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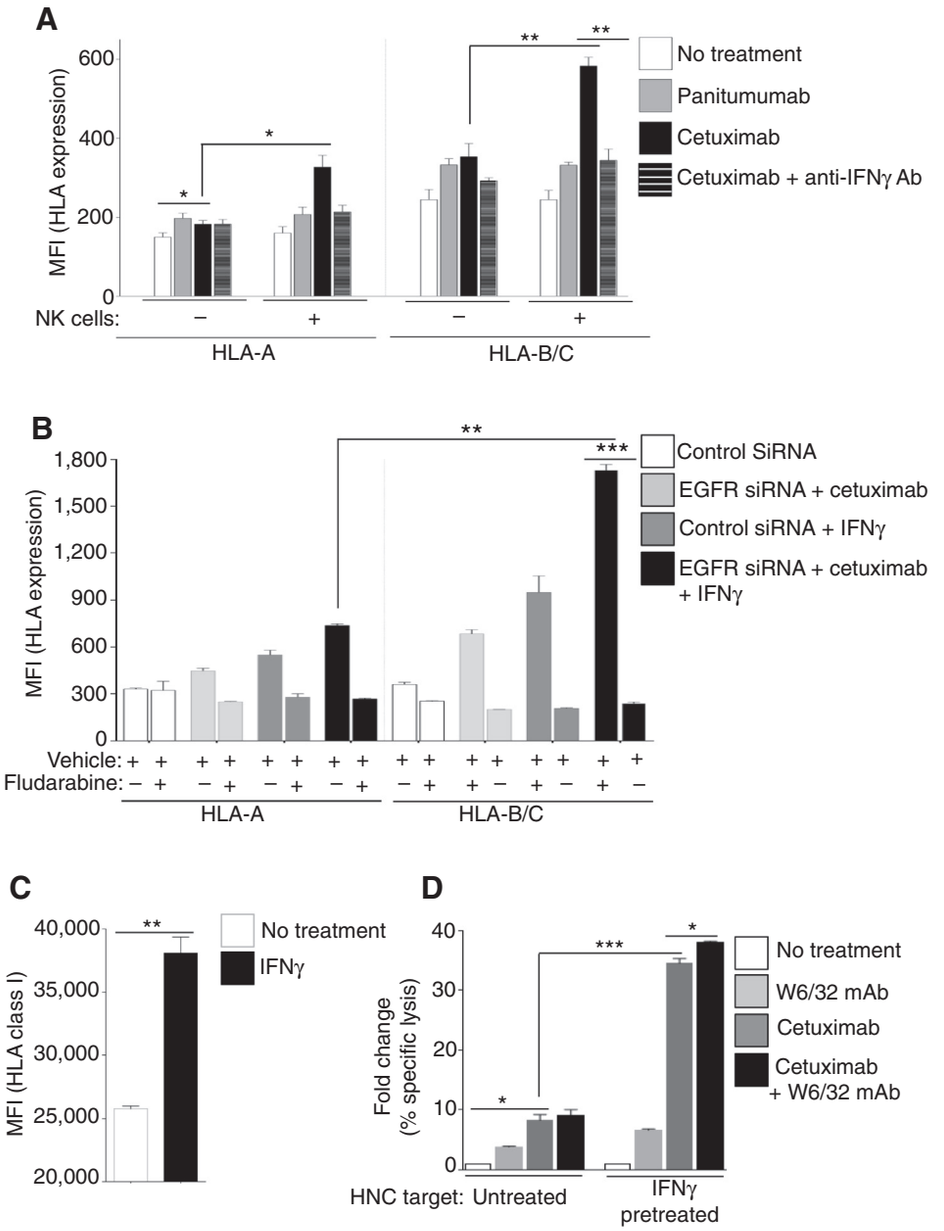


**Figure 4.**
Cetuximab-activated NK cells and IFNγ increase expression of the HLA class I APM pathway. A, JHU-029 HNC cells were cultured alone, or JHU-029 plus NK cells in coculture (1:1 ratio) were left untreated or were treated for 48 hours with panitumumab (IgG2, 10 μg/mL), cetuximab (IgG1, 10 μg/mL). Levels of HLA-A (left) or HLA-B/C (right) were determined by FACS. In parallel, polyclonal anti-IFNγ Ab (10 μg/mL) was added at indicated conditions to determine the effect of IFNγ released from cetuximab-activated NK cells. B, HNC cells were pretreated with fludarabine (20 μmol/L), and after EGFR siRNA or cetuximab treatment (48 hours, 10 μg/mL) levels of surface HLA-A, HLA-B/C were determined by FACS. C, JHU-029 HNC cells were cultured alone or were treated with IFNγ (10 U/mL, 36 hours), and enhanced levels of HLA class I (mAb W6/32) were verified with FACS. D, NK cell cytotoxicity (4-hour $^{51}$Cr release assay, 40:1 E/T ratio) against untreated or IFNγ pretreated HNC targets (C), were independently evaluated in presence of mAb W6/32 (50 μg/mL), cetuximab (10 μg/mL) or combination of mAb W6/32 plus cetuximab. The ratio of NK cell cytotoxicity against untreated HNC targets, and IFNγ-treated HNC targets is shown. Results represent mean ± SEM from three independent experiments; *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$. MFI, mean fluorescence intensity.

HLA-A. In support of a common pathway, the STAT1 inhibitor fludarabine (23) abrogated HLA-A and HLA-B/C upregulation in response to cetuximab, EGFR siRNA, or IFNγ treatment (Fig. 4B). We further evaluated the contribution of IFNγ-induced HLA class I expression to NK-cell–mediated antitumor effects (Fig. 4C). Cetuximab-mediated antibody-dependent cellular cytotoxicity (ADCC) was significantly enhanced against IFNγ-treated HNC targets, and blocking HLA class I with W6/32 mAb (pan-HLA class I mAb) augmented cetuximab-mediated ADCC (Fig. 4D).

### SHP2 inhibition robustly enhances cetuximab-mediated tumor antigen presentation

Next, we evaluated the combined effect of cetuximab and IFNγ on the expression of free HLA-A (HCA-2 mAb) or free HLA-B (HC-10 mAb), surface HLA-A and HLA-B/C, surface pan-HLA class I (HLA-A/B/C), or β2-m. Cetuximab alone increased HLA-A expression by approximately 1.25-fold, in comparison with an approximately 1.45-fold increase for HLA-B/C (Fig. 5A); IFNγ alone increased HLA-A expression by approximately 3.9-fold in comparison with an approximately 6.9-fold increase for HLA-B/C when compared with that of no treatment. The most prominent upregulation of HLA-A and HLA-B/C (~5 fold and ~9.5 fold vs. untreated, $P < 0.0001$) was observed when the combination of IFNγ and cetuximab was used. We also found greater HLA-B versus HLA-A allele transcripts after treatment with cetuximab, with IFNγ alone, or with cetuximab plus IFNγ (Fig. 5B and Supplementary Fig. S3A–S3E).

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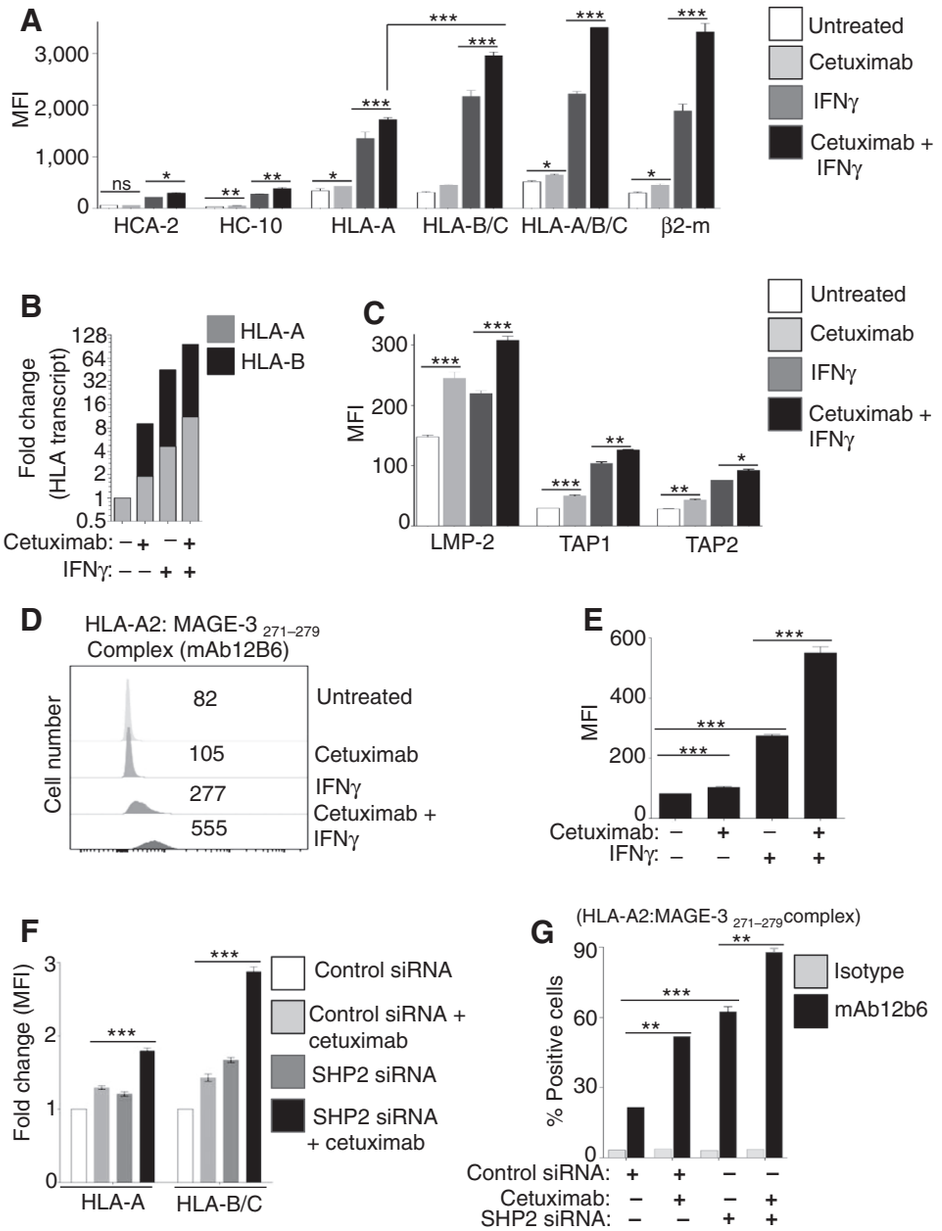


**Figure 5.**
SHP2 inhibition robustly enhances cetuximab-mediated TA presentation. A, JHU-029 cells were treated with cetuximab (10 μg/mL, 72 hours), IFNγ (10 U/mL, 72 hours), cetuximab plus IFNγ (10 μg/mL, 10 U/mL, respectively, 72 hours) and levels of intracellular-free HLA class I A or B, HLA-A, HLA-B/C, HLA-A/B/C, β2-m were determined by FACS. B, under above described conditions the transcript levels of HLA-A and HLA-B were determined by qPCR. C, similarly, in the above described conditions, levels of LMP2, TAP1, and TAP2 were determined by FACS. In HLA-A2$^+$ JHU-022 cells presentation of HLA-A2:MAGE-3$_{271–279}$–peptide complex (probed by mAb 12B6, followed by FITC-(Fab)$^2$ was determined with flow cytometry after treatment with cetuximab (10 μg/mL), IFNγ (10 U/mL), cetuximab plus IFNγ (10 μg/mL, 10 U/mL, respectively, 72 hours). D, representative histogram. E, graphical presentation of MFI values. F, HNC JHU-029 cells were treated with SHP2 siRNA or control siRNA. After 24 hours, cells were treated with cetuximab (10 μg/mL, 48 hours), and levels of HLA-A and HLA-B/C were evaluated by FACS. G, levels of HLA-A2:MAGE-3$_{271–279}$–peptide complex (clone mAb 12b6) presentation were determined by FACS after treatment with control siRNA, control siRNA plus cetuximab (5 μg/mL), SHP2 siRNA, and SHP2 siRNA plus cetuximab (additional 48 hours) in MAGE-3$_{271–279}$ TA-positive HLA-A2$^+$- PCI-13. Results represent mean ± SEM from three independent experiments; *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$). MFI, mean fluorescence intensity.

Because cetuximab treatment enhanced the expression of LMP2, and TAP1/2 (Fig. 5C and Supplementary Fig. S4A–S4B), we investigated whether the enhanced HLA class I APM components enhanced surface presentation of tumor antigens (TA). We used a novel mAb (12b6), recognizing the HLA-A2:MAGE-3$_{271–279}$ complex (Supplementary Fig. S5A–S5B), to quantitatively measure levels of surface HLA–TA complexes. Cetuximab enhanced the HLA-A2:MAGE-3$_{271–279}$ complex ($P < 0.001$; Fig. 5D and E), which was even more robust after IFNγ treatment ($P < 0.0001$). Interestingly, the combination of cetuximab and IFNγ treatment evoked the highest level of HLA-A2:MAGE-3$_{271–279}$–peptide complexes ($P < 0.0001$). Indeed, the combination of SHP2 siRNA and cetuximab treatment strongly enhanced cetuximab-induced HLA-A and HLA-B/C expression, most prominently in the latter alleles (Fig. 5F and Supplementary Fig. S5C). SHP2 depletion in HLA-A2$^+$ HNC cells also enhanced HLA-A2:MAGE-3$_{271–279}$–peptide presentation after cetuximab treatment (Fig. 5G and Supplementary Fig. S5D), whereas no binding was observed in HLA-A2$^-$ or TA$^-$ HNC cells (Supplementary Fig. S5E–S5F).

### Cetuximab neoadjuvant therapy enhances expression of HLA class I in HNC patients

In a novel phase II prospective clinical trial, tumors from HNC patients were biopsied before and after 4 weeks of single-agent neoadjuvant cetuximab therapy. HLA class I expression was measured semiquantitatively using IHC and digital image analysis, and correlated with clinical response by paired pre/post CT scans to identify clinical "responders." After cetuximab therapy,

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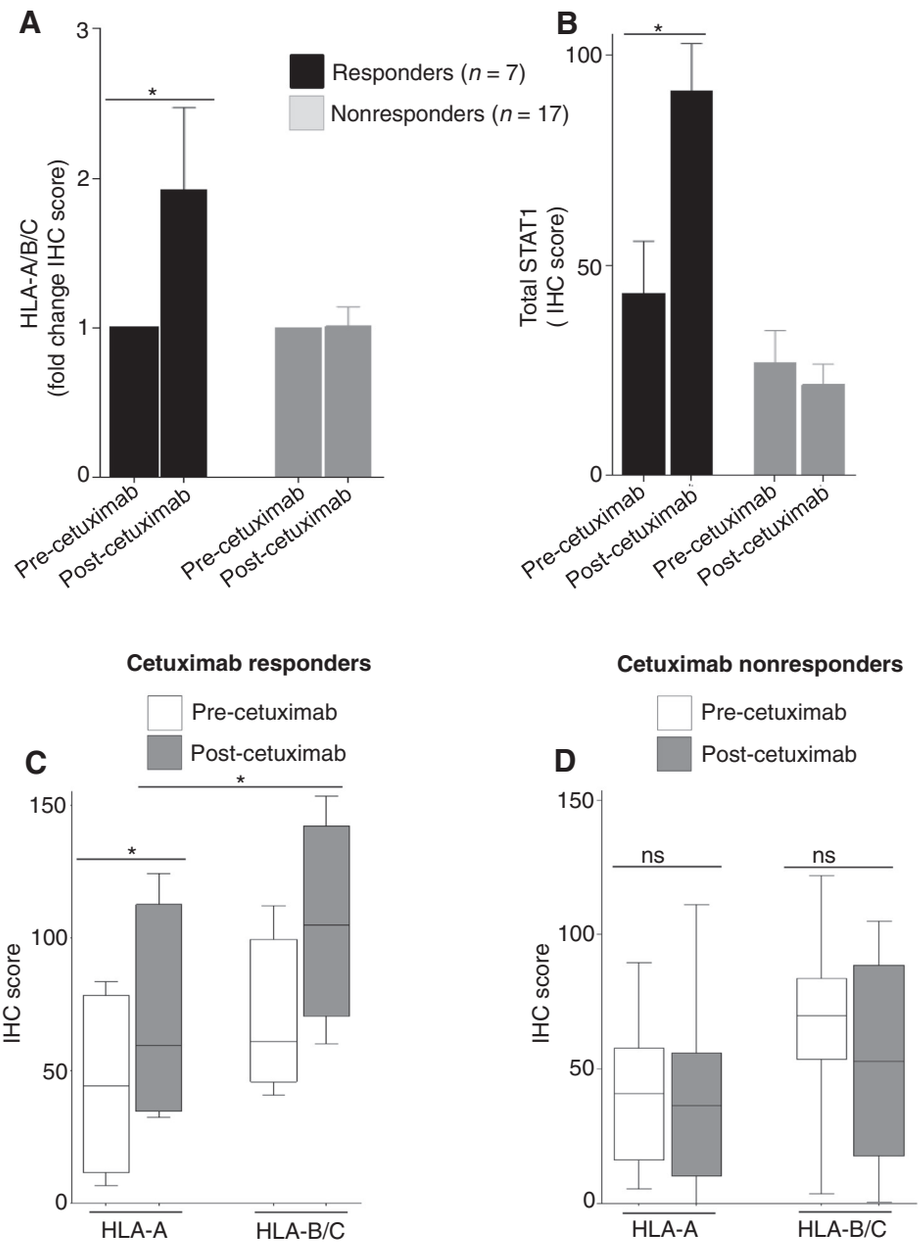


**Figure 6.**
Cetuximab neoadjuvant therapy enhances HLA class I expression in patients' tumors in clinical responders but not in nonresponder HNC patients. A–D, tumor specimens from HNC patients ($n = 24$) enrolled on UPCI trial 08-013 were biopsied pretreatment and after neoadjuvant treatment (cetuximab, i.v., 400 mg/m$^2$ day 1, then 250 mg/m$^2$ alone days 8, 15, and 22), and a tissue microarray was prepared from paired tumor biopsies. Expression of HLA-A (mAb HCA2), HLA-B (mAb HC10), and STAT1 (mAb C-24) was determined in neoadjuvant cetuximab-treated HNC patients by semiquantitative IHC, and correlated with tumor shrinkage (CT scan "responders") after 4 weeks of cetuximab therapy; *, $P \leq 0.05$; ns, not statistically significant.

both HLA alleles and STAT-1 were upregulated in the clinical responders ($n = 7$) but not in nonresponders ($n = 17$; Fig. 6A–D) to EGFR-specific mAb therapy.

## Discussion

In HNC, low levels of HLA class I and APM component expression preclude effectiveness of CTL responses in mediating tumor elimination (11), and this mechanism of immune escape is a consequence of diminished STAT1 activation generated by the overexpression of SHP2 (17). Multiple pathways are linked with SHP2 functions in HNC, primarily the EGFR–SHP2 pathway. Because of the frequent overexpression of EGFR, which is a poor prognostic factor in HNC, constitutive activation of this pathway may greatly facilitate an "immune-escape" phenotype through suppression of p-STAT1–mediated expression of the HLA–APM pathway. This study sheds light on the mechanism(s) responsible for the diminished TA processing and presentation due to suppression of STAT1 and HLA class I APM components in HNC, which may be reversed through EGFR blockade, IFNγ release due to cetuximab-activated NK cells, or both. The effect is likely to have a beneficial impact on the clinical course of the disease in HNC patients treated with cetuximab.

Recently, we have shown that in HNC patients, cetuximab induces cross-priming of EGFR-specific CTLs by NK:DC cross-talk (18, 19). However, the determinants of TA recognition by CTLs

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may benefit cetuximab-mediated clinical responses. Intriguingly, processing and presentation of HLA class I peptide complex is an intricate process (28), and polymorphism in HLA class I alleles (29), their differential levels, and the dynamic role of APM components represent important immune-escape mechanisms from adaptive immunity in cancer (19, 29). Using a novel HLA-A2:MAGE-3–specific mAb, we demonstrated quantitatively enhanced TA presentation in HNC cells, which is critical for CTL lysis. Thus, the likelihood of generation of a greater repertoire of TAs ("antigen spreading") appears to result from EGFR blockade using cetuximab, perhaps due to IFNγ-induced antigen presentation along with upregulated HLA alleles. Enhanced recognition of the peptide–HLA-A2 complex using the combination of cetuximab and IFNγ could be monitored diagnostically as a measure of Th1-biased immune responses. However, in light of superior restoration of HLA-B with cetuximab, greater characterization of HLA-B–restricted TAs is also warranted, particularly during cetuximab-based immunotherapy. These effects could be overcome by a greater drop in HLA class I expression after EGF treatment of HNC cells. The effect of HLA upregulation on reduced cetuximab-mediated ADCC supports a moderate impact of NK-cell inhibitory, killer immunoglobulin–like receptors (KIR) and the tumor cell/HLA class I interaction during cetuximab-mediated ADCC.

Polymorphism of HLA class I alleles may play a dominant role in regulating NK-cell effector function. Interaction of a few NK-cell inhibitory receptors with a specific HLA allele plays an important role in NK-cell–mediated antitumor responses. Inhibitory KIRs have higher affinity for HLA-B than for HLA-A (29). This suggests the strong possibility that HLA-B, which interacts with KIR, may have a negative impact on NK-cell stimulation (29), and HNC may become more resilient to further NK-cell attack (30, 31). HLA-B is most often loaded with antigenic peptides (32). In the HNC cell lines used in our study, IFNγ induced HLA-B/C more strongly than HLA-A (32, 33), a result that had been rarely reported previously. This observation reflects the fact that EGFR signaling in combination with p-STAT1 suppression by SHP2 has overwhelmingly negative effects on immunogenic TA-peptide presentation, abrogating the generation, loading, and presentation on surface HLA class I:TA peptide complexes, which are necessary for CTL lysis.

Cetuximab induces EGFR-specific CTL responses in some HNC patients (18), whereas clinical response to cetuximab is only observed in a subset of patients (∼20%; refs. 34, 35). The effects of concerted antitumor immune responses involving NK cells, NK–DC cross-talk, and CTL responses (18, 19, 36, 37), along with upregulation of HLA class I and IFNRI, may contribute to response to cetuximab therapy. Indeed, we observed STAT-1, HLA class I upregulation in cetuximab-treated patients in a novel neoadjuvant trial, suggesting that EGFR inhibition and/or IFNγ release contributes to the reversal of HLA downregulation. Results from recent studies also indicate an immunosuppressive effect of EGFR signaling on STAT1-dependent HLA class I and CIITA genes (38, 39). However, HLA class I and CIITA are modulated by both total STAT1 and p-STAT1 (40, 41), and a recent report also indicates that EGFR inhibitors reduce PD-L1 expression in lung tumors (42), suggesting multiple immune-escape mechanisms mediated by EGFR signaling.

## Disclosure of Potential Conflicts of Interest

R.L. Ferris is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, and Merck. No potential conflicts of interest were disclosed by the other authors.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** R.M. Srivastava, S. Trivedi, F. Concha-Benavente, J. Hyun-bae, L. Wang, R.R. Seethala, R.L. Ferris
**Writing, review, and/or revision of the manuscript:** R.M. Srivastava, B.F. Branstetter IV, S. Ferrone, R.L. Ferris
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** R.R. Seethala, B.F. Branstetter IV, R.L. Ferris

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