obtained at each concentration of mAb. **C**, whole PBMCs from healthy donors were cocultured with JHU-029 cells and treated with 10 μg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) for 24 hours and then surface expression of activation markers, CD69, CD16, CD107a, and CD137, was measured by flow cytometry. PBMCs treated with cetuximab express significantly higher activation markers compared with cells treated with either control antibodies or panitumumab. Data are mean ± SEM. **, $P < 0.01$; ****, $P < 0.0001$ cetuximab compared with panitumumab.



cells by flow cytometry. The results shown in Fig. 1A demonstrate that binding of both mAbs to EGFR are similar at all concentrations of mAb tested ($P > 0.05$). Binding of both panitumumab and cetuximab to EGFR resulted in a similar extent of blocking of the active EGFR-binding site at all the mAb concentrations tested (Fig. 1B).

Whole PBMCs are activated to a greater extent by cetuximab than by panitumumab

Aside from inhibiting EGFR signaling, anti-EGFR mAbs play an important role in activating cellular components of the immune system, in particular CD16/FcγRIIIa-bearing NK cells. We investigated whether there were differences in activation of CD3⁻CD56⁺ NK cells incubated with cetuximab or panitumumab. After isolating PBMCs from healthy donors, NK cells were purified by positive selection from PBMCs and cocultured with JHU-029 HNSCC cells plus cetuximab, IgG1 isotype control Ab, panitumumab, or IgG2 isotype control Ab (10 μg/mL) for 24 hours at 37°C. We measured NK cell activation markers, CD69, CD16, CD107a, and CD137 on CD56⁺CD3⁻ NK cells by flow cytometry. The activation markers CD69, CD107a, and CD137 were upregulated by both mAbs (Fig. 1C). CD16 activation, as indicated by CD16 internalization and downregulation of surface expression, was also enhanced by both mAb over isotype controls. Interestingly, cetuximab significantly activated all NK cell surface markers and downregulated CD16 to a greater extent than panitumumab ($P < 0.01$).

ADCC mediated by whole PBMCs and NK cells is significantly greater with cetuximab than panitumumab

Previous studies demonstrate that cetuximab activation of NK cells, and subsequent ADCC is an important feature of antitumor therapy (10, 19). We sought to establish whether panitumumab-activated NK cells could function in ADCC to the same extent as cetuximab-activated NK cells. Whole PBMCs isolated from healthy donors were cocultured for 4 hours with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with cetuximab, panitumumab, or isotype (IgG1 or IgG2) control mAbs (10 μg/mL) at different E:T ratios (2.5:1, 5:1, and 10:1). Panitumumab-activated PBMCs did not appear to induce ADCC above isotype control. Conversely, cetuximab significantly enhanced ADCC compared with panitumumab at E:T ratio of 10:1 ($P < 0.05$; Fig. 2A). As NK cells are thought to be the primary mediators of cetuximab-mediated ADCC, we repeated similar ⁵¹Cr-release assays using positively isolated CD56⁺ cells (Fig. 2B). Again, panitumumab-activated NK cells did not appear to induce ADCC above isotype control. Indeed, cetuximab-activated NK cells significantly induced ADCC to a greater extent than panitumumab at all E:T ratios tested.

Cetuximab-activated neutrophil-mediated ADCC is enhanced in donors who are homozygous for FcγIIIa VV genotype and FcγIIa HH genotype

Although panitumumab did not appear to activate NK cell-mediated ADCC, it has been suggested that it may be effective in mediating ADCC by myeloid or granulocytic effector cells (13). To determine whether mAb-activated neutrophils could mediate ADCC against HNSCC cells, we cocultured negatively selected neutrophils with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with cetuximab, panitumumab (10 μg/mL), or isotype (IgG1 or IgG2) control mAb for 4 hours at different E:T ratios (10:1, 20:1, and

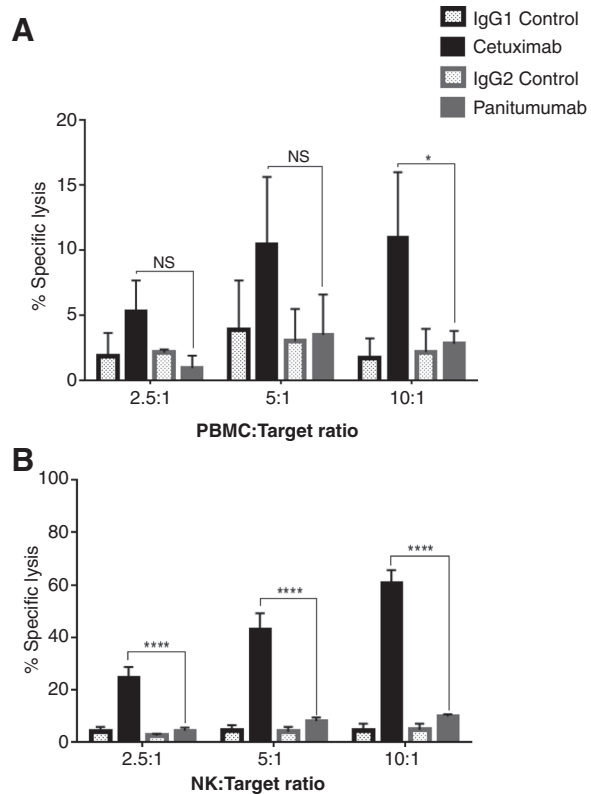


Figure 2.

Greater triggering by cetuximab than by panitumumab of PBMCs or NK cell-dependent ADCC against HNSCC cells. **A**, whole PBMCs cocultured for 4 hours with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with 10 μg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) at different E:T ratios (2.5:1, 5:1, and 10:1). Cetuximab significantly enhanced ADCC compared with panitumumab at an E:T ratio of 10:1. When this experiment was repeated using isolated NK cells (**B**), cetuximab significantly enhanced ADCC in comparison with panitumumab at all E:T ratios. Data are mean + SEM. *, $P < 0.05$; ****, $P < 0.0001$ cetuximab compared with panitumumab.

80:1). Cetuximab-activated neutrophils significantly induced ADCC over isotype controls at all E:T ratios ($P = 0.0004$; Fig. 3A). Panitumumab-activated neutrophils did not mediate ADCC over isotype control. This effect was dependent on CD16 but not on CD32, as demonstrated by significant reduction of cetuximab-activated neutrophil mediated ADCC only when a CD16-blocking mAb was used (Fig. 3B). By genotyping the neutrophils used in the ADCC assays at the FcγRIIIa and FcγRIIIa loci, we found that neutrophils from donors homozygous for FcγRIIIa VV demonstrated significantly enhanced ADCC activity than VF and FF donors (Fig. 3C). A similar trend was noted in patients homozygous for FcγRIIa HH genotype compared with HR and RR donors (Fig. 3D).

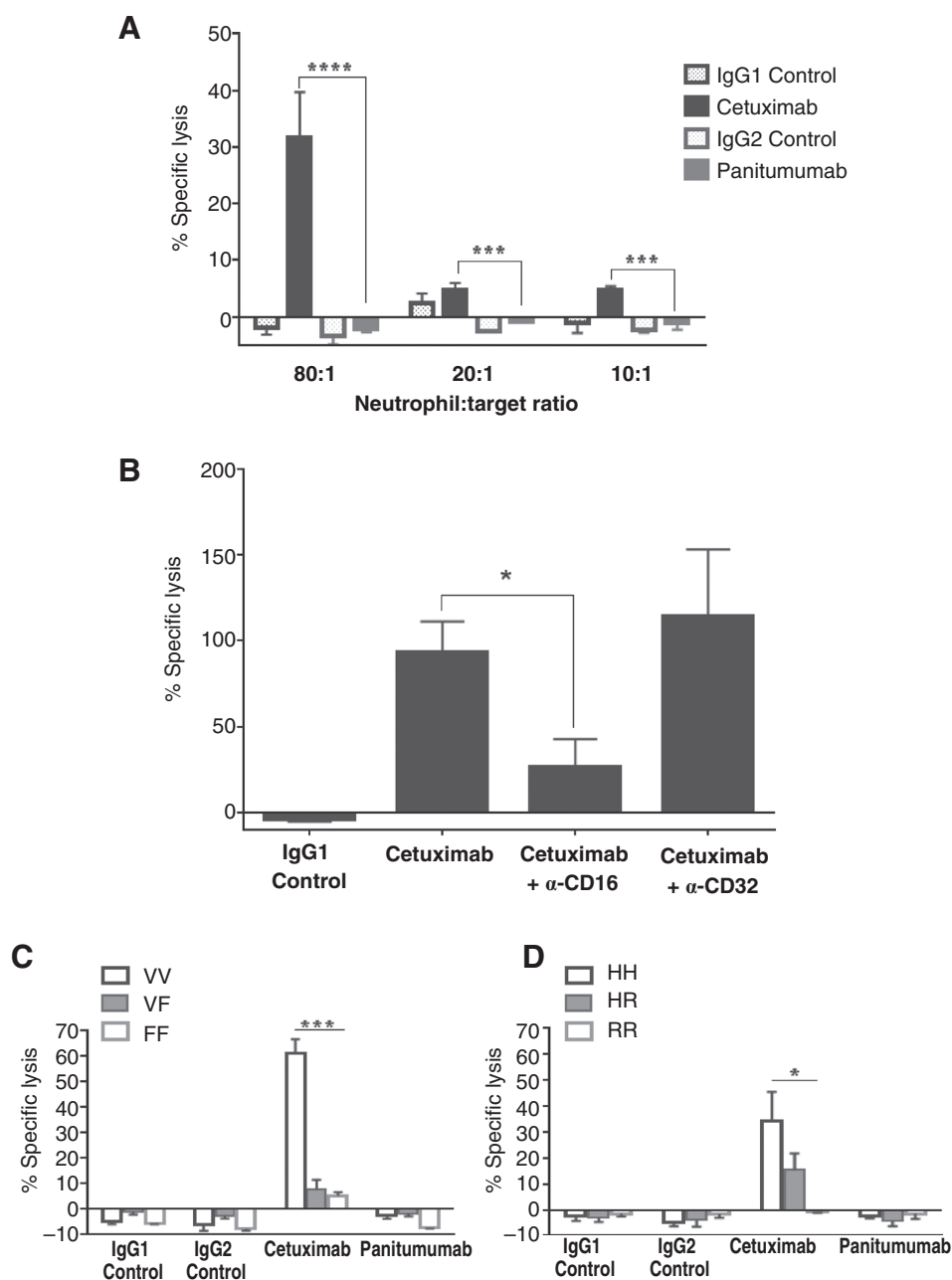
Panitumumab activates CD32 receptors on monocytes to a greater degree than cetuximab but does not induce ADCC against HNSCC cells

We next sought to determine whether CD32 activation on CD14⁺ monocytes, as measured by internalization of CD32, could be enhanced by either cetuximab or panitumumab. We cocultured CD14⁺ monocytes with HNSCC cell lines treated with

Figure 3.

Cetuximab-activated neutrophil-mediated ADCC is enhanced in donors who are homozygous for FcγIIIa VV genotype and FcγIIa HH genotype.

A, negatively isolated neutrophil cocultured for 4 hours with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with 10 μg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) at different E:T ratios (10:1, 20:1, and 80:1). Cetuximab-activated neutrophils mediate ADCC above isotype controls, whereas panitumumab-activated neutrophils do not. **B**, JHU-029 HNSCC cells pretreated with either CD16 or CD32 blocking antibodies for 30 minutes, washed once, then labeled with ⁵¹Cr, and then cocultured with negatively isolated neutrophils for 4 hours in the presence of cetuximab or IgG1 control antibody (10 μg/mL) at an 80:1 E:T ratio. Cells pretreated with CD16 blocking antibody show a reduction in cetuximab-activated neutrophil-mediated ADCC compared with non-pretreated cells and cells pretreated with CD32 blocking antibody. **C**, neutrophils from donors separated by FcγRIIIa and FcγRIIa genotype cocultured for 4 hours with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with 10 μg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) at 80:1 E:T ratio. Neutrophils from FcγRIIIa VV donors demonstrate significantly enhanced ADCC activity compared with VF and FF donors. **D**, neutrophils from FcγRIIa HH donors mediate enhanced ADCC compared with HR and RR donors. Data are mean ± SEM. *, *P* < 0.05; ***, *P* < 0.001; ****, *P* < 0.0001.



cetuximab, panitumumab, or isotype control Ab and then measured surface CD32 expression by flow cytometry (Fig. 4A). As a measure of activation, CD32 internalization in monocytes cocultured with JHU-029 cells occurred to a greater extent with panitumumab, as compared with that with cetuximab (*P* < 0.05). Positively isolated CD14⁺ cells were again cocultured with JHU-029 cells and incubated with cetuximab, panitumumab, or isotype control mAbs for 72 hours at 37°C. Then, CD80 and CD86 surface expression was measured by flow cytometry (Fig. 4B). We observed no significant difference in the expression of CD80 or CD86 on cells treated with either cetuximab or panitumumab.

To determine whether mAb-activated CD14⁺ cells could mediate ADCC, we cocultured CD14⁺ cells with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with cetuximab, panitumumab (10 μg/mL),

or isotype(IgG1 or IgG2) control mAb for 4 hours at different E:T ratios (5:1, 10:1 and 20:1). We found that CD14⁺ cells did not mediate ADCC above isotype control mAb in the presence of either cetuximab or panitumumab (Fig. 4C).

Cetuximab enhances cross-presentation and adaptive immune responses to a greater extent than panitumumab

We next investigated the effect of these 2 mAbs on adaptive cellular immunity. Although we had established that cetuximab activates NK cells to a greater extent than panitumumab, we wished to compare their ability to enhance NK cell-mediated DC maturation. NK cells were cocultured with CD11c⁺ DC and either cetuximab or panitumumab for 48 hours, and then HLA-DR expression on the DC was analyzed by flow cytometry

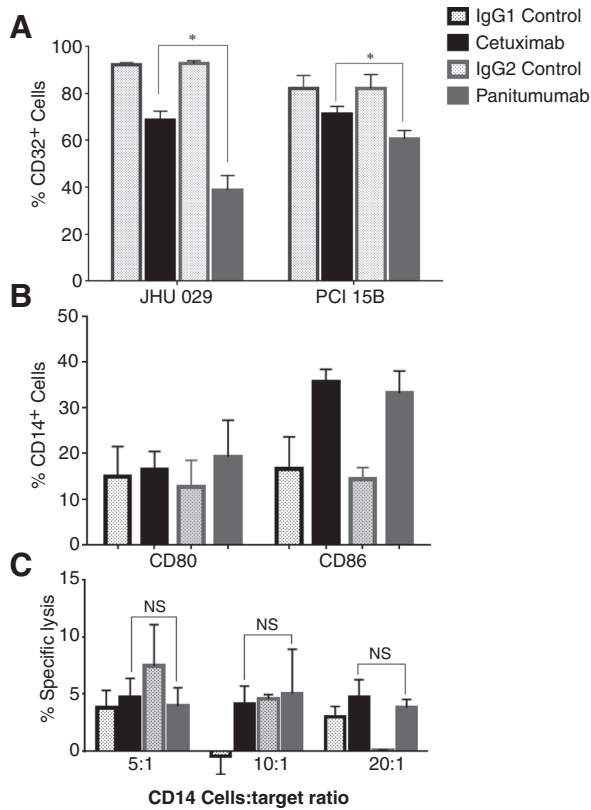


Figure 4.

Panitumumab activates CD32 receptors on monocytes to a greater degree than cetuximab but does not induce ADCC against HNSCC cells. Surface activation markers, CD32 (A) and CD80, CD86 (B) of isolated monocytes (CD14⁺ selection) cocultured with JHU029 and PCI15B HNSCC cells and treated with 10 µg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) for 72 hours were measured by flow cytometry. Monocytes treated with panitumumab activate surface CD32 to a greater extent than those treated with cetuximab as demonstrated by downregulation of surface CD32. There is no significant difference in CD80 or CD86 expression on monocytes treated with cetuximab or panitumumab. C, CD14⁺ cells cocultured for 4 hours with ⁵¹Cr-labeled JHU-029 HNSCC cells coated with 10 µg/mL of cetuximab, panitumumab, or isotype controls (IgG1 or IgG2) at different E:T ratios (5:1, 10:1, and 20:1). Results demonstrate that monocytes did not mediate ADCC above isotype controls in the presence of either cetuximab or panitumumab. Data are mean ± SEM. *, *P* < 0.05 cetuximab compared with panitumumab.

(Fig. 5A). Cetuximab-treated NK cells mediated significantly higher HLA-DR expression on DC than panitumumab (*P* < 0.001).

Next, we investigated whether increased DC maturation corresponded with improved presentation of tumor antigens in the presence of cetuximab or panitumumab. NK cells, DC, and JHU-029 cells were cocultured with cetuximab, panitumumab, or isotype control mAbs. Using a novel antibody (12B6), we measured the expression of HLA-A2:MAGE-3₃₂₇₁₋₂₇₉ complex on mature DC (mDC) to compare efficiency of antigen processing and presentation by the DC (Fig. 5B). NK cells treated with cetuximab induced significantly higher surface MAGE-3₃₂₇₁₋₂₇₉ presentation on DC compared with those treated with panitumumab (*P* < 0.05), indicating that cetuximab improves cross-presentation of endogenous tumor antigen compared with panitumumab.

We then assessed whether there were differences in EGFR-specific CTL frequencies in PBMCs from 2 prospective chemoradiotherapy clinical trials, combined with either cetuximab (UPCI # 08-013, NCT 01218048) or panitumumab (UPCI 06-120, NCT00798655). Using paired PBMCs from HLA-A2⁺ patients with HNSCC treated on these 2 clinical trials, we measured EGFR-specific CTL by flow cytometry, as a measure of adaptive immunity due to cross-presentation induced by the respective mAbs (10, 11). There was no increase in EGFR-specific CTL frequencies in patients post-panitumumab treatment (*n* = 7) (Fig. 5C and D). Interestingly, in all the tested paired samples from the cetuximab clinical trial (*n* = 8), we observed that EGFR-specific CTL frequencies increased significantly following cetuximab therapy (*P* = 0.003; Fig. 5E and F).

Discussion

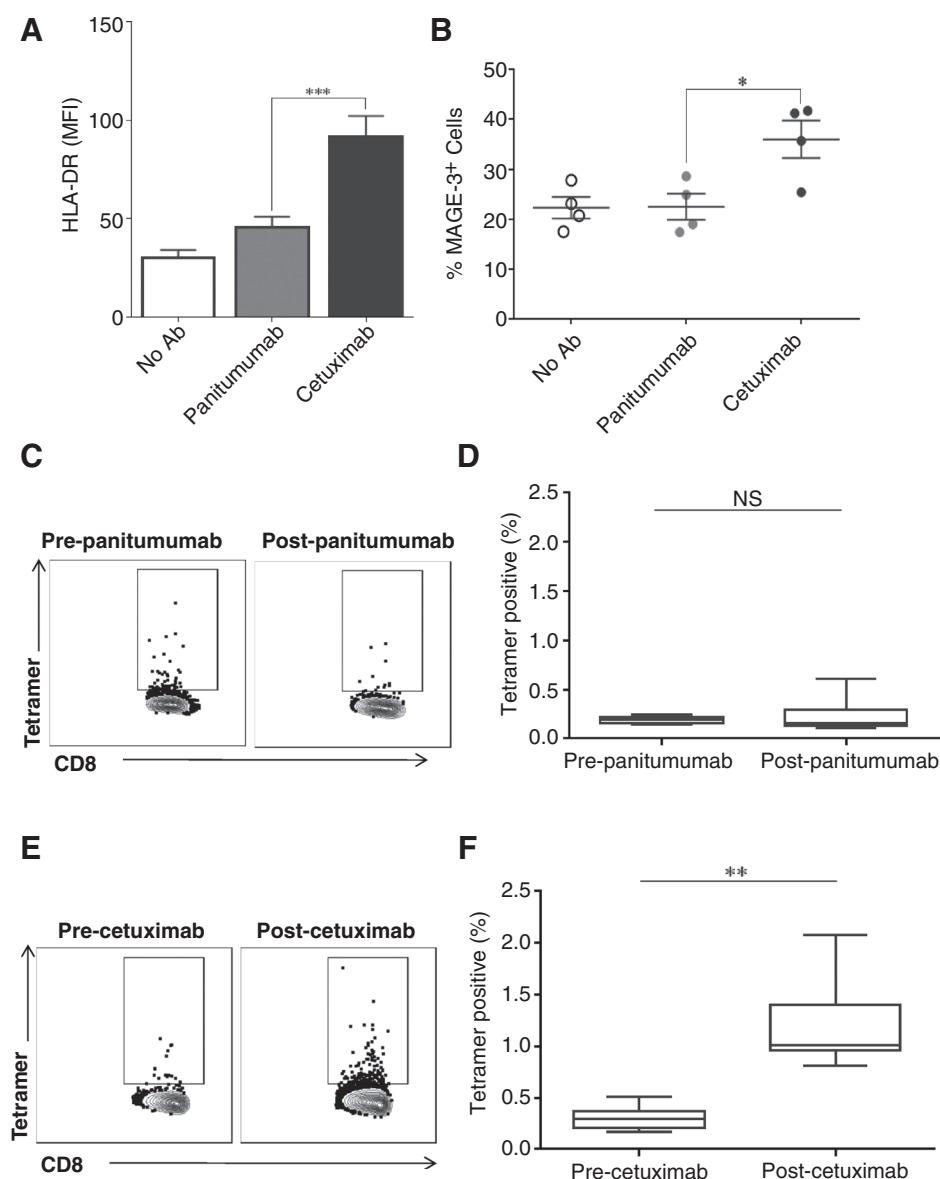
EGFR overexpression and subsequent downstream signaling activates tumor proliferative and prosurvival pathways in HNSCC (20). Despite near-universal EGFR expression, 2 FDA-approved mAb have modest activity, and cetuximab appears more clinically effective for unclear reasons. We hypothesized that mAb isotype may underlie this differential clinical activity, as IgG1 isotype mAbs, such as cetuximab, are more apt to induce cellular immunity than IgG2 isotype mAb like panitumumab. Cetuximab, an EGFR-targeted mAb, has been shown to improve outcomes in patients with recurrent or metastatic HNSCC (21, 22). Studies using panitumumab in HNSCC have shown lower clinical activity than those using cetuximab (7, 8).

Inhibition of downstream EGFR signaling, by blocking ligand binding, is an important function of mAbs. Indeed, panitumumab has been shown to bind the EGFR with an 8-fold greater affinity than cetuximab (23). On the basis of our prior studies (9) and this study using HNSCC cells, we observed that both panitumumab and cetuximab bind to EGFR and HNSCC cells with similar dilution curves and, importantly, both mAbs appeared to inhibit EGFR internalization and ligand-induced activation at similar doses. Thus, EGFR-binding affinity is of uncertain clinical significance.

The activation of Fcγ receptor (FcγR) bearing innate immune cells plays a role in effective antitumor responses generated by mAbs. Following cetuximab-mediated ADCC, antibody-bound EGFR-positive tumor cells are recognized by immune cells via FcγR. This results in the release of lytic granules from the effector immune cell (24). NK cells are the most frequently studied facilitators of cetuximab-mediated ADCC through surface CD16/FcγRIIIa and there is some evidence correlating FcγRIIIa polymorphisms with clinical outcomes (25, 26). IgG2 isotype therapeutic mAbs are believed to be relatively inert in their Fc functions, and this isotype may even be preferred when Fc-mediated effector functions were not desired (27). The role of anti-EGFR mAbs on myeloid cells in the context of HNSCC is yet undetermined, some studies suggest that panitumumab may mediate ADCC through CD32/FcγRIIa expressed by monocytes (13). Furthermore, recent data suggest that cetuximab may ameliorate suppressive phenotypes of myeloid cells in patients with HNSCC (28). However, no significant difference in activation markers on isolated CD14⁺ monocytes was noted between cetuximab and panitumumab. Interestingly, CD32 appeared to be partially activated and internalized by panitumumab as compared with cetuximab. However, neither cetuximab-activated nor

Figure 5.

Cetuximab enhances adaptive cellular immune responses to a greater extent than panitumumab. **A**, HLA-DR expression on CD11c⁺ DC cocultured with NK cells and cetuximab or panitumumab for 48 hours, measured by flow cytometry. HLA-DR expression on DC cocultured with cetuximab is significantly higher than on DC cocultured with panitumumab. **B**, mDC, NK cells, and JHU-029 (MAGE-3⁺) tumor cells were cocultured in the presence of no antibody, cetuximab (10 µg/mL), or panitumumab (10 µg/mL) at a 1:1:1 ratio for 48 hours. DC were then stained using 12B6 antibody and expression of MAGE-3 was analyzed by flow cytometry. MAGE-3 expression on DCs cocultured in the presence of cetuximab was significantly higher than those cocultured in the presence of panitumumab. EGFR-specific CTL frequencies of PBMCs from patients on 2 clinical trials employing chemoradiotherapy combined with either cetuximab or panitumumab measured by flow cytometry. **C**, representative plots illustrating the frequency of EGFR tetramer-positive CD8⁺ T cells in patient PBMCs pre- and post-panitumumab. **D**, percentage of EGFR-specific CTL in patients treated with panitumumab did not significantly change posttreatment. **E**, representative plots illustrating the frequency of EGFR tetramer-positive CD8⁺ T cells in patient PBMCs pre- and post-cetuximab. **F**, patients treated with cetuximab demonstrate significantly greater percentage of EGFR-specific CTL posttreatment. Data are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



panitumumab-activated CD14⁺ monocytes were effective mediators of ADCC. Schneider-Merck and colleagues demonstrated that anti-EGFR IgG2 mAbs can effectively trigger neutrophil-mediated ADCC, and this effect was enhanced in neutrophils from FcγRIIa HH donors (13). Interestingly, we found that cetuximab but not panitumumab-activated neutrophils were able to mediate ADCC against HNSCC cells. We additionally found that both functional FcγRIIa and FcγRIIIa polymorphisms may additionally affect cetuximab-activated neutrophil ADCC. In the clinical setting, the significance of lymphoid versus myeloid-mediated ADCC is yet unknown, these data suggest that anti-EGFR mAbs may exert their effect through the combined enhancement of both innate and adaptive immune systems.

Enhanced adaptive cellular immunity is the ultimate goal of any therapeutic agent. Our prior *in vitro* studies show that through its immunologic effects on NK cells and DC, which lead to enhanced DC maturation and tumor antigen processing, cetux-

imab therapy ultimately results in priming of T-cell-based immunity (10, 11). Here, we compared the ability of cetuximab- and panitumumab-activated NK cells to enhance DC maturation and NK:DC cross-talk. We found that panitumumab-activated NK cells showed modest increase in DC maturation. However, cetuximab-activated NK cells were significantly better at maturing DC and thus, cross-presenting tumor antigen to T cells. In HNSCC patient samples, we noted increased EGFR-positive CTL following treatment with cetuximab, this was not seen in patients treated with panitumumab. Given that recently published data indicated that mDC phenotype and myeloid-derived suppressor cells induction correlated with clinical response to cetuximab, a potential mechanism of myeloid-DC priming may underlie this enhancement of EGFR-specific T-cell expansion (28).

Taken together, our data show that although panitumumab is capable of binding EGFR and inhibiting its activation to the same extent as cetuximab, its ability to activate innate immune cells and

enhance cellular immune responses is inferior in HNSCC. Future studies examining other factors which may affect the efficacy of panitumumab in the clinical setting are warranted including the effect of genomic heterogeneity of HNSCC as well as novel combination immunotherapies available.

A recent phase III trial of single-agent panitumumab or cetuximab in chemotherapy-refractory, wild-type KRAS exon 2 metastatic colorectal cancers determined that overall survival following panitumumab treatment was noninferior to cetuximab (29), suggesting that colorectal cancer and HNSCC could manifest differences in therapeutic response to EGFR inhibition based on unique features of this pathway in each disease. Indeed, it has been established that in colorectal tumors, the presence of activating KRAS mutations, specifically in exon 2 (codons 12 and 13), predicts resistance to anti-EGFR mAbs (30). The majority of HNSCC contain wild-type KRAS with less than 5% harboring KRAS mutations compared with 35% to 45% of colorectal cancers (31, 32). In contrast, the HNSCC MAPK mutational pathway is primarily characterized by HRAS mutations (33). Therefore, it remains to be seen whether these mutations could impact clinical responses to panitumumab treatment in patients with head and neck cancer. Clinical trials examining the effect of cetuximab in combination with novel antibodies targeting immune checkpoint inhibitors and toll-like receptor agonists with various radiotherapy schedules in HNSCC are currently underway. The effect of panitumumab may be enhanced when given in similar combinations and this remains an area of future investigation.

References

- Saltz LB, Meropol NJ, Loehrer PJ Sr, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004;22:1201–8.
- Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalberg JR, Tu D, Au HJ, et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;357:2040–8.
- Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–92.
- Shin DM, Donato NJ, Perez-Soler R, Shin HJ, Wu JY, Zhang P, et al. Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. *Clin Cancer Res* 2001;7:1204–13.
- Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* 2011;11:9–22.
- Mesia R, Henke M, Fortin A, Minn H, Yunes Ancona AC, Cmelak A, et al. Chemoradiotherapy with or without panitumumab in patients with unresected, locally advanced squamous-cell carcinoma of the head and neck (CONCERT-1): a randomised, controlled, open-label phase 2 trial. *Lancet Oncol* 2015;16:208–20.
- Giralt J, Trigo J, Nuyts S, Ozsahin M, Skladowski K, Hatoum G, et al. Panitumumab plus radiotherapy versus chemoradiotherapy in patients with unresected, locally advanced squamous-cell carcinoma of the head and neck (CONCERT-2): a randomised, controlled, open-label phase 2 trial. *Lancet Oncol* 2015;16:221–32.
- Vermorken JB, Stohlmacher-Williams J, Davidenko I, Licitra L, Winquist E, Villanueva C, et al. Cisplatin and fluorouracil with or without panitumumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck (SPECTRUM): an open-label phase 3 randomised trial. *Lancet Oncol* 2013;14:697–710.
- Lopez-Albaitero A, Ferris RL. Immune activation by epidermal growth factor receptor specific monoclonal antibody therapy for head and neck cancer. *Arch Otolaryngol Head Neck Surg* 2007;133:1277–81.
- Lee SC, Srivastava RM, Lopez-Albaitero A, Ferrone S, Ferris RL. Natural killer (NK): dendritic cell (DC) cross talk induced by therapeutic monoclonal antibody triggers tumor antigen-specific T cell immunity. *Immunol Res* 2011;50:248–54.
- Srivastava RM, Lee SC, Andrade Filho PA, Lord CA, Jie HB, Davidson HC, et al. Cetuximab-activated natural killer and dendritic cells collaborate to trigger tumor antigen-specific T-cell immunity in head and neck cancer patients. *Clin Cancer Res* 2013;19:1858–72.
- Lopez-Albaitero A, Lee SC, Morgan S, Grandis JR, Gooding WE, Ferrone S, et al. Role of polymorphic Fc gamma receptor IIIa and EGFR expression level in cetuximab mediated, NK cell dependent *in vitro* cytotoxicity of head and neck squamous cell carcinoma cells. *Cancer Immunol Immunother* 2009;58:1853–64.
- Schneider-Merk T, Lammerts van Bueren JJ, Berger S, Rossen K, van Berkel PH, Derer S, et al. Human IgG2 antibodies against epidermal growth factor receptor effectively trigger antibody-dependent cellular cytotoxicity but, in contrast to IgG1, only by cells of myeloid lineage. *J Immunol* 2010;184:512–20.
- Srivastava RM, Trivedi S, Concha-Benavente F, Hyun-Bae J, Wang L, Seethala RR, et al. STAT1-induced HLA class I upregulation enhances immunogenicity and clinical response to anti-EGFR mAb cetuximab therapy in HNC patients. *Cancer Immunol Res* 2015;3:936–45.
- Andrade Filho PA, Lopez-Albaitero A, Gooding W, Ferris RL. Novel immunogenic HLA-A*0201-restricted epidermal growth factor receptor-specific T-cell epitope in head and neck cancer patients. *J Immunother* 2010;33:83–91.
- Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, et al. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res* 2011;17:7248–64.
- Heo DS, Snyderman C, Gollin SM, Pan S, Walker E, Deka R, et al. Biology, cytogenetics, and sensitivity to immunological effector cells of new head and neck squamous cell carcinoma lines. *Cancer Res* 1989;49:5167–75.

Disclosure of Potential Conflicts of Interest

R.L. Ferris reports receiving commercial research grants from Amgen, Astra Zeneca, Bristol-Meyers Squibb, and Venti RX and is a consultant/advisory board member for Astra Zeneca, Bristol-Meyers Squibb, Celgene, and ONO Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Trivedi, S. Ferrone, R.L. Ferris

Development of methodology: S. Trivedi, F. Concha-Benavente, J. Li, R.L. Ferris

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Trivedi, R.M. Srivastava, F. Concha-Benavente, J. Li, R.L. Ferris

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Trivedi, R.M. Srivastava, F. Concha-Benavente, R.L. Ferris

Writing, review, and/or revision of the manuscript: S. Trivedi, F. Concha-Benavente, S. Ferrone, T.M. Garcia-Bates, R.L. Ferris

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.M. Garcia-Bates, R.L. Ferris

Study supervision: R.L. Ferris

Grant Support

This work was supported by NIH grants R01 DE 019727, P50 CA097190, T32 CA060397 and used the University of Pittsburgh Cancer Institute award P30 CA047904.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 7, 2015; revised April 9, 2016; accepted May 13, 2016; published OnlineFirst May 23, 2016.

18. Day KE, Sweeny L, Kulbersh B, Zinn KR, Rosenthal EL. Preclinical comparison of near-infrared-labeled cetuximab and panitumumab for optical imaging of head and neck squamous cell carcinoma. *Mol Imaging Biol* 2013;15:722–9.
19. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003;21:3940–7.
20. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res* 1993;53:3579–84.
21. Vermorken JB, Mesia R, Rivera F, Remenar E, Kaweck A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med* 2008;359:1116–27.
22. Burtneß B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. *J Clin Oncol* 2005;23:8646–54.
23. Kim GP, Grothey A. Targeting colorectal cancer with human anti-EGFR monoclonal antibodies: focus on panitumumab. *Biol Targets* 2008;2:223–8.
24. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. *Lancet* 2009;373:1033–40.
25. Patel D, Guo X, Ng S, Melchior M, Balderes P, Burtrum D, et al. IgG isotype, glycosylation, and EGFR expression determine the induction of antibody-dependent cellular cytotoxicity in vitro by cetuximab. *Hum Antibodies* 2010;19:89–99.
26. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol* 2009;27:1122–9.
27. Carter PJ. Potent antibody therapeutics by design. *Nat Rev Immunol* 2006;6:343–57.
28. Li J, Srivastava RM, Etyreddy A, Ferris RL. Cetuximab ameliorates suppressive phenotypes of myeloid antigen presenting cells in head and neck cancer patients. *J Immunother Cancer* 2015;3:54.
29. Price TJ, Peeters M, Kim TW, Li J, Cascinu S, Ruff P, et al. Panitumumab versus cetuximab in patients with chemotherapy-refractory wild-type KRAS exon 2 metastatic colorectal cancer (ASPECCT): a randomised, multicentre, open-label, non-inferiority phase 3 study. *Lancet Oncol* 2014;15:569–79.
30. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626–34.
31. Langer CJ. Exploring biomarkers in head and neck cancer. *Cancer* 2012;118:3882–92.
32. Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001;85:692–6.
33. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. *Cancer Discov* 2013;3:761–9.