

## Cetuximab-Activated Natural Killer and Dendritic Cells Collaborate to Trigger Tumor Antigen-Specific T-cell Immunity in Head and Neck Cancer Patients

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### Abstract

**Purpose:** Tumor antigen-specific monoclonal antibodies (mAb) block oncogenic signaling and induce Fcγ receptor (FcγR)-mediated cytotoxicity. However, the role of CD8<sup>+</sup> CTL and FcγR in initiating innate and adaptive immune responses in mAb-treated human patients with cancer is still emerging.

**Experimental Design:** FcγRIIIa codon 158 polymorphism was correlated with survival in 107 cetuximab-treated patients with head and neck cancer (HNC). Flow cytometry was carried out to quantify EGF receptor (EGFR)-specific T cells in cetuximab-treated patients with HNC. The effect of cetuximab on natural killer (NK) cell, dendritic cell (DC), and T-cell activation was measured using IFN-γ release assays and flow cytometry.

**Results:** FcγRIIIa polymorphism did not predict clinical outcome in cetuximab-treated patients with HNC; however, elevated circulating EGFR<sub>853-861</sub>-specific CD8<sup>+</sup> T cells were found in cetuximab-treated patients with HNC ( $P < 0.005$ ). Cetuximab promoted EGFR-specific cellular immunity through the interaction of EGFR<sup>+</sup> tumor cells and FcγRIIIa on NK cells but not on the polymorphism *per se*. Cetuximab-activated NK cells induced IFN-γ-dependent expression of DC maturation markers, antigen processing machinery components such as TAP-1/2 and T-helper cell (T<sub>H</sub>1) chemokines through NKG2D/MICA binding. Cetuximab initiated adaptive immune responses via NK cell-induced DC maturation, which enhanced cross-presentation to CTL specific for EGFR as well as another tumor antigen, MAGE-3.

**Conclusion:** Cetuximab-activated NK cells promote DC maturation and CD8<sup>+</sup> T-cell priming, leading to tumor antigen spreading and T<sub>H</sub>1 cytokine release through "NK-DC cross-talk." FcγRIIIa polymorphism did not predict clinical response to cetuximab but was necessary for NK-DC interaction and mAb-induced cross-presentation. EGFR-specific T cells in cetuximab-treated patients with HNC may contribute to clinical response. *Clin Cancer Res*; 19(7); 1858-72. ©2013 AACR.

### Introduction

Tumor antigen-specific monoclonal antibodies (mAb) are clinically effective in a variety of malignancies. However, these results are only seen in a subset (~20%) of patients (1). The large majority of treated patients, who are not likely to benefit from this therapy, emphasizes the need to char-

acterize the mechanisms underlying the activity of tumor antigen-specific mAb and to optimize the selection of patients to be treated. In the present study, we have used cetuximab, an EGF receptor (EGFR)-specific, chimeric immunoglobulin G (IgG)1 isotype mAb that is U.S. Food and Drug Administration (FDA)-approved for colorectal carcinoma (CRC) and head and neck cancer (HNC), as a model. Despite overexpression of EGFR in 80% to 90% of HNC, cetuximab is effective in only in 10% to 20% of patients (2, 3).

A growing body of experimental and clinical evidence suggests that mAb-induced antitumor immunity underlie treated patients' clinical responses and may play a role in the differential clinical responses. Specifically, the statistically significant correlations of mAb-binding Fcγ receptor (FcγR) polymorphisms with clinical outcome in patients treated with rituximab (4, 5) trastuzumab (6, 7), and cetuximab (8, 9) argue in favor of a role of immune cell activation in the clinical responses, which have been described. Recently,

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### Translational Relevance

The anti-EGF receptor (EGFR) monoclonal antibody (mAb) cetuximab may act through blocking oncogenic signals and by inducing Fc $\gamma$  receptor (Fc $\gamma$ R)-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). We show that polymorphism of Fc $\gamma$ RIIIa [expressed by natural killer (NK) cells] does not correlate with clinical outcome of cetuximab-treated patients with head and neck cancer (HNC). Interestingly, however, cetuximab induces NK cell–dendritic cell (DC) cross-talk, which promotes DC maturation and CD8<sup>+</sup> T-cell priming, leading to tumor antigen spreading and T-helper cell (T<sub>H</sub>1) cytokine release. The identification of EGFR-specific T cells in cetuximab-treated patients with HNC now permits a biomarker of response to be correlated with outcome in prospectively collected specimens. The implications of this finding include potential combinatorial vaccines with EGFR-specific mAbs or in combination with immune checkpoint inhibitors to provide sustained CD8<sup>+</sup> T activity after their induction by cetuximab-activated NK–DC priming.

trastuzumab emtansine (T-DM1) an antibody–drug conjugate showed significantly prolonged progression-free and overall survival in patients with breast cancer (10), and such a strategy may be enhanced by immune cell activation. However, the nonabsolute nature of the correlations in these malignancies (8, 9), the strong influence of k-ras mutation (8) on clinical response to cetuximab in CRC, and conflicting information about the beneficial effect of Fc $\gamma$ RIIIa genotype in patients with CRC (8, 9), raise the possibility of additional immunologic mechanisms in the clinical benefit from mAb-based immunotherapy. This possibility is supported by the growing evidence in animal model systems (11) and in clinical settings (12) that tumor antigen–specific mAb may elicit cellular immunity.

Therefore, in this study, we have investigated whether the Fc $\gamma$ RIIIa polymorphism on natural killer (NK) cells correlates with the antitumor activity of cetuximab using human HNC cells and with the clinical course of the disease in patients with HNC. We have selected these patients for our investigations, as in recent studies, we and others have shown in *in vitro* experiments a significant correlation of Fc $\gamma$ RIIIa polymorphism with the antitumor activity of cetuximab (13). Furthermore, we have tested whether the interaction of cetuximab with Fc $\gamma$ RIIIa on NK cells was required to trigger dendritic cell (DC) maturation and tumor antigen–specific cellular immune responses in patients with HNC. We show for the first time that cetuximab-activated NK cells trigger cross-talk and maturation of DC in an Fc $\gamma$ R and NK group 2, member D (NKG2D)-dependent manner, and this results in tumor antigen–specific priming of CTL in cetuximab-treated patients with HNC. Finally, we have analyzed the mechanism(s) under-

lying the tumor antigen–specific immune response elicited by cetuximab and its potential clinical relevance.

### Materials and Methods

#### Tumor cell lines

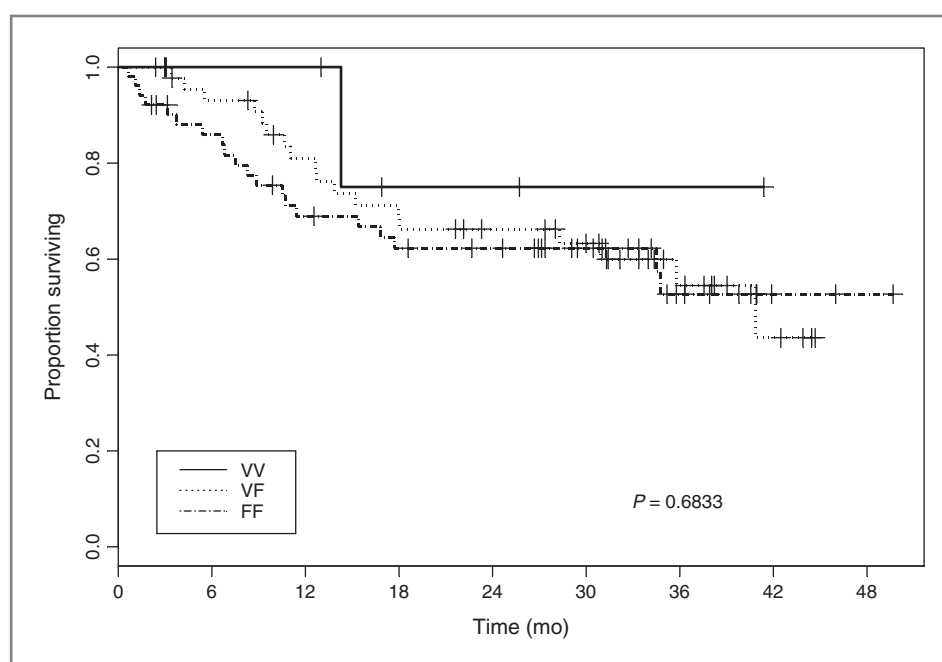
The HNC cell lines HLA-A2<sup>−</sup>EGFR<sup>+</sup> PCI-15B, HLA-A2<sup>−</sup>EGFR<sup>+</sup>, and MAGE-3<sup>+</sup>-JHU-029 (14–16), the breast cancer cell line MCF-7, and the lymphoid T2 cell line were grown in Iscove's modified Dulbecco's medium (IMDM; Sigma) supplemented with 10% FBS (Cellgro), 2% l-glutamine, and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO<sub>2</sub>, 95% humidity. Adherent tumor cells were detached by warm Trypsin–EDTA (0.25%) solution (Invitrogen).

#### Patients and demographics

The cohort of 107 cetuximab-treated patients with stage III/IV HNC described in Fig. 1 combined 60 patients enrolled on 2 prospective, cetuximab containing clinical trial regimens, UPCI-05-003 and UPCI-05-005 and 47 additional patients treated with cetuximab off protocol, as described in Table 1. The majority of these patients were treated with cetuximab plus cisplatin/paclitaxel/radiotherapy (UPCI-05-003; ref. 17) or cetuximab plus pemetrexed/radiotherapy (UPCI-05-005; ref. 18). Both trial cohorts were single arm phase II trials for locoregionally advanced, previously untreated disease. Patients were assigned to either trial by the treating physician at the time. The remainder of the patients was treated off-trial with cetuximab alone or in conjunction with palliative radiotherapy. EGFR tetramer measurements were conducted on protocol patients who were receiving single-agent cetuximab during the 6-month cetuximab maintenance phase of UPCI-05-003 (Table 1) or other newly diagnosed patients with HNC with stage III–IV disease while receiving cetuximab alone as primary treatment on a newly initiated, prospective phase II trial of single-agent cetuximab (UPCI-08-013). The comparison (cetuximab-naïve) HNC cohorts were gender and age-matched, previously cetuximab-untreated patients with HNC. No patients were excluded as a result of prior treatments or performance status. Blood from cetuximab-naïve patients with HNC was drawn within the same period after completing therapy without cetuximab.

#### Antibodies and cytokines

The EGFR-specific chimeric IgG1 mAb cetuximab (Erbix; BMS Imclone) and the EGFR-specific human IgG2 mAb panitumumab (Vectibix; Amgen) were purchased from the University of Pittsburgh Hillman Cancer Center Pharmacy (Pittsburgh, PA). The nonspecific control, human isotype IgG1 was purchased from Axxora LLC. Fluorescein isothiocyanate (FITC) or PE-Cy7-anti-CD11c mAb (R&D Systems), anti-CD80 mAb, anti-CD86 mAb, anti-CD83 mAb, anti-HLA-DR mAb, anti-CCR7 mAb (BD Biosciences Pharmingen) were purchased. Neutralizing anti-Fc Fc $\gamma$ RIIIa antibody and phycoerythrin (PE)- and FITC-conjugated IgG isotypes for flow cytometry were purchased from BD Biosciences. FITC-goat anti-human Fc-specific IgG and FITC-



**Figure 1.** Kaplan-Meier estimates of DSS in cetuximab-treated patients with HNC. Lack of correlation between Fc $\gamma$ RIIIa polymorphisms (based on VV, VF, and FF genotype) and survival of cetuximab-treated patients with HNC. Genomic DNA of patients with HNC treated with cetuximab at the University of Pittsburgh Cancer Institute was tested for Fc $\gamma$ RIIIa (at codon 158) polymorphisms. Patients with HNC ( $n = 107$ ) were treated in 2 prospective cetuximab-based clinical trial regimens or with cetuximab + radiotherapy, as described in Materials and Methods. DSS was measured and a Kaplan-Meier plot was constructed to determine whether the tested Fc $\gamma$ R polymorphisms were correlated with clinical outcome. No differences were observed in patients' DSS based on Fc $\gamma$ RIIIa genotype ( $P = 0.683$ ).

goat anti-mouse IgG were purchased from Invitrogen. The antigen processing machinery (APM) components TAP-1-specific mAb NOB1 and TAP-2-specific mAb NOB2 were developed and characterized as described (19). Anti-CXCL10 antibody, recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), rec. human IFN- $\alpha$ , rec. human interleukin (IL)-1 $\beta$ , rec. human IL-2, rec. human IL-4, rec. human IL-6, rec. human IL-7, PGE2, poly I: C, and TNF- $\alpha$  were purchased from R&D Systems Inc.. IFN- $\gamma$  was purchased from InterMune.

#### Lymphocyte isolation from blood and PBMC

After approval by our Institutional Review Board [University of Pittsburgh Cancer Institute (UPCI; Pittsburgh, PA) protocol 99-069], informed consent was obtained from each subject. Blood from healthy donors (Western PA blood bank) or patients with HNC treated with cetuximab during or within 1 month of treatment. Lymphocytes were purified by Ficoll-Paque PLUS centrifugation (Amersham Biosciences) and stored frozen. DC were generated as described previously (19). NK cells and CD8<sup>+</sup> T cells were purified using EasySep kits (Stem cell technologies) and purity was more than 95% Fc $\gamma$ RIIIa<sup>+</sup>, CD56<sup>+</sup>, and CD3<sup>-</sup> (13).

Peripheral blood mononuclear cell (PBMC) were genotyped at Fc $\gamma$ RIIIa (codon 158) using quantitative PCR (Applied Biosystems). Genomic DNA was extracted using the DNeasy Kit (Qiagen) following the manufacturer's protocol. Genomic DNA (5–50 ng) was added to a 25- $\mu$ L reaction using 2 $\times$  TaqMan master mix (Applied Biosystems). Plates were analyzed using an ABI prism 7700 (13).

#### In vitro stimulation of EGFR-specific CD8<sup>+</sup> T cells

Mature DC were incubated with autologous negatively isolated CD8<sup>+</sup> T cells for 48 hours at 37°C with rhIL-2 (20 U/mL) and rhIL-7 (5 ng/mL). On day 7 and weekly

thereafter, lymphocytes were restimulated with autologous irradiated DC pulsed with peptide in culture medium supplemented with IL-2 (20 U/mL) and IL-7 (5 ng/mL). The stimulated CD8<sup>+</sup> T cells were analyzed for tumor antigen specificity in IFN- $\gamma$  ELISPOT at day 21 and then every 7 days thereafter. We have previously published a novel wild-type EGFR<sub>853–861</sub>-specific tetramer that recognizes EGFR<sub>853–861</sub>-specific CTL and also showed that this specific tetramer recognizes EGFR<sub>853–861</sub>-specific CTL clone that was generated by pulsing DC with EGFR<sub>853–861</sub> peptide *in vitro* (14).

#### CTL lines and ELISPOT assay

IFN- $\gamma$  ELISPOT assays were conducted as described previously (14). The MAGE-3<sub>271–279</sub> (FLWGPRALV) peptide and wild-type EGFR<sub>853–861</sub> peptide (ITDFGLAKL) were produced by the peptide synthesis facility at the University of Pittsburgh (Pittsburgh, PA), using F-moc technology (19, 20). The HLA-A2-MAGE-3<sub>271–279</sub> tetramer was synthesized by the NIH Tetramer Facility (Emory University, Atlanta, GA). CTL lines were sorted using PE-labeled HLA-A2-EGFR<sub>853–861</sub> peptide-loaded tetramers (14) and restimulated every 7 to 10 days. For ELISPOT assays, CD8<sup>+</sup> T cells were seeded in triplicate ( $5 \times 10^4$ ) for bulk CD8<sup>+</sup> T cells and  $1 \times 10^3$  for CD8<sup>+</sup> T-cell clones) in multiscreen HTS plates. Synthetic peptides were then added to ELISPOT assays after APCs were seeded. After 18 hours, spots were enumerated using computer-assisted image analysis software.

#### Flow cytometry

Lymphocytes were prepared for flow cytometry by washing with PBS (Sigma-Aldrich) and fluorescence-activated cell sorting (FACS0 buffer (2% FBS in PBS). For flow-cytometric analysis using HLA-A2-peptide tetramers, PE-labeled HLA-A2-EGFR<sub>853–861</sub> tetramers were obtained from the Tetramer

**Table 1.** Demographics of FcγRIIIa-genotyped cohort

| Demographics of cetuximab-treated, FcγRIIIa-genotyped cohort |                 |                 |    |             |       |         |
|--|-----------------|-----------------|----|-------------|-------|---------|
| Regimen  | No. of patients | Tumor site      |    | Mean age, y | Males | Females |
| UPCI 05-003 <sup>a</sup>                                     | 36              | OC              | 3  | 52.9        | 32    | 4       |
|  |                 | OP              | 23 |             |       |         |
|  |                 | L               | 4  |             |       |         |
|  |                 | HP              | 3  |             |       |         |
|  |                 | Other           | 1  |             |       |         |
|  |                 | Unknown primary | 2  |             |       |         |
| UPCI 05-005 <sup>b</sup>                                     | 24              | OC              | 0  | 58.2        | 20    | 4       |
|  |                 | OP              | 9  |             |       |         |
|  |                 | L               | 5  |             |       |         |
|  |                 | HP              | 4  |             |       |         |
|  |                 | Other           | 4  |             |       |         |
|  |                 | Unknown primary | 2  |             |       |         |
| Cetuximab  | 11              | OC              | 4  | 65.4        | 10    | 1       |
|  |                 | OP              | 1  |             |       |         |
|  |                 | L               | 1  |             |       |         |
|  |                 | HP              | 0  |             |       |         |
|  |                 | Other           | 5  |             |       |         |
|  |                 | Unknown primary | 0  |             |       |         |
| Cetuximab + radiotherapy                                     | 36              | OC              | 10 | 62.5        | 30    | 6       |
|  |                 | OP              | 12 |             |       |         |
|  |                 | L               | 3  |             |       |         |
|  |                 | HP              | 2  |             |       |         |
|  |                 | Other           | 8  |             |       |         |
|  |                 | Unknown primary | 1  |             |       |         |

Abbreviations: OC, oral cavity; OP, oropharynx; L, larynx; HP, hypopharynx.

<sup>a</sup>Cetuximab 250 mg/m<sup>2</sup> days weekly (after an initial dose of 400 mg/m<sup>2</sup>), docetaxel 75 mg/m<sup>2</sup> day 1, cisplatin 75 mg/m<sup>2</sup> day 1, repeated every 21 days × 3 cycles, then radiotherapy to 70 Gy (2 Gy/d) with concurrent cetuximab 250 mg/m<sup>2</sup> weekly + cisplatin 30 mg/m<sup>2</sup>, then maintenance cetuximab for 6 months. Blood was drawn during single-agent cetuximab maintenance.

<sup>b</sup>Cetuximab 250 mg/m<sup>2</sup> (after an initial dose of 400 mg/m<sup>2</sup>) weekly + pemetrexed on days 1, 22, and 43 with concurrent radiotherapy (2 Gy/d).

Facility of the NIH (Atlanta, GA). Cells were analyzed on cyan flow cytometer (Beckman Coulter) using Summit v4.3 software. Intracellular IFN-γ staining of CD56<sup>+</sup> NK cells was conducted by using cytofix/cytoperm fixation/permeabilization kit (BD Biosciences). Briefly, cells were treated with BD GolgiSTOP reagent and stained with FITC-anti-CD56 antibody. After 4 hours, cells were washed, fixed, permeabilized, and stained with FITC-anti-IFN-γ antibody.

#### Cytokine and chemokine analysis by Luminex

A standard calibration curve was generated by serial dilutions of recombinant cytokine. Cytokine concentrations in culture supernatants were determined by a multiplexed ELISA (Luminex) at the UPCI facility. Hu30 Plex kit (Invitrogen, catalog number LHC 6003) detected IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MCP-1, MIP-1β, CXCL-10, MIG, Eotaxin, RANTES, VEGF, granulocyte colony-stimulating factor (G-CSF), EGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF).

#### T-cell chemotaxis assay

Cell migration assays was conducted in 5.0-μm pore size 96-well polycarbonate filtration plate (Millipore). Briefly, the lower chambers were filled with cell-free culture supernatant collected from various experimental conditions. T cells ( $5 \times 10^4/100 \mu\text{l}$  of fresh media) were plated in the upper well. Following 4-hour incubation at 37°C, the upper wells containing filtration unit were gently removed and the cells in bottom chamber were counted by hemocytometer. After 4 hours incubation and counting, cell numbers were plotted as the percentage migrated cells.

#### Statistical and patient follow-up analysis

Disease-specific survival (DSS) of patients with HNC was defined as the time elapsed from the first treatment with cetuximab until death from HNC. Patients were censored if they were alive at last follow-up or had died but were cancer-free at the time of death. FcγR genotype subclasses were tested for association with disease-free survival with the log-rank test. Other factors were tested for association with FcγR

subclasses including cancer type (primary vs. recurrent), disease site and whether the patient was treated on protocol. These results were used to insure the conclusions about the influence of FcγR upon disease-specific mortality were not confounded by other potentially influential covariates. Two-tailed unpaired *t* test was conducted for statistical analysis to compare the significant difference between 2 groups and  $P < 0.05$  was considered significant. T-cell reactivity as measured by the ELISPOT assay was considered positive if the number of spots in test wells was significantly higher than that in background wells when using a one-tailed permutation test for  $\alpha \leq 0.05$ .

## Results

### Survival of cetuximab-treated HNC patients does not correlate with the FcγRIIIa polymorphism

We have previously shown that NK cells expressing an FcγRIIIa V–encoding allele (at codon158) are more potent than NK cells expressing an FcγRIIIa F–encoding allele in antibody-dependent cell-mediated cytotoxicity (ADCC) against HNC cells *in vitro* (13). The clinical significance of our previously published *in vitro* data (13, 15) was then investigated in a cohort of 107 consecutive patients with HNC treated with cetuximab at our institution and genotyped for polymorphic FcγRIIIa position 158 VV, VF, or FF. Interestingly, FcγRIIIa genotype was not associated with DSS in this large cohort of cetuximab-treated patients with HNC (Fig. 1 and Table 1). The low frequency of the beneficial VV genotype (5%; ref. 13), in light of the higher observed rate of clinical response (~15%–20%; ref. 21), raised the likelihood that an additional immune mechanism may play a role in the antitumor activity of cetuximab. Because cetuximab can activate NK cells in the presence of EGFR<sup>+</sup> tumor cells, and subsequently NK cells may provide a crucial DC maturation signal, we hypothesized that cetuximab-activated NK cells might facilitate the generation of tumor antigen-specific CTL.

### Enhancement by cetuximab of EGFR-specific CTL in HNC patients

To evaluate whether induction of CD8<sup>+</sup> T cells by cetuximab occurred *in vivo*, we measured the frequency of EGFR<sub>853–861</sub>-specific CTL in the circulation of cetuximab-treated patients with HNC. PBMC were obtained from HLA-A2<sup>+</sup> cetuximab-treated ( $n = 17$ ) and HLA-A2<sup>+</sup> cetuximab-naïve patients with HNC ( $n = 39$ ), and compared with HLA-A2<sup>+</sup> healthy donors ( $n = 24$ ). On the basis of the analysis of CD3<sup>+</sup>CD8<sup>+</sup> T cells stained with an EGFR<sub>853–861</sub>-specific tetramer (14), a significantly higher frequency of EGFR<sub>853–861</sub>-specific T cells was found in cetuximab-treated patients with HNC than in cetuximab-naïve patients with HNC (Fig. 2A). As a control, we observed low background of EGFR<sub>853–861</sub>-specific precursor CTL in healthy HLA-A2<sup>+</sup> donors. These data strongly support that the administration of cetuximab to patients with HNC leads to EGFR<sub>853–861</sub> peptide cross-presentation by DC, triggering expansion of EGFR-specific T cells.

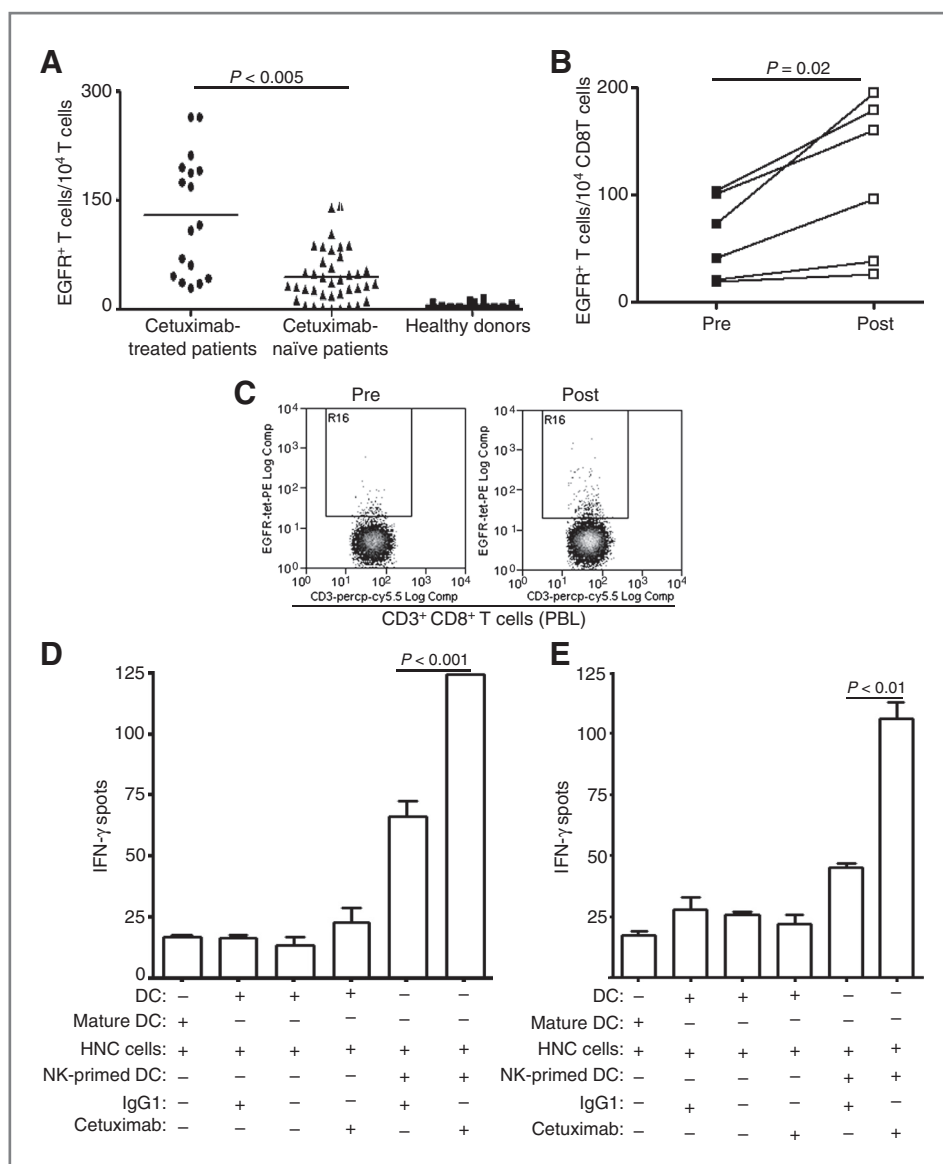
Similarly, higher frequency of EGFR<sub>853–861</sub>-specific T cells was also found in cetuximab-treated patients with HNC who received 4 weeks of single-agent cetuximab treatment (Fig. 2B and C). Taken together, these results indicate that cetuximab alone or in combination with chemotherapy induces expansion of EGFR-specific T cells.

### Enhancement by cetuximab of tumor antigen cross-presentation to cognate CTL in the presence of NK cells

Having observed a higher level of EGFR-specific T cells in cetuximab-treated patients with HNC, we investigated *in vitro* whether cetuximab enhanced EGFR-specific T-cell immunity and whether this effect was modulated by NK cells. We studied the effect of cetuximab with or without NK cells on DC cross-presentation using EGFR<sup>+</sup>, HLA-A2<sup>−</sup> HNC cells to stimulate cognate CTL (19). First, DC alone (HLA-A2<sup>+</sup> donors) were incubated with cetuximab and HLA-A2<sup>−</sup> PCI-15B HNC cells, which did not show enhanced cross-presentation. We furthermore included FcγRIIIa<sup>+</sup> NK cells with DC to evaluate their effect on the cross-presentation capacity of DC. Although addition of NK cells alone showed a 3-fold increase in the cross-presentation by DC, the addition of cetuximab + NK cells resulted in a 5-fold enhancement in the EGFR<sub>853–861</sub>-specific CTL activation in comparison with IgG1 control mAb + NK cells ( $P < 0.001$ ). The cross-presentation by NK cell-stimulated, HLA-A2<sup>+</sup> DC was quantified using IFN-γ ELISPOT assays and HLA-A2<sup>+</sup>, EGFR<sub>853–861</sub>-specific CTL (14). DC incubated with HLA-A2<sup>−</sup> PCI-15B HNC cells and cetuximab enhanced NK cell-dependent cross-presentation to EGFR-specific CTL. A low basal level of cross-presentation was observed with apoptotic PCI-15B HNC cells incubated with an isotype control IgG1 or without NK cells (Fig. 2D) or without HNC cells. A positive control included CTL recognition of EGFR<sub>853–861</sub> peptide loaded DC.

### Enhancement by cetuximab-activated NK cells of non-EGFR (MAGE-3) cross-presentation to MAGE-specific CTL

We selected another HNC cell line JHU-029, which expresses EGFR as well as MAGE-3 (14, 20), to investigate whether cross-presentation of MAGE-3 by DC could also be enhanced by cetuximab-activated NK cells. DC incubated with HLA-A2<sup>−</sup> JHU-029 HNC cells and cetuximab significantly enhanced the cross-presentation to MAGE-3<sub>271–279</sub> peptide-specific CTL, primarily in the presence of NK cells. Addition of NK cells alone showed a 2-fold increase in EGFR cross-presentation by DC. Moreover, addition of cetuximab with NK cells resulted in approximately 4-fold enhancement in the MAGE-3<sub>271–279</sub> peptide-specific CTL activation in comparison with control IgG1 mAb ( $P < 0.01$ ; Fig. 2E). Cytokine-matured DC alone were used as a control in the cross-presentation assay. An isotype control IgG1 failed to facilitate enhancement of cross-presentation of EGFR or MAGE-3 antigens in the presence or absence of NK cells. The importance of FcγRIIIa-activated NK cells to promote the activation of EGFR-specific (Fig. 2D) as well as non-EGFR



**Figure 2.** Enhancement by cetuximab of EGFR<sub>853-861</sub>-specific CTL in the patients with HNC. **A**, higher frequency of EGFR<sub>853-861</sub> peptide-specific tetramer<sup>+</sup> CD8<sup>+</sup> T cells in HLA-A2<sup>+</sup> cetuximab-treated patients with HNC ( $n = 17$ ) compared with HLA-A2<sup>+</sup> cetuximab-naïve patients with HNC ( $n = 39$ ) was found after EGFR<sub>853-861</sub> tetramer staining of CD3<sup>+</sup> CD8<sup>+</sup> T cells by using flow cytometry. The data comparing mean frequencies between cetuximab-treated (●) and -naïve (▲) patient groups are presented. As a negative control, staining of EGFR<sub>853-861</sub> peptide-specific tetramer<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> T cells (from HLA-A2<sup>+</sup> healthy donors ( $n = 24$ )) was conducted. A two-tailed unpaired  $t$  test was conducted for statistical analysis. **B**, expansion of EGFR<sub>853-861</sub>-specific CTL in single-agent cetuximab treated, newly diagnosed patients with HNC. Higher frequency of EGFR<sub>853-861</sub>-specific tetramer<sup>+</sup> CD8<sup>+</sup> T cells in HLA-A2<sup>+</sup>, single-agent cetuximab-treated patients with HNC ( $n = 6$ ) after 4 weekly doses using flow cytometry. Mean frequencies were compared from the same patients before (■) and post (□)-cetuximab therapy. **C**, a representative dot plot is shown for a single-agent cetuximab-treated patient (pre- and post-cetuximab therapy). **D**, enhancement by cetuximab of EGFR cross-presentation to EGFR<sub>853-861</sub>-specific CTL by DC that was preincubated with cetuximab-activated NK cells. EGFR<sub>853-861</sub> peptide-specific CTL were stimulated for 36 hours at 37°C with DC (from a HLA-A 2.1<sup>+</sup> healthy donor) fed with UV-irradiated PCI-15B HNC cells (EGFR<sup>+</sup>, HLA-2.1<sup>-</sup>) coated with IgG1 or cetuximab (each at 10  $\mu$ g/mL), with or without autologous NK cells (DC:NK:PCI-15B at 1:1:1 ratio). Cetuximab (IgG1 isotype) binds Fc $\gamma$ R1IIa expressed by NK cells avidly. EGFR<sub>853-861</sub> peptide-specific CTL activated under the indicated conditions were examined for IFN- $\gamma$  production by ELISPOT assay. Mature DC generated by 36-hour incubation with cytokines IL-1 $\beta$ , IL-6, PGE2, and TNF- $\alpha$  were used as a negative control in the assay. Data are representative of 3 individual experiments. A two-tailed unpaired  $t$  test was conducted for statistical analysis. **E**, enhancement by cetuximab of MAGE-3 cross-presentation to MAGE-3<sub>271-279</sub>-specific CTL by DC that was preincubated with cetuximab-activated NK cells. MAGE-3<sub>271-279</sub> peptide-specific CTL were stimulated for 36 hours at 37°C with DC that were prior (from a HLA-A 2.1<sup>+</sup> healthy donor) fed with UV-irradiated JHU-029 HNC cells (MAGE-3<sup>+</sup>, HLA-2.1<sup>-</sup>) coated with IgG1 or cetuximab (each at 10  $\mu$ g/mL), with or without autologous NK cells (DC:NK:JHU-029 at 1:1:1 ratio). MAGE-3<sub>271-279</sub> peptide-specific CTL activated under the indicated conditions were examined for IFN- $\gamma$  production by ELISPOT assay. Mature DC generated by 36-hour incubation with cytokines IL-1 $\beta$ , IL-6, PGE2, and TNF- $\alpha$  were used as a negative control in the assay. We previously showed that an immunogenic EGFR-encoded CTL could be generated by pulsing DC with EGFR<sub>853-861</sub> peptide that could stimulate cognate CTL *in vitro* (14). Total recombinant EGFR protein was not used in this study as a source of tumor antigen as this would not mimic Fc $\gamma$ R1IIa-mediated effects and NK:DC cross-talk that cetuximab stimulates. Data are representative of 2 independent, repeated experiments. A two-tailed unpaired  $t$  test was conducted for statistical analysis.

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antigen (MAGE-3)-specific CD8<sup>+</sup> T cells, highlights the beneficial effect of enhanced DC maturation and antigen presentation after NK cell activation by cetuximab (Fig. 2D and E).

To model cetuximab-dependent cross-priming of EGFR to CTL, we conducted *in vitro* stimulation for 2 weeks, with DC matured using NK cells activated by PCI-15B HNC cells coated with cetuximab. Consequently, we measured the efficiency of cross-priming of EGFR-specific CTL by tetramer quantification by flow cytometry. DC matured with cetuximab-activated NK cells and PCI-15B HNC cells induced significantly higher levels of EGFR-specific CTL than panitumumab. The latter IgG2 mAb was used as control mAb (Supplementary Fig. S1) due to its reduced FcγRIIIa binding. Taken together, these data strongly support that the administration of cetuximab to patients with HNC leads to EGFR<sub>853–861</sub> peptide cross-presentation by DC, triggering expansion of EGFR-specific T cells.

#### Enhancement of DC maturation by cetuximab in the presence of NK cells

To determine whether enhanced tumor antigen cross-presentation by cetuximab was mediated through enhanced DC maturation (19), we analyzed the expression level of surface maturation markers by DC incubated with cetuximab, PCI-15B HNC cells and NK cells. FACS analysis of DC showed significant upregulation of HLA-DR, costimulatory molecule CD80, CD83, and CD86 (Fig. 3A and Supplementary Fig. S2), and APM component TAP-1 (Fig. 3B). The latter is highly associated with efficient DC cross-presentation (19). Similarly, increased HLA-DR<sup>+</sup>/CD86<sup>+</sup> expression was observed on the cetuximab-activated, NK cell-treated DC (Fig. 3C). Enhanced frequencies of HLA-DR<sup>+</sup>CD83<sup>+</sup> DC or HLA-DR<sup>+</sup>/CD86<sup>+</sup> DC were also observed after culture with cetuximab-activated NK cells (Supplementary Fig. S3A and S3B). Interestingly, enhanced expression of CD137 by DC was also observed after culture with cetuximab-activated NK cells (Fig. 3D and E; ref. 22).

Although differences in lytic activity between NK cells expressing the FcγRIIIa VV genotype versus those expressing the FF genotype were reported (13), both VV and FF expressing, cetuximab-activated NK cells showed a similar effect on the expression of the DC maturation markers, CD80 and TAP-1 (Unpublished Data). Despite the lack of influence of codon 158 polymorphism on NK cell-induced DC maturation, the absolute dependence of FcγRIIIa binding by NK cells was evident, as the EGFR-specific mAb panitumumab, which binds very poorly to FcγRIIIa due to its IgG2 isotype (23), did not mediate downstream DC maturation (Fig. 3B and Supplementary Fig. S2) or NK cell activation (Fig. 4A).

#### Enhancement by cetuximab of IFN-γ secretion by NK cells

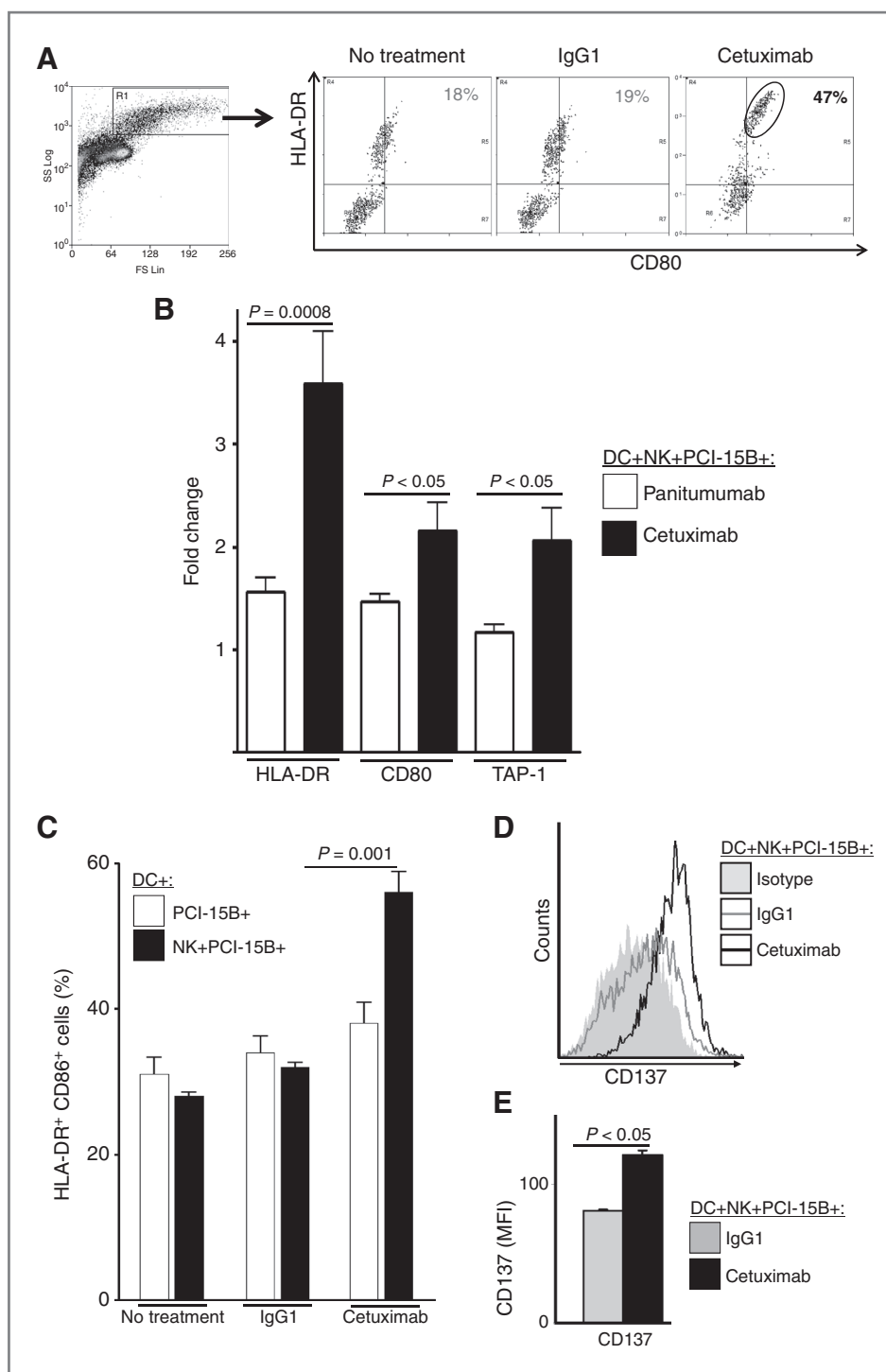
Next, we investigated the cytokine(s) secreted by cetuximab-activated helper NK cells, which could be respon-

sible for this DC maturation. Cetuximab treatment of NK cells in the presence of PCI-15B HNC cells was found to induce a high level of IFN-γ secretion by NK cells (Fig. 4A). Moreover, cisplatin does not negatively affect cetuximab-induced activation and secretion of IFN-γ by NK cells (Fig. 4A). Notably, cetuximab-activated, NK cell-treated DC further stimulated these NK cells in a reciprocal fashion, leading to significantly increased secretion of IFN-γ by cetuximab-activated NK cells when incubated with DC. Control IgG1 did not activate NK cells (Fig. 4B), consistent with the need for mAb binding of both EGFR and FcγRIIIa. Furthermore, the absence of cetuximab in the NK:DC coculture (without HNC cells) or DC:PCI-15B coculture (without NK cells) abrogated the IFN-γ secretion (Supplementary Figs. S4 and S5). We then determined that DC maturation was mediated through IFN-γ, as an IFN-γ-specific neutralizing antibody blocked the induction of HLA-DR (Fig. 4C) on DC by cetuximab-activated NK cells. Blockade of other cytokines, such as IL-12p40/70 showed a slight ability to promote HLA-DR expression on DC, however, no significant differences were found on DC maturation (Fig. 4C). Similarly, a notable effect of the blocking of IFN-γ was observed on the maturation markers HLA-DR, CD80, HLA-DR<sup>+</sup>/CD86<sup>+</sup>, or HLA-DR/CD83<sup>+</sup> on DC by cetuximab-activated NK cells (Supplementary Fig. S3A and S3B). These changes in the expression of DC maturation markers are specific, as no significant increase in their expression level was detected on DC after incubation with autologous NK cells (Supplementary Fig. S5), PCI-15B cells alone or isotype control IgG1 (Supplementary Figs. S2 and S3).

#### Role of FcγRIIIa in the enhancement by cetuximab and NK cells of TAP-1 upregulation in DC

In the experiments described earlier (Fig. 2D), we observed that DC cross-priming of EGFR-specific T cells was enhanced in the presence of cetuximab-activated NK cells. The latter cells had been previously shown to secrete high levels of T<sub>H</sub>1 cytokines and chemokines (13, 24). Because these cytokines strongly upregulate the expression of certain APM components, such as TAP-1 and TAP-2, which are crucial for tumor antigen-derived peptide presentation to cognate CTL (19), we investigated whether these APM components were upregulated in DC incubated with cetuximab-activated NK cells. Consistent with this notion, the enhanced level of TAP-1 was observed in DC that were cocultured with cetuximab-activated NK cells, even if they were physically separated from the NK cells:PCI-15B cells coculture (in the presence of cetuximab) through a Transwell membrane (Fig. 4D). The increase in TAP-1 expression in cetuximab-activated, NK cell-treated DC was found after incubation with PCI-15B HNC cells (EGFR<sup>high</sup>) but not with MCF-7 breast cancer cells. These results indicate that high EGFR expression on tumor cells is necessary for cetuximab-induced NK cell activation to induce DC maturation (Fig. 4E). Also, the upregulation of TAP-1 in DC cocultured with

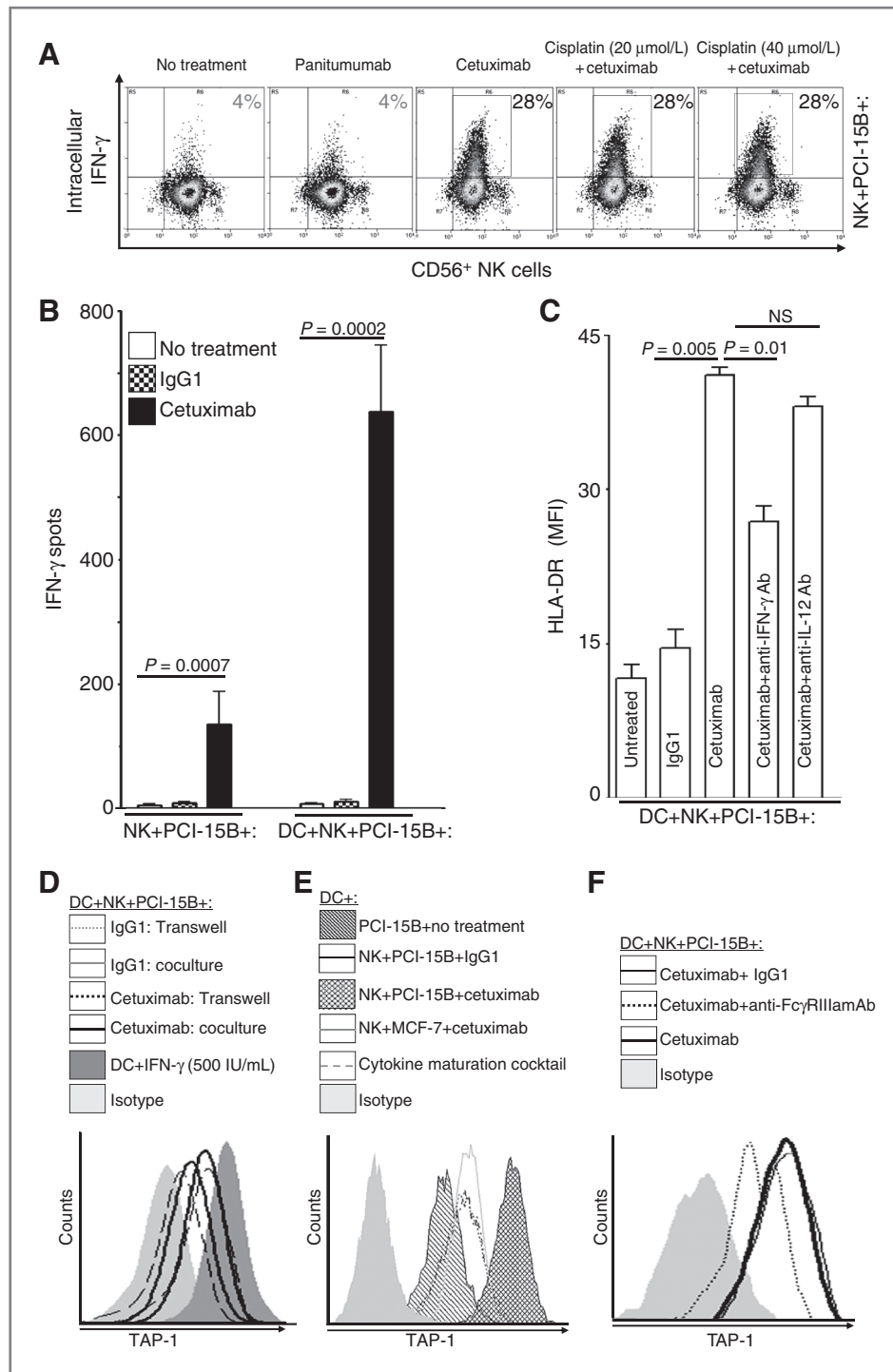
**Figure 3.** Enhancement of DC maturation by cetuximab-activated NK cells. **A**, histogram analysis of the upregulation of maturation markers HLA-DR<sup>+</sup>/CD80<sup>+</sup> on DC cocultured with NK:PCI-15B in the presence of cetuximab. **B**, analysis of upregulation of maturation markers on DC cocultured with NK:PCI-15B in the presence of cetuximab or panitumumab. Expression level of HLA-DR [represented in mean fluorescence intensity (MFI), *n* = 13 donors], CD80 (represented in percentage positive cells, *n* = 8 donors), and TAP-1 (represented in MFI, *n* = 4 donors) on DC cocultured with NK:PCI-15B (1:1:1 ratio) with no treatment or panitumumab or cetuximab (each 10 μg/mL, 48 hours) was measured. The fold change of DC marker expression were related to levels on control DC cocultured with NK:PCI-15B alone are shown. A two-tailed unpaired *t* test was conducted for statistical analysis. **C**, percentages of DC (HLA-DR<sup>+</sup> CD86<sup>+</sup>) that were cocultured with PCI-15B (1:1 ratio) or with NK:PCI-15B (1:1:1 ratio) with no treatment or with IgG1 or cetuximab (each at 10 μg/mL, 48 hours) were measured. Data are representative of 3 experiments from 3 different donors. Enhancement of CD137 expression on DC by cetuximab-activated NK cells. **D** and **E**, histogram analysis of the upregulation of CD137 expression on DC cocultured with NK:PCI-15B in the presence of IgG1 or cetuximab (each 10 μg/mL, 48 hours). **B**, the quantitative bar diagram showing the significant differences in CD137 expression is also shown. A two-tailed unpaired *t* test was conducted for statistical analysis.



cetuximab-activated NK cells was abrogated by the blocking FcγRIIIa-specific mAb 3G8 but was not affected by a control isotype IgG1 (Fig. 4F). Furthermore, incubation of NK cells with the EGFR-specific, IgG2 isotype mAb panitumumab which binds poorly to FcγRIIIa on NK cells (15, 23, 25), failed to upregulate the expression of costimulatory molecules on DC, in accordance

with the lack of effect on EGFR cross-priming (Supplementary Figs. S1–S3). Thus, cetuximab-bound HNC cells activate NK cells through an FcγRIIIa-dependent mechanism, leading to APM component and costimulatory molecule upregulation in DC, manifested by their enhanced ability to mediate tumor antigen cross-presentation.





**Figure 4.** Enhancement by cetuximab of IFN- $\gamma$  secretion by NK cells. **A**, flow-cytometric analysis of intracellular IFN- $\gamma$  in CD56<sup>+</sup> NK cells, which were cocultured with PCI-15B (1:1 ratio) with no treatment or with panitumumab or cetuximab (each 10  $\mu$ g/mL, 12 hours) in the presence or absence of cisplatin (20 and 40  $\mu$ mol/L). Representative figure of 2 independent experiments is shown. **B**, enhancement by cetuximab of IFN- $\gamma$  secretion by NK-DC cross-talk in the presence of PCI-15B. The level of IFN- $\gamma$  in the coculture of NK:PCI-15B (1:1 ratio) or DC:NK:PCI-15B (1:1:1 ratio) with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL) was measured after 12 hours of incubation by ELISPOT assay. Cumulative data of 2 donors are shown. A two-tailed unpaired *t* test was conducted for statistical analysis. **C**, importance of IFN- $\gamma$  released by cetuximab-activated NK cells in the enhancement of DC maturation. Percentages of HLA-DR<sup>+</sup> DC in DC preparations cocultured with NK:PCI-15B (at 1:1:1 ratio) with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL, 48 hours) were measured by flow cytometry. In parallel, anti-IFN- $\gamma$  mAb or anti-IL-12p40/70 mAb (each at 10  $\mu$ g/mL) were added along with cetuximab to the coculture of DC:NK:PCI-15B, and DC maturation markers (HLA-DR<sup>+</sup>) were analyzed. Data are representative of 2 experiments from different donors. Enhancement by cetuximab of TAP-1 upregulation by DC in the

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### Enhancement by cetuximab of NK–DC cross-talk is NKG2D mediated

In light of the reciprocal activation of NK cells by DC (Fig. 4B), we investigated the molecular mechanism underlying the interaction of cetuximab-activated NK cells cultured with mature DC. Specifically blocking mAb for known modulators of NK–DC cross-talk were used to disrupt NK:DC interactions. Interestingly, only a blocking NKG2D-specific mAb abrogated the reciprocal activation of cetuximab-activated NK cells and DC, leading to significantly reduced IFN- $\gamma$  secretion (Fig. 5A). Blockade of other important contributors to NK–DC cross-talk, including NKP30 expressed by NK cells, as well as IL-12 and IL-18, were not responsible for this effect. We observed upregulation of MHC class I chain-related protein A (MICA) on DC but not on HNC cells, suggesting a major role of NKG2D in the reciprocal NK cell activation mediated by cetuximab-induced DC maturation (Fig. 5B).

### Enhancement by cetuximab of T<sub>H</sub>1 cytokines and chemokines in NK cell–DC cocultures

The consequences of NK cell–DC stimulation include functional and phenotypic activation, such as secretion of chemokines and cytokines. We conducted multiplex ELISA (Luminex) analysis to profile the cytokines and chemokines in the supernatants of coculture (DC:PCI-15B or DC:NK:PCI-15B) untreated or treated with IgG1 or cetuximab. Secretion of the cytokines and chemokines, MCP-1, MIP-1 $\beta$ , IL-12p40/70, (CCR5 ligand), CXCL10, and MIG (CXCR3 ligands) was enhanced only in the cetuximab-activated NK cell–treated, DC (Figs. 6A–E). Because cetuximab-activated, NK cell–treated DC supernatants contained higher amounts of chemokines responsible for recruiting and activating CD8<sup>+</sup> T cells, we confirmed the enhanced migratory ability of naïve CD8<sup>+</sup> T cells under the influence of chemotactic factor/s present in supernatant from cetuximab-activated NK cell–treated DC. The induced T-cell migration was completely abrogated by a CXCL10-specific mAb (Fig. 6F).

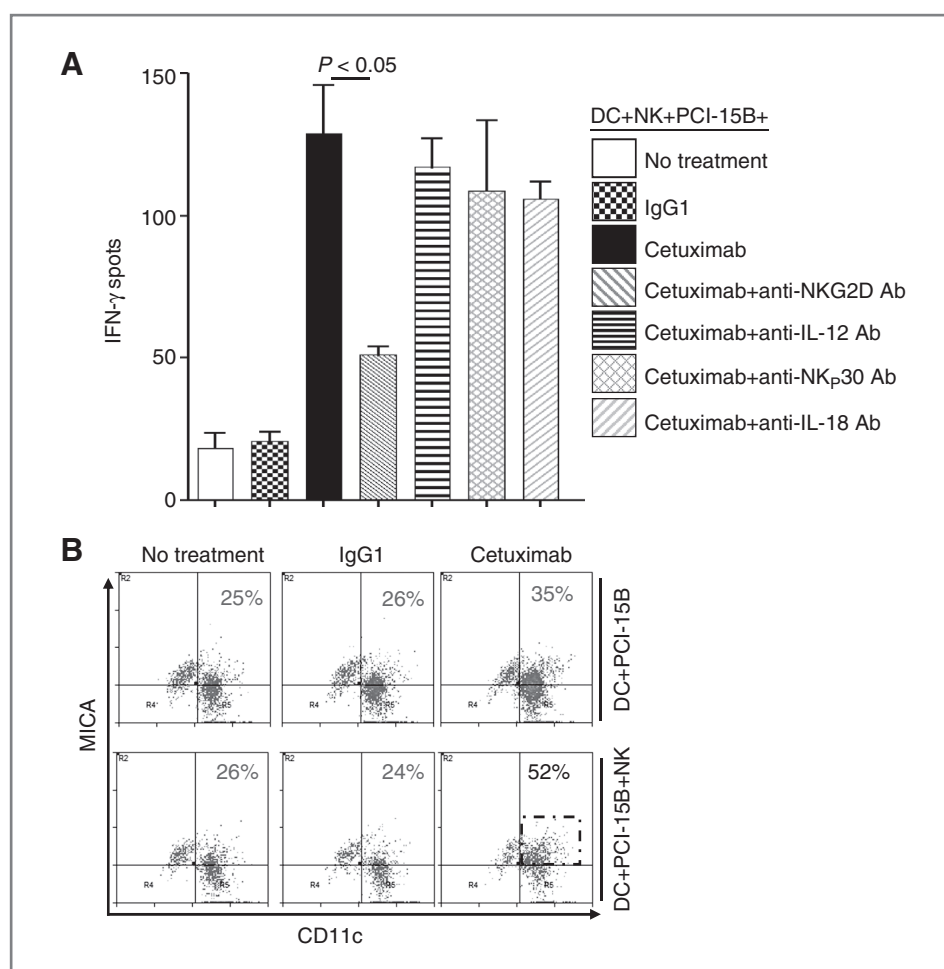
## Discussion

While blockade of proliferative and antiapoptotic pathways in tumor cells plays a major role in the clinical

efficacy of therapeutic mAb, such as rituximab, cetuximab, and trastuzumab, the role of Fc $\gamma$ RIIIa-dependent innate immunity (ADCC) induced by these antitumor mAbs have generated conflicting findings (8, 9, 26–29). In addition, using murine models, the role of adaptive antitumor immune mechanisms in the clinical activity of these mAb has been suggested (30, 31), though data from treated patients with cancer is generally lacking. Our *in vitro* data showed that NK cells significantly enhance the antitumor effects of cetuximab via interaction with Fc $\gamma$ RIIIa (13), which parallel similar results obtained with trastuzumab in breast carcinoma bearing mice (7, 32). Taken together, these results indicated that Fc $\gamma$ RIIIa on NK cells could play a critical role in an immuno-naïve environment. However, this effect becomes less marked in "immunocompetent" patients with cancer, as we did not find a significant correlation between Fc $\gamma$ RIIIa genotype and disease-free survival in patients with HNC treated with cetuximab and radiotherapy and chemotherapy (Fig. 1), as seen in patients with other cancer (26–29). Nevertheless, the data are in agreement with the dominant role of Fc $\gamma$ RIIIa on NK cells, although not absolutely dependent on the index of affinity of cetuximab, but rather to initiate proximity of Fc $\gamma$ R bearing NK cells and DC with EGFR<sup>+</sup> HNC cells and with each other, leading to NK–DC cross-talk mediated through NKG2D–MICA. NK–DC cross-talk is crucial to cetuximab-mediated DC maturation and cross-presentation, which may potentially lead to CTL expansion, and potentially clinical effects, *in vivo* in cetuximab-treated patients with HNC. While the sample size of this retrospective analysis was insufficient to correlate T-cell frequencies or phenotype with clinical outcome, such a study is ongoing as part of a separate, prospectively treated cohort. Nevertheless, higher frequency of EGFR-specific CD8<sup>+</sup> T cells was also found in prospectively treated patients with HNC who received cetuximab alone (without chemotherapy), strongly supporting our conclusions (Fig. 2B and C).

Interestingly, that adaptive immune responses might contribute to therapeutic efficacy, based on higher frequency of EGFR-specific CD8<sup>+</sup> T cells, must be validated prospectively in a larger cohort cetuximab-treated patients (Fig. 2) with sufficient follow up (2–3 years) for

presence of NK cells. D–F, enhancement of DC maturation by soluble factor released from cetuximab-activated NK cells. D, TAP-1 upregulation in DC cocultured with cetuximab-activated NK cell is mediated by a soluble factor. Flow-cytometric analysis of intracellular TAP-1 expression (and TAP-2 expression, data not shown) was conducted for DC incubated with NK:PCI-15B (1:1:1 ratio) with IgG1 or cetuximab (each at 10  $\mu$ g/mL, 48 hours). DC were physically separated from NK:PCI-15B coculture using a Transwell micropore system. TAP-1 expression in DC was enhanced after coculture with NK:PCI-15B with cetuximab in both contact dependent and Transwell culture conditions. Isotype (gray filled histogram), IgG1 Transwell (gray dotted line), IgG1 coculture (gray line), cetuximab Transwell (black dotted line), cetuximab coculture (black line), and IFN- $\gamma$  (black filled histogram). Data are representative of 2 experiments from 2 different donors. E, enhancement by cetuximab of TAP-1 upregulation in DC in the presence of NK cells with EGFR<sup>high</sup> HNC cells (PCI-15B) but not with breast cancer cells (MCF-7). By flow cytometry, intracellular TAP-1 expression (and TAP-2 expression, data not shown) was measured in DC that were incubated with NK:PCI-15B (at 1:1:1 ratio) or NK:MCF-7 (1:1:1 ratio) with IgG1 or cetuximab (each at 10  $\mu$ g/mL, 48 hours). No upregulation of TAP-1 was detected in DC that was incubated with NK:MCF-7 breast cancer cells (1:1:1 ratio) in the presence of cetuximab (each at 10  $\mu$ g/mL, 48 hours). As a control, the level of TAP-1 was measured in DC matured with cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, PGE2 for 48 hours). F, enhancement by cetuximab of TAP-1 upregulation by DC in the presence of NK cells is Fc $\gamma$ RIIIa-dependent. TAP-1 expression was measured in DC incubated with NK:PCI-15B (1:1:1 ratio) in the presence of IgG1 isotype or cetuximab (each at 10  $\mu$ g/mL, 48 hours). A blocking Fc $\gamma$ RIIIa-specific mAb (3G8) or IgG1 isotype control were used to show dependence of the observed TAP-1 upregulation (in DC) on Fc $\gamma$ R expressed by NK cells but not Fc $\gamma$ R expressed by DC. Isotype (filled histogram), cetuximab plus IgG1 (thin line), cetuximab plus anti-Fc $\gamma$ RIIIa-specific mAb (dotted line), and cetuximab (thick line).



**Figure 5.** Enhancement by cetuximab of NK-DC cross-talk is NKG2D mediated. A, levels of IFN- $\gamma$  were measured after coculture of NK:PCI-15B (1:1 ratio) or DC:NK:PCI-15B (1:1:1 ratio) with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL) after 24 hours by ELISPOT assay. Furthermore, the molecular mechanism that may modulate NK-DC cross-talk was assessed by using anti-NKG2D antibody (Ab) or anti-IL-12 antibody or anti-NKp30 antibody or anti-IL-18 antibody along with cetuximab (each at 10  $\mu$ g/mL) in parallel assays. A two-tailed unpaired t test was conducted for statistical analysis. Cumulative data of 2 donors are shown. B, enhancement by cetuximab of NK-DC cross-talk is dependent on MICA upregulation by DC. The level of NKG2D ligand MICA, on CD11c<sup>+</sup> DC in cocultures of NK:PCI-15B (1:1 ratio) or DC:NK:PCI-15B (1:1:1 ratio) with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL, 48 hours) was measured by flow cytometry. A representative histogram is also shown.

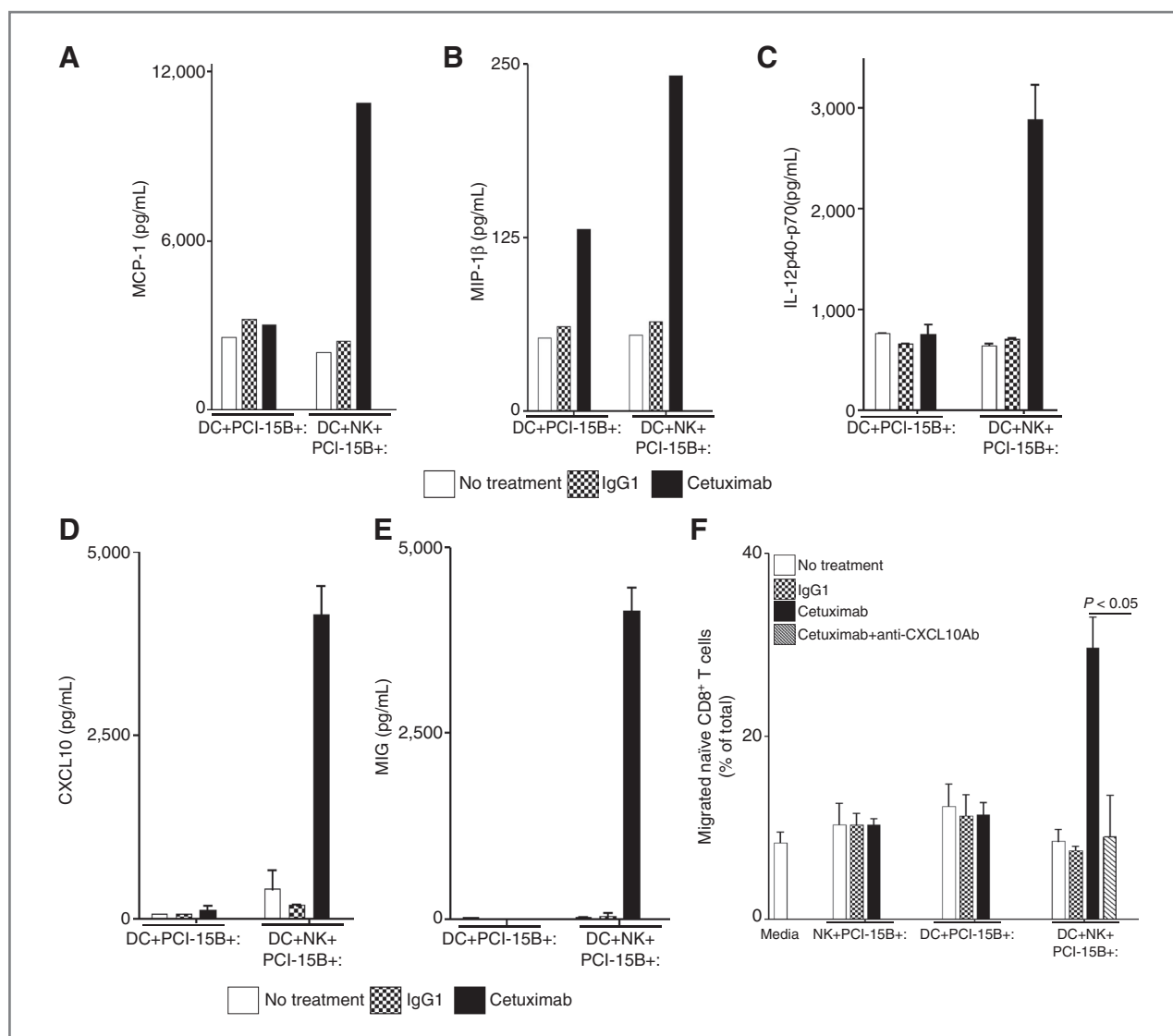
outcome measurements. This extends recent reports of rituximab and anti-HER2/neu inducing long lasting antitumor adaptive immune responses in murine systems, as support the plausible role of adaptive antitumor immune responses triggered by antitumor specific mAb in treated patients (30, 31, 33). The dependence on IFN- $\gamma$  secretion by NK cells supported that NK cells provide helper function in DC cross-presentation, extending our previous finding that an immunogenic EGFR-encoded CTL could be generated by pulsing DC with EGFR<sub>853-861</sub> peptide that could stimulate cognate CTL *in vitro* (14). However, total recombinant EGFR protein was not used in this study as a source of tumor antigen as this would not mimic Fc $\gamma$ RIIIa-mediated effects and NK:DC cross-talk that cetuximab stimulates.

Although Fc $\gamma$ RIIIa polymorphism-dependent survival of patients with cancer were reported in CRC cohorts (8, 9), adaptive immune responses probably contribute in the cetuximab-mediated immune responses and may better predict clinical outcome of immunotherapy, because NK cells also provide helper function in shaping host adaptive immune responses. Moreover, the low frequency of VV patients often "neg-

atively" influences statistical analysis (26, 29). These data clearly support the paradigm that antitumor mAb participate in the long-term control of tumor in patients with HNC, probably accounting for the clinical activity (30, 31, 33-35).

To analyze the importance of Fc $\gamma$ RIIIa, we used the 2 FDA-approved, EGFR-specific mAb cetuximab and panitumumab, which bind the same epitope on EGFR (23, 25) but differ in their isotype and Fc $\gamma$ RIIIa affinity, to show that only cetuximab (IgG1)-induced NK-DC cross-talk and DC cross-presentation ability, whereas panitumumab (IgG2) does not, despite their equal EGFR binding (Fig. 3B and Supplementary Figs. S1 and S2). It is notable that this IgG2 isotype mAb, despite shown potency in EGFR signaling blockade, has failed multiple prospective HNC trials of clinical efficacy, supporting the proposition that cetuximab imparts greater clinical activity due to the IgG1 isotype-induced immunologic effects.

We have recently shown that cetuximab-naïve patients with HNC possess elevated levels of EGFR<sub>853-861</sub>-specific T cells confirming that EGFR expressed on HNC cells induces a specific immune response *in vivo* (36). Although antigen-specific CD4<sup>+</sup> T-cell activation has



**Figure 6.** Enhancement by cetuximab of  $T_H1$  polarizing cytokines in the NK:DC:PCI-15B coculture. A–C, Luminex analysis for the estimation of level of MCP-1, MIP-1 $\beta$ , and IL-12 in the supernatant of DC:PCI-15B (1:1 ratio) or DC:NK:PCI-15B (1:1:1 ratio) coculture with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL for 48 hours). Data are representative of 2 different donors. D and E, enhancement by cetuximab of CXC chemokines in the NK–DC coculture. The levels of CXC chemokines CXCL10 and MIG were determined in the supernatant from coculture of DC:PCI-15B (1:1 ratio) or DC:NK:PCI-15B (1:1:1 ratio) incubated with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL for 48 hours). Values are mean  $\pm$  SEM of 2 independent experiments from separate donors. F, enhancement by cetuximab of the migration of CD8<sup>+</sup> T cells under the influence of CXCL10 in the supernatant of NK:DC:PCI-15B cocultures. Migration of CD8<sup>+</sup> T cells under the influence of migratory factor(s) present in the fresh media or in the supernatant of NK:PCI-15B (at 1:1 ratio) coculture, or in the supernatant of DC:PCI-15B (1:1 ratio) coculture, or in the supernatant of DC:NK:PCI-15B coculture (1:1:1 ratio), with no treatment or with IgG1 or cetuximab (each at 10  $\mu$ g/mL for 48 hours) was quantified. After 4 hours of incubation of CD8<sup>+</sup> T cells with coculture supernatants in the upper chamber of Transwell plate, cells were collected from lower chamber and counted. Each condition was plated in triplicate. In parallel to the cetuximab condition anti-CXCL10 antibody (10  $\mu$ g/mL) was used for the blocking experiments. Data shown are from a representative donor carried out in triplicate. Two-tailed unpaired *t* test was conducted for statistical analysis.

been noted in a high proportion of patients with breast cancer treated with trastuzumab and correlated with clinical responses (7), this is the first demonstration of enhanced tumor antigen-specific CTL in mAb-treated patients (Fig. 2), which are crucial to mediating strong antitumor immunity *in vivo*. This effect provides a general yet novel immune mechanism of enhanced antitumor

activity relevant to clinical response, as well as a potential biomarker to monitor cetuximab effects in mAb-treated patients with cancer. Because the frequency of HLA-A2.1 in patients with HNC in different regions varies (ranging from 30%–50% worldwide), additional HLA-restricted, EGFR-specific T-cell epitopes should be identified to expand application of such a biomarker.

Our results indicate that cetuximab:EGFR<sup>+</sup> tumor cell complexes originating in the presence of NK cells are provided to DC to generate polyclonal tumor antigen (MAGE-3 as well as EGFR) processing and presentation (36–38). In addition, activated-NK cells facilitate DC maturation, as indicated by the concomitant upregulation of the costimulatory molecules CD80, CD86, and CD137 on the DC surface (Fig. 3A–E and Supplementary Figs. S2 and S3). At the same time, cytokines released by NK cells activated through binding of EGFR-cetuximab complexes via FcγRIIIa induce activation markers and TAP-1 (refs. 39–42; Figs. 3B and 4A–F), which we have shown to predict cross-priming capacity of DC (19). Our data show that with the release of cytokines from cetuximab-activated NK cells DC becomes highly responsive to the tumor microenvironment and undergoes maturation along with uptake of dead tumor materials (37). This finding is in strong accordance with the established fact (24, 37) that release of cytokines from cetuximab-activated NK cells may trigger T<sub>H</sub>1 responses against tumor microenvironment. The ability of activated/mature DC to generate or activate EGFR-specific CTL is an attractive opportunity to subvert immunosuppressive environment, which could be easily achieved with antitumor mAb administrations (43). Through this mechanism, therapeutic mAb may enhance antigen cross-presentation by DC to T cells, resulting in augmentation of tumor antigen-specific CTL generation (43–45). If this interpretation is correct, cetuximab administration should be probably combined with vaccines targeting EGFR (14) or other tumor antigen, as well as adjuvant(s) to specifically stimulate cellular immunity.

In addition to their ability to mediate ADCC, cetuximab-activated NK cells, secrete cytokines, such as IFN-γ, MCP-1, MIP-1β that inhibit tumor cell proliferation, enhance antigen presentation, and chemokines such as IP10 and MIG that aid in the chemotaxis of T cells (13, 39). The resulting potent activating bi-directional signaling between NK cells and DC can shape both the innate immune response within inflamed peripheral tissues and the adaptive immune response in secondary lymphoid organs (46) enhancing cross-presentation and priming of T cells, as we observed (Figs. 2 and 3). Through direct cellular interactions and secretion of cytokines/chemokines NK cells function as helper cells (40, 47, 48) and enhance DC cross-presentation and T-cell induction, with the potential to strengthen tumor antigen-specific cellular immune response and to spread this priming to multiple tumor antigen, including private antigens. The effects observed can be blocked using a neutralizing IFN-γ mAb consistent with our previous findings about optimal APM upregulation in mature DC mediating cross-presentation (19). However, the beginning of DC maturation is clearly dependent on secreted IFN-γ from NK cells, whereas IL-12 might contribute for the cross-priming activities and reciprocal NK cell activation (Figs. 4B and C and 5A and B). We have also observed that NKG2D, a potent tumor recognition molecule on NK cells may interplay in shaping the NK-DC cross-

talk and reciprocal activities, subsequently NKG2D can also interact with its ligand expressed on tumor cells and or activated DC (Fig. 5).

However, the generation and functional activation of NK cells and CTL for elimination of tumor cells might be influenced by the impact of concomitant chemotherapy and/or radiotherapy (8, 22), often administered in conjunction with cetuximab (49). We observed that cetuximab-induced IFN-γ secretion by NK cells activation is not negatively influenced by cisplatin (Fig. 4A). Recently, trastuzumab emtansine (T-DM1) an antibody–drug conjugate showed a significantly prolonged progression-free and overall survival in patients with breast cancer (10). The induction of T cells might be more relevant and efficacy even further enhanced, when mAb-conjugated cytotoxics can induce immunogenic cell death. Currently, we are investigating the combination of cetuximab with NK cell-activating molecules and with cytotoxic drugs to further enhance the adaptive immune responses in patients and functional properties of HLA class I or APM components as potential immune escape mechanisms, especially in HNC cells (50). These mechanisms and variables are now being studied in prospective clinical trials using single-agent cetuximab, as well as cetuximab in combination with chemoradiotherapy, as in both situations clinical efficacy is observed.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

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