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CD137 Stimulation Enhances Cetuximab-Induced Natural Killer: Dendritic Cell Priming of Antitumor T-Cell Immunity in Patients with Head and Neck Cancer

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Abstract

Purpose: Cetuximab, an EGFR-specific antibody (mAb), modestly improves clinical outcome in patients with head and neck cancer (HNC). Cetuximab mediates natural killer (NK) cell:dendritic cell (DC) cross-talk by cross-linking FcγRIIIa, which is important for inducing antitumor cellular immunity. Cetuximab-activated NK cells upregulate the costimulatory receptor CD137 (4-1BB), which, when triggered by agonistic mAb urelumab, might enhance NK-cell functions, to promote T-cell–based immunity.

Experimental design: CD137 expression on tumor-infiltrating lymphocytes was evaluated in a prospective cetuximab neoadjuvant trial, and CD137 stimulation was evaluated in a phase Ib trial, in combining agonistic urelumab with cetuximab. Flow cytometry and cytokine release assays using NK cells and DC were used *in vitro*, testing the addition of urelumab to cetuximab-activated NK, DC, and cross presentation to T cells.

Results: CD137 agonist mAb urelumab enhanced cetuximab-activated NK-cell survival, DC maturation, and tumor antigen cross-presentation. Urelumab boosted DC maturation markers, CD86 and HLA DR, and antigen-processing machinery (APM) components TAP1/2, leading to increased tumor antigen cross-presentation. In neoadjuvant cetuximab-treated patients with HNC, upregulation of CD137 by intratumoral, cetuximab-activated NK cells correlated with FcγRIIIa V/F polymorphism and predicted clinical response. Moreover, immune biomarker modulation was observed in an open label, phase Ib clinical trial, of patients with HNC treated with cetuximab plus urelumab.

Conclusions: These results suggest a beneficial effect of combination immunotherapy using cetuximab and CD137 agonist in HNC. *Clin Cancer Res; 23(3); 707–16. ©2016 AACR.*

Introduction

Immunotherapy against head and neck cancer (HNC) is an important and rapidly expanding field (1). Using a phase II clinical trial of neoadjuvant cetuximab, we studied the therapeutically relevant, antitumor immune effects of this EGFR-targeted mAb (2, 3). Indeed, cetuximab treatment induced innate and adaptive immunity in a subset of patients who generated objective clinical responses (4, 5). Previous studies have shown that cetuximab-coated HNC cells induce NK cells (4, 5), promote NK cell–dendritic cell (DC) cross-talk (2, 6), and expand EGFR-specific cytotoxic T cells (CTL; refs. 2, 7, 8, 9, 10). Combining the tumor targeting effects of cetuximab with a specific, immune cell targeting mAb may be a useful therapeutic strategy (11).

CD137 (4-1BB), a member of the TNF-receptor superfamily, is broadly induced on activated $CD4^+$ T cells (12), $CD8^+$ T cells (13), B cells (14), NK cells (15), monocytes (16), and DCs (17). The introduction of the fully human, clinical grade CD137-agonist mAb, urelumab (BMS-663513) has enabled modulation of CD137 function in immune-oncology, including evaluating its role in combination with tumor targeting mAb (11).

Previous studies have established the effects of the CD137 pathway on activated T and B lymphocytes (13, 18, 19); however, the potential mechanism of action of CD137 targeting to enhance NK: DC cross-talk is only recently emerging (15). NK cells are subdivided in to $CD56^{dim}$ and $CD56^{bright}$ subsets, which differ significantly in their effector function. Cetuximab-activated $CD56^{dim}$ NK cells appear to upregulate CD137 receptor in higher magnitude than $CD5^{bright}$ NK cells (2, 6, 15). Depletion of DC abrogates CD137 agonist mAb induced therapeutic benefits in mice; however, antigen presentation, cross-presentation of TA were not tested in patients with HNC (20, 21). Here, we have investigated the factors that modulate CD137 expression on cetuximab-activated NK cells (2, 4). We additionally investigated the effect of stimulating CD137 expressed by cetuximab activated NK:DC cross-talk in the tumor microenvironment using clinical specimens from a neoadjuvant cetuximab clinical trial

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Translational Relevance

The anti–EGFR mAb cetuximab acts through blocking oncogenic signals and by inducing Fcγ receptor (FcγR)–mediated cytotoxicity and cross-priming of T-cell responses. However, cetuximab only modestly improves clinical outcome in patients with head and neck cancer (HNC). Therefore, a suitable combinatorial agent, which can boost cetuximab-mediated antitumor cellular immunity is warranted. Stimulation of CD137 delivers a robust costimulatory signal to both NK and DC, potentially improving adaptive, T-cell–based antitumor immune responses. The implication of these finding includes identification of biomarkers for combination therapy.

(NCT01218048). These findings have important implications for biomarkers of response to cetuximab based immunotherapy (18, 22).

Materials and Methods

Lymphocyte isolation, DC generation, and HNC cell lines culture

Following Institutional Review Board (UPCI protocol 99-069) approval and informed consent, blood was obtained from healthy donors (Western PA blood bank) or HNC patients treated with cetuximab (NCI-2011-02479, NCT01218048). Lymphocytes were purified by Ficoll-Paque PLUS centrifugation (Amersham Biosciences) and stored frozen. DC were generated as described previously (2). NK cells were purified using EasySep kits (Stem Cell Technologies), and purity was >95% $CD16^+$, $CD56^+$, $CD3^-$ evaluated with flow cytometry (23).

The HNC cell lines PCI-15B ($HLA\text{-}A2^-EGFR^+$) were isolated from patients treated at the University of Pittsburgh Cancer Institute (Pittsburgh, PA) through the explant/culture method. The JHU-029 ($HLA\text{-}A2^-EGFR^+$ and $MAGE\text{-}3^+$) cell line was a kind gift from Dr. James Rocco (Harvard Medical School) in January 2007. All cell lines were authenticated and validated as unique using STR profiling and HLA genotyping every 6 months (24, 25). Cell lines were grown in IMDM (Sigma) supplemented with 10% FBS (Cellgro), 2% L-glutamine and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2, 95% humidity atmosphere. Adherent tumor cells were detached by warm Trypsin-EDTA (0.25%) solution (Invitrogen).

Antibodies, cytokines, and FcγR IIIa genotyping

The EGFR-specific chimeric IgG1 mAb cetuximab (Erbitux) and CD137-specific human IgG4 mAb urelumab (BMS-663513) were obtained from Bristol-Myers Squibb (Imclone). FITC-CD56, AF-700 CD56, PE-Texas Red-CD56, PercpCy5.5-CD3, AF700-CD3, PE-CRTAM, PECy5-CD137, PE-Texas Red-CD16, APC-PD-1, FITC-CD69, PE-Cy7-NKG2D, BV421-NKp46, APC-Cy7 IFNγ, AF-700 TNFα, FITC-Ki67, PE-CD107a, AF647-Granzyme B, PE-Cy5-CD8, APC-Cy7-CD8, PerCPCy5.5-CD4, BV421-TIM3, APC-CD14, BV-421-PD-L1 were purchased from Biolegend. FITC or PE-Cy7–anti-CD11c mAb (R&D Systems), anti-CD80 mAb, anti-CD86 mAb, anti–PD-L1 mAb, anti–HLA-DR mAb, and EPCAM mAb (BD Biosciences Pharmingen) were purchased. PE-Cy5 anti-CD137 Ab and PE- and FITC-conjugated IgG isotypes for flow cytometry were purchased from BD Biosciences. FITC- goat-anti–human Fc-specific IgG and FITC-goat anti-mouse IgG were purchased from Invitrogen. The antigen-processing machinery components (APM) TAP-1–specific mAb (clone NOB1) and TAP-2–specific mAb (clone NOB2) were developed and characterized as described previously (26). Recombinant human GM-CSF and recombinant human IL-4, were purchased from R&D Systems Inc. Frozen patient PBMC were thawed and subjected to viability testing by using the Zombie-Aqua Fixable Viability Kit (Biolegend) for multicolor flow cytometry.

FcγR IIIa-158 genotype was determined using a quantitative PCR-based assay kit from Applied Biosystems. Briefly, genomic DNA was extracted using the DNeasy Kit (Qiagen) following the manufacturer's protocol. Five to 50 ng of genomic DNA were added to a 25-μL reaction using 2× TaqMan master mix (Applied Biosystems). Plates were run and analyzed for allelic expression using an ABI prism 7700 sequence detection system (2, 4).

Flow cytometry

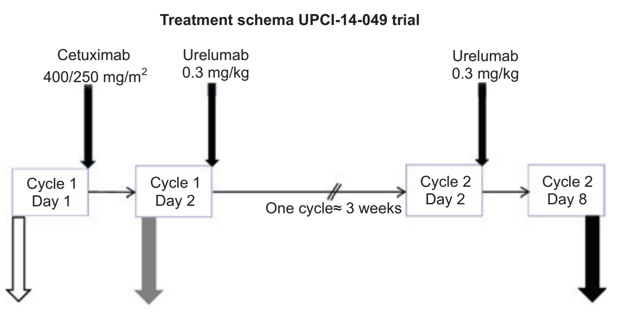
Lymphocytes, NK cells were prepared for flow cytometry by washing with PBS (Sigma-Aldrich) and FACS buffer (2% FBS in PBS). Cells were analyzed on BD LSR Fortessa flow cytometer (Becton Dickinson) using FACS DIVA software, and data were analyzed by using FlowJo software. Intracellular APM component (TAP-1, TAP-2, LMP-2–specific mAbs, 1:500 dilutions) staining of DC was performed by using cytofix/cytoperm fixation/permeabilization kit (BD Biosciences; ref. 26).

Tumor and lymphocyte specimens

Peripheral venous blood samples were obtained from patients with HNC with stage III/IVA disease (Table 1), receiving neoadjuvant cetuximab on a prospective phase II clinical trial (UPCI 08-013, NCT 01218048). Tumors were biopsied immediately before, and again after 4 weeks, of cetuximab therapy. Clinical response

Table 1. Demographics table of cetuximab, cetuximab plus urelumab–treated cohorts

Regimen	No. of patients	Tumor site		Mean age (y)	Males	Females
UPCI 08-013[1]	18	OC	8	59.7	12	6
		OP	8			
		L	2			
		HP				
		Other				
		Unknown primary				
UPCI 14-049[2]	6	OC		55.6	4	2



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was analyzed by comparing paired CT scans pre-/post-cetuximab and quantifying tumor measurement by a dedicated head and neck radiologist blinded to patient status. Anatomic tumor measurements were recorded in two dimensions and the cohort segregated into clinical responders, or nonresponders. Peripheral venous blood samples were obtained from patients with HNC receiving cetuximab plus urelumab on a phase Ib, open-label trial (UPCI-14-049, NCT02110082).

Statistical Analysis

Data were analyzed statistically using GraphPad Prism 4.0. A two-way ANOVA, a two-tailed unpaired or paired t test was used to calculate whether observed differences were statistically significant, defined as *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Results

Cetuximab-mediated NK-cell expression of CD137 is dependent on FcγRIIIa polymorphism

CD137 is an activation marker for NK cells after exposure to cetuximab. Although cetuximab-coated HNC cells significantly enhanced CD137 expression on all NK cells, CD137 expression was significantly higher on NK cells expressing FcγRIIIa VV/VF ($n = 8$) compared with FcγRIIIa FF ($n = 8$) genotype (Fig. 1A). Upregulation of CD137 on both groups coincided with decreased expression of surface CD16 (Fig. 1B). To validate the clinical importance of these *in vitro* findings, we used specimens from a phase II clinical trial (UPCI 08-013) in which tumors from patients with HNC were biopsied before and after 4 weeks of single-agent neoadjuvant cetuximab. CD137 expression was measured on intratumoral $CD56^{+}CD3^{-}$ NK cells by flow cytometry from freshly isolated TIL, and correlated with radiologic clinical response. Following cetuximab therapy, CD137 expression was significantly upregulated in clinical responders ($n = 5$, $P = 0.02$) but not in nonresponders ($n = 12$; Fig. 1C).

We then measured the expression of CD137 in peripheral blood and intratumoral NK cells before and after therapy with prospective neoadjuvant cetuximab clinical trial (UPCI 08-013). Significant induction of CD137 was observed on intratumoral, but not circulating NK cells, primarily in FcγRIIIa VV/VF patients ($P = 0.03$; Fig. 1D). In contrast, no significant increase in CD137 expression was observed on intratumoral or peripheral blood NK cells in FcγRIIIa FF patients (Fig. 1D) consistent with the correlation of CD137 upregulation with clinical response to neoadjuvant cetuximab. We next compared CD137 expression on intratumoral NK cells in HPV (+) and HPV (−) HNC patients treated on the neoadjuvant cetuximab trial. Although cetuximab treatment raised CD137 expression on both HPV (−) and HPV (+) patients' NK cells, the mean induction of CD137 on HPV (+) patients was significantly higher than HPV (−) patients (Fig. 1E).

Urelumab enhances cetuximab-mediated DC maturation

In addition to stimulating better innate immunity through activated NK cells, we investigated whether cetuximab modulates the expression of CD137 on DC during cetuximab-mediated NK:DC cross-talk. We measured surface activation/maturation markers on DC co-cultured with PCI-15B cells and NK cells in the presence of cetuximab. FACS analysis of DC showed significant upregulation of CD137 and CD86 in the presence of cetuximab plus NK cells (Fig. 2A; Supplementary Fig. S1). Furthermore, we examined whether the addition of urelumab could enhance cetuximab-mediated DC maturation in the presence of NK cells. Compared with cetuximab alone, the addition of urelumab significantly enhanced HLA-DR and CD86 expression (Fig. 2B–C). Because both DC and NK express CD137, we investigated whether enhanced DC maturation is the direct effect of urelumab, or if this was mediated by NK cells activated by urelumab. Purified NK cells were incubated with cetuximab-coated PCI-15B cells (24 hours) then isolated. These cetuximab-activated NK cells were then cocultured with autologous immature DC and PCI-15B cells in the presence of urelumab, cetuximab, or a combination of both mAbs for 48 hours. Although urelumab alone failed to induce CD80 (Fig. 2D), CD86 (Fig. 2E), the combination of urelumab and cetuximab augmented cetuximab-mediated DC maturation markers (Fig. 2D–E). To determine the additive effect of urelumab on cetuximab-mediated NK:DC cross-talk, we co-cultured DC and PCI-15B cells, in absence or presence of NK cells. Whole PBMC was incubated with cetuximab-coated HNC cells for 24 hours. NK cells were then purified and co-cultured with autologous DC and PCI-15B cells for 48 hours without mAb, or co-cultured with autologous DC and PCI-15B cells for 48 hours in the presence of cetuximab, urelumab, or a combination of both mAbs. Urelumab alone failed to upregulate CD80 on DC co-cultured with or without NK cells. However, urelumab in combination increased cetuximab-mediated CD80 expression on DC, when NK cells were present in coculture (Fig. 2F). These results suggest that cetuximab-induced DC maturation, important for NK:DC cross-talk, is enhanced by urelumab, and dependent on NK-cell activation.

Urelumab enhances cetuixmab-mediated cross-presentation of TA in the presence of NK cells by augmenting antigen-processing machinery in DC

We observed that urelumab augments cetuximab-mediated DC maturation (Fig. 2 B–F), a crucial mechanism for cross-priming of TA specific T cells (2). Moreover, type I DC have previously been shown to secrete high levels of Th1 cytokines and chemokines (26), which may augment the expression of certain APM components, such as TAP-1/2 and LMP-2, which are important for TA-derived peptide presentation to cognate CTL. Thus, investigated whether intracellular APM components were upregulated in DC incubated with cetuximab-activated NK cells plus HNC cells (JHU-029) in the presence of urelumab. Interestingly, the addition of urelumab enhanced expression of TAP-1 (Fig. 3A), TAP-2 (Fig. 3B), and LMP-2 (Fig. 3C) by DC that were co-cultured with cetuximab-activated NK cells.

We then used a novel mAb (clone 12B6), recognizing the HLA-A2:MAGE-$3_{271\text{-}279}$ complex (27), to investigate whether the enhanced HLA class I APM components resulted in elevated levels of surface HLA–TA complexes. Indeed, cetuximab treatment enhanced HLA-A2:MAGE-$3_{271\text{-}279}$ complexes on DC in the presence of JHU-029 and NK cells (Fig. 3D–E), but not in urelumab alone-treated cells, or those co-cultured without MAGE-$3_{271\text{-}279}$–positive HNC cells (data not shown). Furthermore, the combination of urelumab plus cetuximab further augmented TA presentation in a quantitative function (Fig. 3D–E; $P = 0.02$).

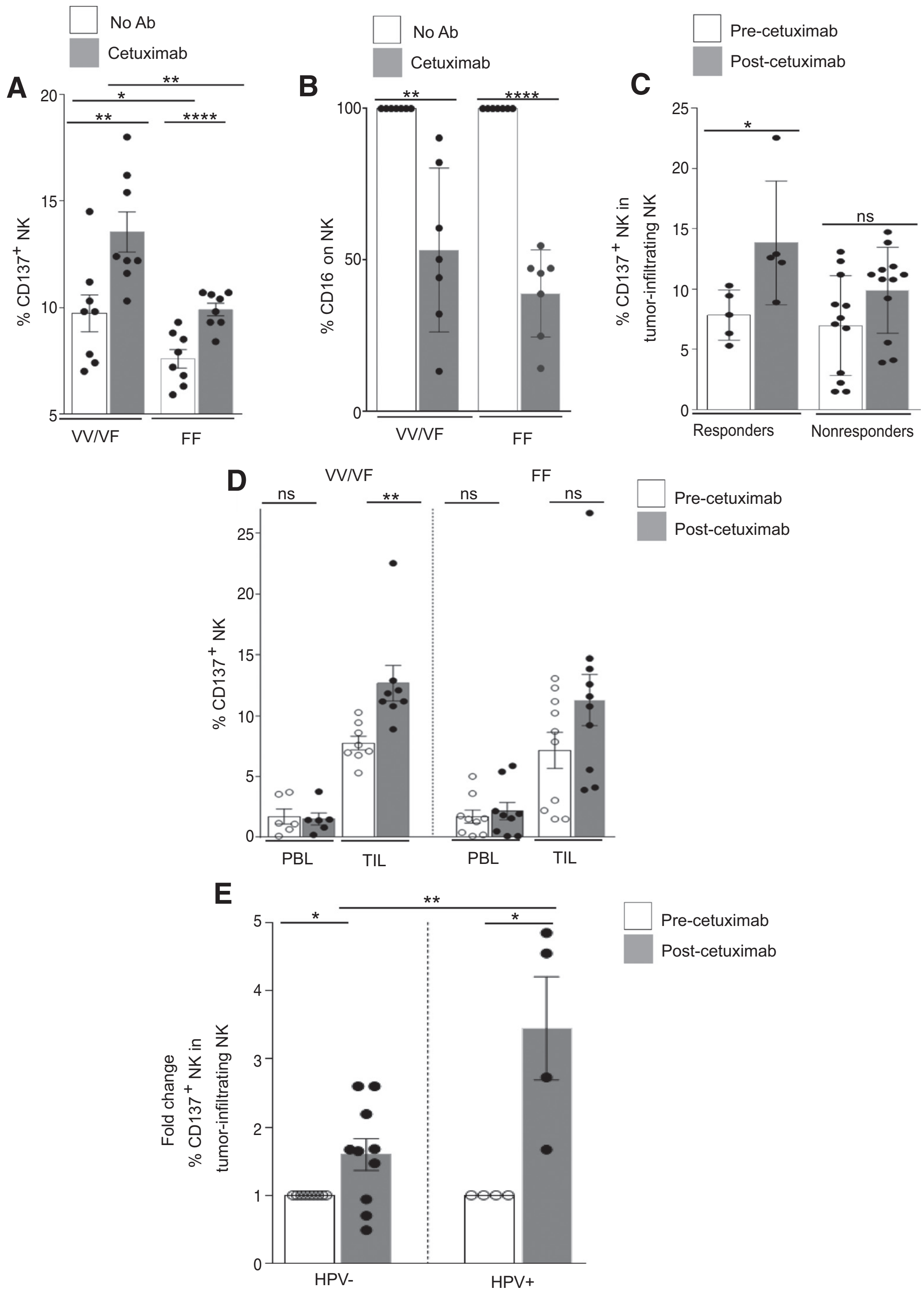

Figure 1.
Cetuximab-mediated NK-cell expression of CD137 is dependent on FcγRIIIa polymorphism. Expression of CD137 (VV/VF $n = 8$, FF $n = 8$; **A**), and CD16 (VV/VF $n = 7$, FF $n = 7$; **B**) on healthy donor peripheral blood NK cells from high (VV/VF) and low affinity (FF) FcγRIIIa genotypes was determined after co-culture with PCI-15B cells in the presence of cetuximab (10 μg/mL, 24 hours) or no Ab. Flow cytometric analysis of CD137 expression in tumor-infiltrating NK cells from patients with HNC before and after cetuximab neoadjuvant therapy (cetuximab i.v. 400 mg/m^2 day 1 then 250 mg/m^2 alone days 8, 15, and 22 (UPCI 08-013). Patients defined as responders ($n = 5$ responders, $n = 12$ nonresponders), demonstrated upregulation of CD137 on tumor-infiltrating NK cells following cetuximab therapy compared with nonresponders (**C**). Frequency of CD137 in peripheral blood NK cells (PBL VV/VF $n = 7$, PBL FF $n = 9$) and tumor-infiltrating NK cells (TINK VV/VF $n = 8$, TINK FF $n = 10$) were determined in VV/VF and FF patients with HNC before and after cetuximab neoadjuvant therapy (UPCI 08-013; **D**). The percentage of CD137 in tumor-infiltrating NK cells was determined before and after cetuximab neoadjuvant therapy (UPCI 08-013) and correlated with HPV status of patients with HNC HPV (−) $n = 10$, HPV (+) $n = 4$ (**E**). A two-tailed unpaired or paired *t* test was performed for statistical analysis; collective data are representative of ±SEM. $P \leq 0.05^{*}$, $P \leq 0.01^{**}$, $P \leq 0.0001^{****}$.

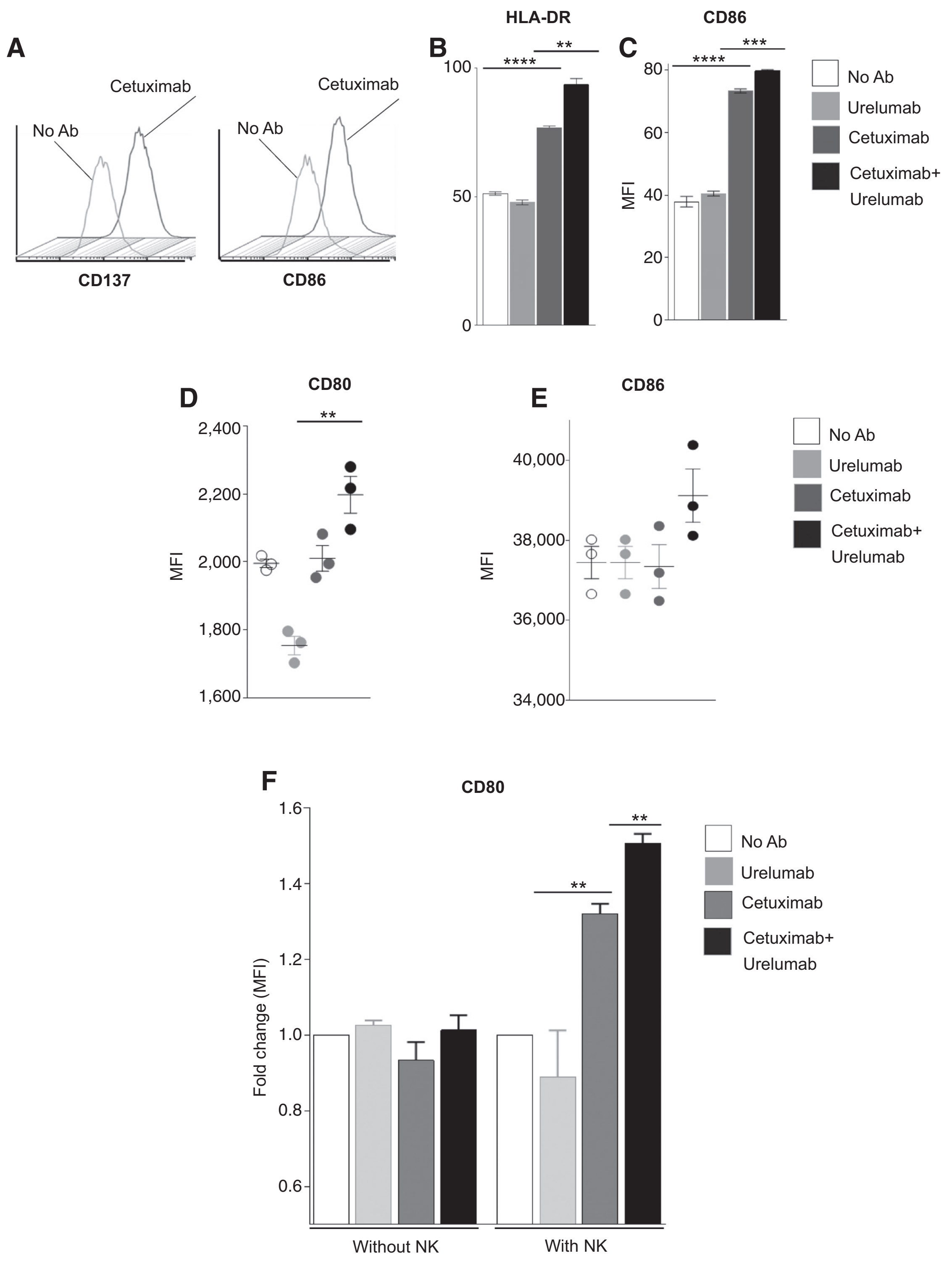

Figure 2.

Urelumab enhances cetuximab-induced DC maturation. Representative histograms demonstrating upregulation of CD86 and CD137 on DC co-cultured with NK cells and PCI-15B (1:1:1 ratio) in the presence of cetuximab (10 μg/mL, 24 hours) or no Ab (**A**). Whole PBMC was incubated with cetuximab-coated PCI-15B, and then NK cells were purified and incubated with DC and PCI-15B cells, in the presence of urelumab (50 μg/mL), cetuximab (10 μg/mL), or cetuximab (10 μg/mL) plus urelumab (50 μg/mL). FACS analysis of upregulation of maturation markers, HLA-DR (**B**) and CD86 (**C**) on DC co-cultured with cetuximab-activated NK cells (in PBMC) and PCI-15B cells in the presence of cetuximab plus urelumab. Expression level of CD80 (**D**), CD86 (**E**) on DC was analyzed by FACS indicates that cetuximab-mediated NK-induced DC maturation is increased by addition of urelumab. Whole PBMCs were incubated with cetuximab-coated PCI-15B cells, and then NK cells were purified and incubated with DC, PCI15B, and expression level of CD80 (**F**) on DC was evaluated after co-culture with PCI-15B cells with or without NK cells treated with cetuximab, urelumab, or both. A two-tailed paired *t* test was performed for statistical analysis, collective data are representative of ±SEM. $P \leq 0.01^{**}$, $P \leq 0.001^{***}$, $P \leq 0.0001^{****}$.

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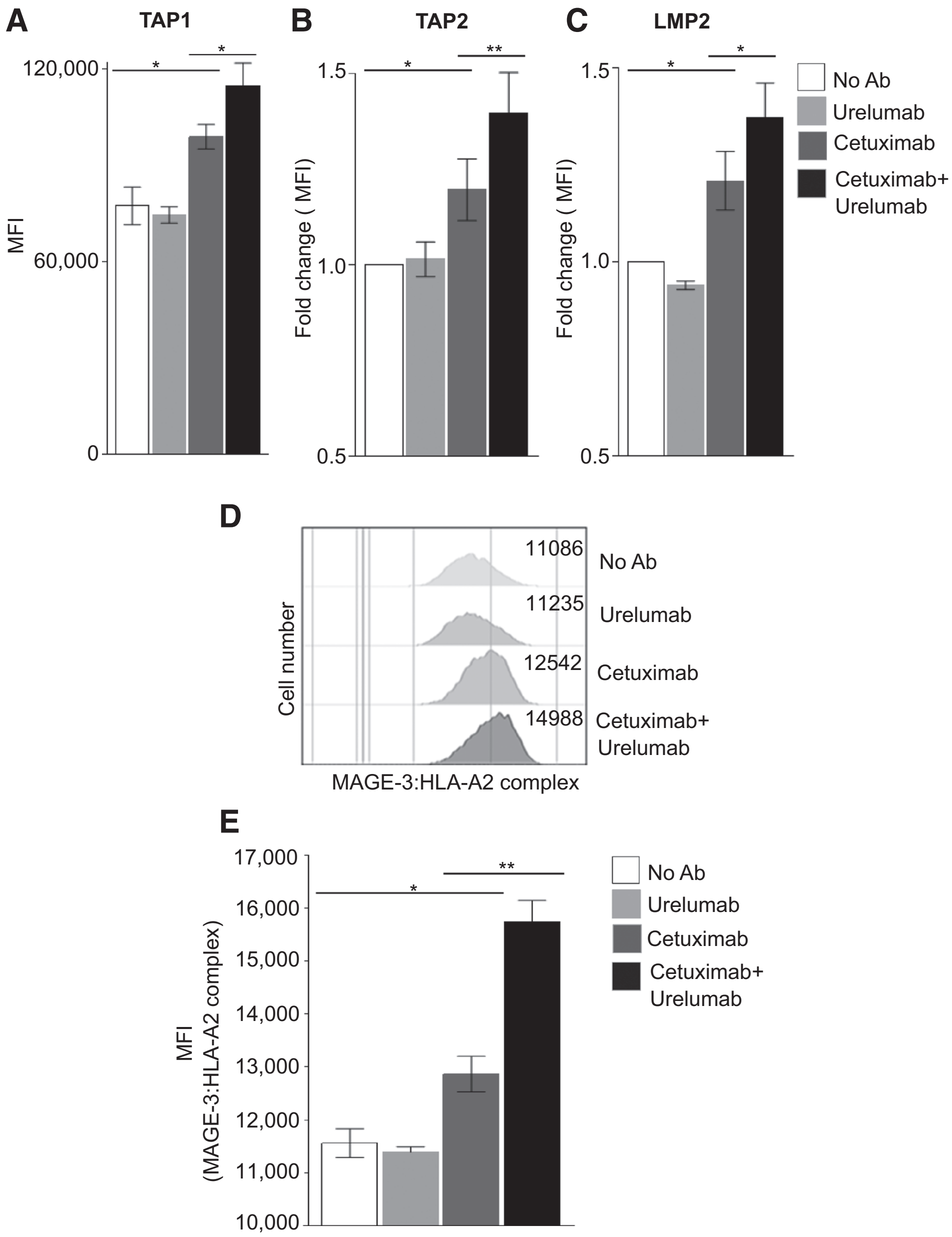

Figure 3.
Enhancement of the DC APM pathway by urelumab. Intracellular levels of antigen-processing machinery components TAP1 (**A**), TAP2 (**B**), LMP-2 (**C**) on DC were evaluated after coculture with NK cells and PCI-15B cells in presence of urelumab (50 μg/mL), cetuximab (10 μg/mL), or cetuximab (10 μg/mL) plus urelumab (50 μg/mL; 48 hours at 1:1:1 ratio). **D** and **E,** Cell surface expression of MAGE-3:HLA-A2 complex on DC was determined after co-culture with DC: NK:JHU-029 (1:1:1 ratio, 48 hours co-culture) with MAGE-3:HLA-A2 complex-specific mAb 12b6. A two-tailed unpaired or paired *t* test was performed for statistical analysis, collective data are representative of ±SEM. $P \leq 0.05^{*}$; $P \leq 0.01^{**}$.

Combination of cetuximab and urelumab enhances antiapoptotic proteins on cetuximab-activated NK cells

We investigated the role of CD137 stimulation in boosting the survival of NK cells in coculture with HNC cells (18, 22). We measured the expression level of the antiapoptotic mitochondrial proteins, Bcl-xL and Bcl-2 on NK cells as an indicator of cell survival. First, we stimulated healthy donor PBMC with cetuximab-coated JHU-029 for 24 hours and purified NK cells. NK cells were then cocultured with urelumab, cetuximab, or a combination of urelumab and cetuximab, in the presence of JHU-029 cells and autologous DC for 36 hours. Intracellular staining of $CD56^{+}$ $Bcl\text{-}xL^{+}$ and $CD56^{+}$ $Bcl\text{-}2^{+}$ NK cells was analyzed by FACS. Urelumab alone enhances Bcl-xL level in both $CD56^{low}$ NK (Fig. 4A) and $CD56^{bright}$ NK (Fig. 4B). Higher levels of Bcl-xL were observed in NK cells activated by cetuximab alone. Interestingly, the combination of urelumab and cetuximab further increased the levels of Bcl-xL on NK cells (Fig. 4A and B). Similarly, urelumab and cetuximab alone enhanced Bcl-2 expression in $CD56^{dim}$, and $CD56^{bright}$ NK cells. Again, the combination of urelumab and cetuximab significantly increased Bcl-2 expression on both NK-cell subsets, and addition of urelumab enhanced viability of NK cells (Fig. 4C–D and Supplementary Fig. S2A and S2B).

Immunophenotypic analysis of urelumab in combination with cetuximab in patients with HNC

To identify modulation of biomarkers in innate and adaptive immune cell types, we performed multicolor flow cytometry in PBMC obtained from patients with advanced stage HNC treated on a phase IB trial of cetuximab plus urelumab (UPCI-14-049,

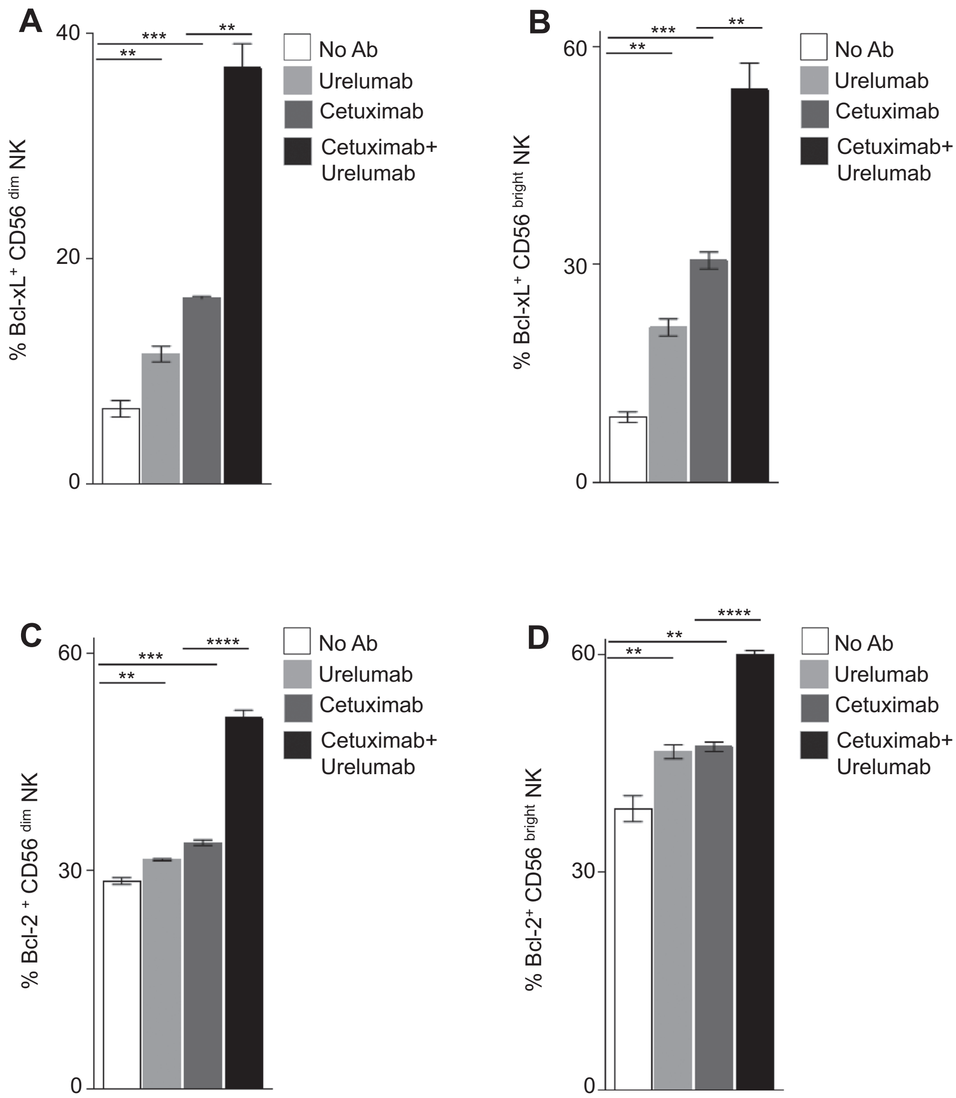

Figure 4.
The combination of cetuximab and urelumab enhances antiapoptotic proteins on cetuximab-activated NK cells. The levels of expression of intracellular antiapoptotic proteins Bcl-xL CD56^{dim} NK (**A**), and CD56^{bright} NK (**B**) were analyzed by intracellular FACS after co-culture with DC:NK: PCI-15B (1:1:1 ratio, 36 hours) in the presence of urelumab (50 μg/mL), cetuximab (10 μg/mL), or cetuximab (10 μg/mL) plus urelumab (50μg/mL). The expression level of intracellular antiapoptotic proteins Bcl-2 in CD56^{low} NK (**C**), and CD56^{bright} NK (**D**), was analyzed by intracellular FACS after coculture with DC:NK:PCI-15B (1:1:1 ratio, 36 hours) in presence of urelumab (50 μg/mL), cetuximab (10 μg/mL), cetuximab (10 μg/mL) plus urelumab (50μg/mL). A two-tailed unpaired or paired *t* test was performed for statistical analysis, collective data are representative of ± SEM. $P \leq 0.01^{**}$, $P \leq 0.001^{***}$, $P \leq 0.0001^{****}$.

NCT02110082; Table 1). PBMC were tested before and 24 hours after cetuximab treatment, and after two cycles of cetuximab plus urelumab treatment (Schema, Table 1). We observed enhancement of CD137 receptor on CD56^{low} and CD56^{bright} NK cells at 24 hours after cetuximab treatment (Fig. 5A and C). Enhancement of cytotoxic marker Granzyme B, proliferation marker Ki67, and natural cytotoxic receptor NKp46 upregulation was apparent after the combination of cetuximab and urelumab in CD56^{low} NK cells (Fig. 5B), whereas NKp46 upregulation is seen in CD56^{bright} NK cells (Fig. 5D); however, no changes in the expression level of, TNFα, CRTAM, IFNγ, PD-1, CD69, NKG2D, CD107a, and CD16 were observed in CD56^{low} cells, similarly no changes in the expression level of TNFα, CRTAM, IFNγ, PD-1, CD69, NKG2D, CD107a, Gr-B, Ki67, and CD16 were observed in CD56^{bright} NK cells (Supplementary Fig. S3A and S3B). Furthermore, in accordance with our *in vitro* results (Fig. 2), we also observed upregulation of HLA-DR in CD11c^{+} myeloid cells (Fig. 5E), whereas no changes in the expression level of CD80, CD86, PD-L1, CD14, CD11c was observed in CD11c^{+} myeloid immune cells (Supplementary Fig. S3C). Interestingly, we also observed upregulation of perforin, and Ki67 expression level in CD8^{+} T cells, and CD4^{+} T cells (Fig. 5F–G), whereas no changes in the expression of TNFα, IFNγ, CRTAM, TIM-3, PD-1, Gr-B, CD69 were observed in these T cells (Supplementary Fig. S3D and S3E).

Discussion

This is the first study to analyze combined triggering of CD137 after cetuximab-induced activation of NK cells and effects on DC processing and presentation to TA-specific T cells. In this study, we investigated the effect of harnessing CD137 expression on NK, DC, and stimulation to enhance innate and adaptive antitumor immune responses. Upregulated expression of CD107a (tumor-infiltrating NK cells), perforin (peripheral blood NK cells), Granzyme B (peripheral blood NK cells; data not shown), and CD137 (tumor-infiltrating NK cells) was observed in a cetuximab neoadjuvant trial (UPCI-08-013 and NCT01218048), and addition of urelumab to cetuximab treatment in a combinatorial clinical trial (UPCI-14-049 and

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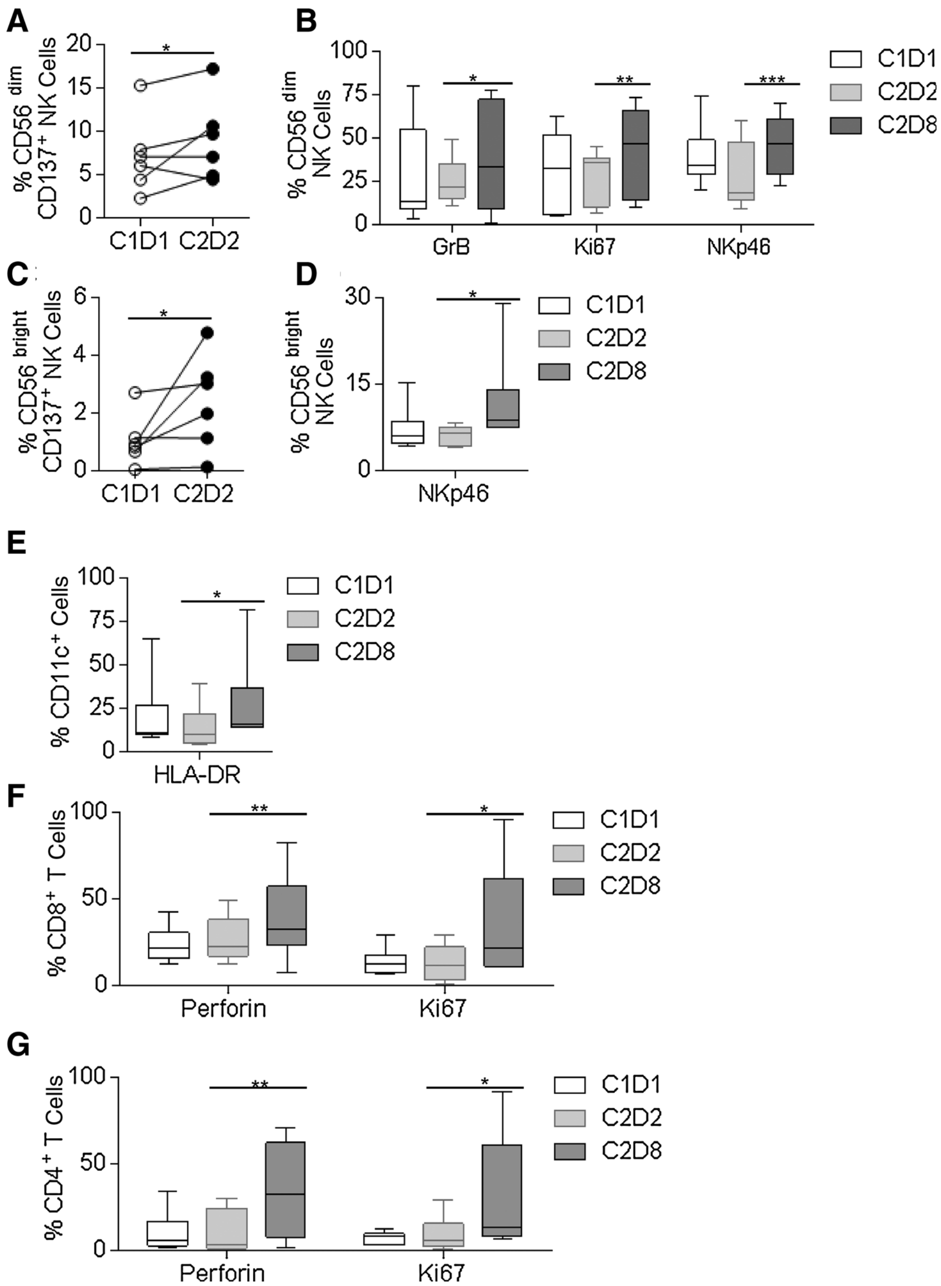

Figure 5.
Immunophenotypic analysis of CD56^{dim}, CD56^{bright}, CD11c^{+} myeloid cells, CD8^{+} T cells, and CD4^{+} T cells in PBMC isolated from UPCI-14-049, an open-label phase Ib clinical trial. The levels of expression of CD137 receptor in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2), were analyzed in CD56^{low} CD3^{-} NK cells (**A**). The levels of expression of intracellular Granzyme B, Ki67, and surface molecule NKp46 were analyzed in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2), cetuximab plus two cycles of urelumab treatment (C2D8) in CD56^{dim} CD3^{-} NK cells (**B**). The levels of expression of CD137 receptor in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2) were analyzed in CD56^{dim} CD3^{-} NK cells (**C**). The levels of expression of surface molecule NKp46 were analyzed in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2), cetuximab plus two cycles of urelumab treatment (C2D8) in CD56^{bright} CD3^{-} NK cells (**D**). The expression level of HLA-DR in CD11c^{+} myeloid cells was analyzed in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2), cetuximab plus two cycles of urelumab treatment (C2D8; **E**). The expression level of perforin, and Ki-67 was analyzed in CD8^{+} T cells (**F**), and CD4^{+} T cells (**G**), in baseline PBMC samples (C1D1), 24 hours after cetuximab treatment (C2D2), cetuximab plus two cycles of urelumab treatment (C2D8). The one-tailed Wilcoxon matched-pair signed rank test was performed for statistical analysis (**A** and **C**). A two-way ANOVA, Tukey multiple comparison test was performed for statistical analysis; collective data are representative of six different donors at different time points, $\pm$ SEM ($n = 6$). $P \leq 0.05^{*}$, $P \leq 0.01^{**}$, $P \leq 0.001^{***}$.

NCT02110082), showed enhancement in NK-cell, DC, and T-cell functionality. We observed that cetuximab-activated NK cells express surface CD137, which correlated with clinical response to neoadjuvant cetuximab. Interestingly, cetuximab-activated CD56^{dim} NK cells upregulate CD137 receptor to a greater extent than CD5^{bright} NK cells. These NK cells could be triggered using agonistic anti-CD137 mAb to potentiate the cytotoxic and helper function of NK cells, thus improving their role in antitumor immunity. We additionally demonstrate that urelumab can uniquely enhance survival of distinct immune cell types by upregulating mitochondrial antiapoptotic proteins Bcl-xL, Bcl-2, modulating the NF-κB pathway (18, 22, 28).

Given the presence of CD137 on cetuximab-activated NK cells (Figs. 1 and 2) and on DC (Fig. 2) and the importance of cetuximab-induced NK:DC cross-talk on the expansion of CTL(2), we tested the impact of CD137 on the function of DC in the tumor microenvironment. The stimulatory CD137 mAb, urelumab alone failed to elevate DC maturation markers or cross-presentation of TA by DC even in the presence of NK cells. Moreover, we see an unexpected decrease in the CD80 expression in urelumab alone treated co-culture (Fig. 2D) Altogether, this inability of the CD137 agonist mAb alone to control HNC had been recently proves in a mouse HPV^{+} HNC model, where stimulatory CD137 neither affected tumor growth nor survival of experimental animals (29, 30). We observed that cetuximab-mediated NK-cell activation in the presence of other lymphocytes, that is, in unfractionated PBMC, are sensitive to a second dose of cetuximab, and the cetuximab plus urelumab combination, to induce DC maturation. Interestingly, cetuximab-mediated NK activation in absence of other immune cells are refractory to a second dose of cetuximab, whereas the combination of cetuximab plus urelumab promotes DC maturation (Fig. 2D–F).

Previously, we showed that cetuximab–HNC cell (JHU-029) complexes, in the presence of NK and DC, generate polyclonal TA (MAGE-3 and EGFR), which are then processed and presented to CTL (2). This is facilitated by DC maturation as identified by upregulation of the maturation/activation molecules, HLA-DR, CD80, CD86, and CD137 (Fig. 2; ref. 6). Interestingly, the addition of urelumab enhanced DC maturation and upregulation of APM components in the presence of cetuximab-activated NK cells. This observation supports the hypothesis of boosting the "vaccinal effect" of cetuximab by cross-linking CD137 mAb to their receptors (Figs. 2–3; ref. 20). Using a novel neoadjuvant trial of single-agent cetuximab and associated paired specimens, we demonstrate that CD137 upregulation is associated with clinical response.

Cetuximab-coated HNC cells induce a higher magnitude of CD137 induction on healthy donor NK cells carrying high-affinity FcγRIIIa VV/VF than low-affinity FcγRIIIa FF (Fig. 1D). This correlates with enhancement of cetuximab-mediated ADCC by urelumab (data not shown). *In vivo*, CD137 induction in tumor-infiltrating NK cells predicted clinical outcomes to neoadjuvant cetuximab therapy and our data suggest better clinical outcomes might be seen in patients if urelumab is added to cetuximab treatment regimens. In circulating NK cells from patients on UPCI-08-013 trial, we did not see induction of CD137 after cetuximab therapy in peripheral blood lymphocytes but only in tumor-infiltrating lymphocytes. We did observe CD137 induction in NK cells after cetuximab therapy in the UPCI-14-049 trial, when blood was available 24 hours after cetuximab treatment. This discrepancy in CD137 induction in two distinct trials could be affected by distinct regimens, where blood collection was performed on different time intervals (30 days vs. 24 hours; ref. 15). Cetuximab-induced tumor-infiltrating NK cells showed CD137 induction in FcγRIIIa VV/VF patients, but not in FcγRIIIa FF patients (Fig. 1D). This suggests that NK-cell recruitment, contact to HNC, and FcγRIIIa affinity are major players in determining overall NK-cell function in response to cetuximab at the tumor site.

HPV (+) HNC are commonly believed to be a separate disease entity and correlates with better prognosis than HPV (−) HNC. Although *in vivo* both HPV(+) and HPV(−) tumors display higher expression of CD137 on tumor-infiltrating NK cells compared with peripheral NK cells after cetuximab, we notice a higher magnitude of CD137 induction on NK cells infiltrating HPV(+) HNC. Increased susceptibility of HPV (+) HNC to NK cells could be attributed to the previously established antiviral functionality of NK cells (29), which may be differentially boosted by cetuximab in HPV (+) tumors. Thus, HPV (+) HNC may result in better clinical outcomes than HPV (−) HNC when treated with a combination of cetuximab and urelumab.

In the phase Ib, open-label, urelumab in combination with cetuximab trial, we observed enhancement in cytotoxic and proliferation markers in NK cells and HLA-DR upregulation in myeloid cells. Similarly, we show upregulation of proliferation marker Ki67 and perforin expression in CD8, CD4 T cells. Although we see enhancement in immune activity with urelumab treatment after cetuximab, the clinical outcome for the sequential combination of urelumab, and cetuximab should be tested in a bigger cohort.

In agreement with several reports (17, 31–34), our findings support the notion of strengthening antitumor immunity with urelumab, albeit in the presence of NK cells already activated by cetuximab in the tumor microenvironment. Taken together, these results suggest that CD137 may present a biomarker of immune and clinical response to cetuximab treatment and provide a novel mechanism of enhancement of cetuximab.

Disclosure of Potential Conflicts of Interest

R.L. Ferris is a consultant/advisory board member for AZ/Medimmune, Bristol-Myers Squibb, Celgene, and Merck, and reports receiving commercial research grants from AZ/Medimmune, Bristol-Myers Squibb and VentiRx. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R.M. Srivastava, R.L. Ferris
Development of methodology: R.M. Srivastava, S. Trivedi, R.L. Ferris
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.M. Srivastava, F. Concha-Benavente, R.L. Ferris
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.M. Srivastava, F. Concha-Benavente, R.L. Ferris
Writing, review, and/or revision of the manuscript: R.M. Srivastava, S. Trivedi, F. Concha-Benavente, S. Ferrone, R.L. Ferris
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.M. Srivastava, S.P. Gibson, C. Reeder, R.L. Ferris
Study supervision: R.L. Ferris
Other (provided reagents): S. Ferrone

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