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Targeting secondary immune responses to cetuximab: CD137 and the outside story

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Targeting secondary immune responses to cetuximab: CD137 and the outside story

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Cetuximab is a murine-human chimeric IgG1 mAb directed against the EGFR that is approved for use in patients with colorectal and head and neck carcinomas. While some patients benefit greatly from cetuximab, many do not; therefore, strategies to increase the efficacy of this drug are of great clinical interest. In this issue of the *JCI*, Kohrt and colleagues report a strategy for enhancing the secondary immune response to cetuximab that involves sequential targeting with an agonist mAb against CD137 expressed on NK and T cells.

The inside story on cetuximab

EGFR, also known as HER1, is a member of the erythroblastic leukemia viral oncogene homolog (ERBB) family of transmembrane receptor tyrosine kinases (RTK). Deregulation of EGFR signaling initiates aberrant growth, proliferation, and angiogenesis. The clinical development of mAbs targeting the extracellular ligand-binding domain and small molecules targeting the intracellular ATPase domain of EGFR was originally compelled by the association of EGFR overexpression with poor prognosis in multiple epithelial malignancies. Despite overexpression of EGFR in the vast major-

ity of human aerodigestive cancers, cetuximab has only gained FDA approval for use in patients with head and neck squamous cell carcinoma (HNSCC) and WT *KRAS* colorectal cancer (CRC), with monotherapeutic activity observed in just 10% to 20% of patients. Clinical selection for cetuximab is confounded by the absence of a reliable biomarker to predict patient response. Mutations that result in constitutively activated EGFR are associated with patient responsiveness to small-molecule EGFR inhibitors in lung cancer; however, these mutations are rarely identified in HNSCC or CRC. Moreover, neither EGFR overexpression nor *EGFR* amplification predicts clinical benefit from cetuximab (1, 2). The discordance between EGFR target expression and the efficacy of target blockade by cetuximab has broadened investigation

into the mechanisms of action and development of therapeutic resistance.

Initial strategies to enhance cetuximab activity have focused on the intracellular signaling hypothesis (Figure 1A), which suggests that de novo or compensatory activation of parallel RTKs (alternate HER family members, cMet, IGF1R, FGFR, VEGFR), downstream EGFR-signaling nodes (RAS, PI3K, STAT3, SRC), or cell cycle promoters (aurora kinase, CDK4/6) circumvents EGFR blockade in HNSCC preclinical models; therefore, coinhibition of these resistance nodes should enhance the activity of cetuximab (3). Cetuximab resistance has also been attributed to heterodimerization of EGFR with other HER proteins that potentially prevent recognition of EGFR by cetuximab as well as acquisition of gain-of-function *PIK3CA* mutations that activate signaling downstream of EGFR. In CRC patients, activating *KRAS* and *BRAF* mutations confer clinical cetuximab resistance. Progressive insight into the complexity and plasticity of the EGFR signaling network has propelled cetuximab-combination trials to evaluate the efficacy of cotargeting these purported resistance nodes (Table 1).
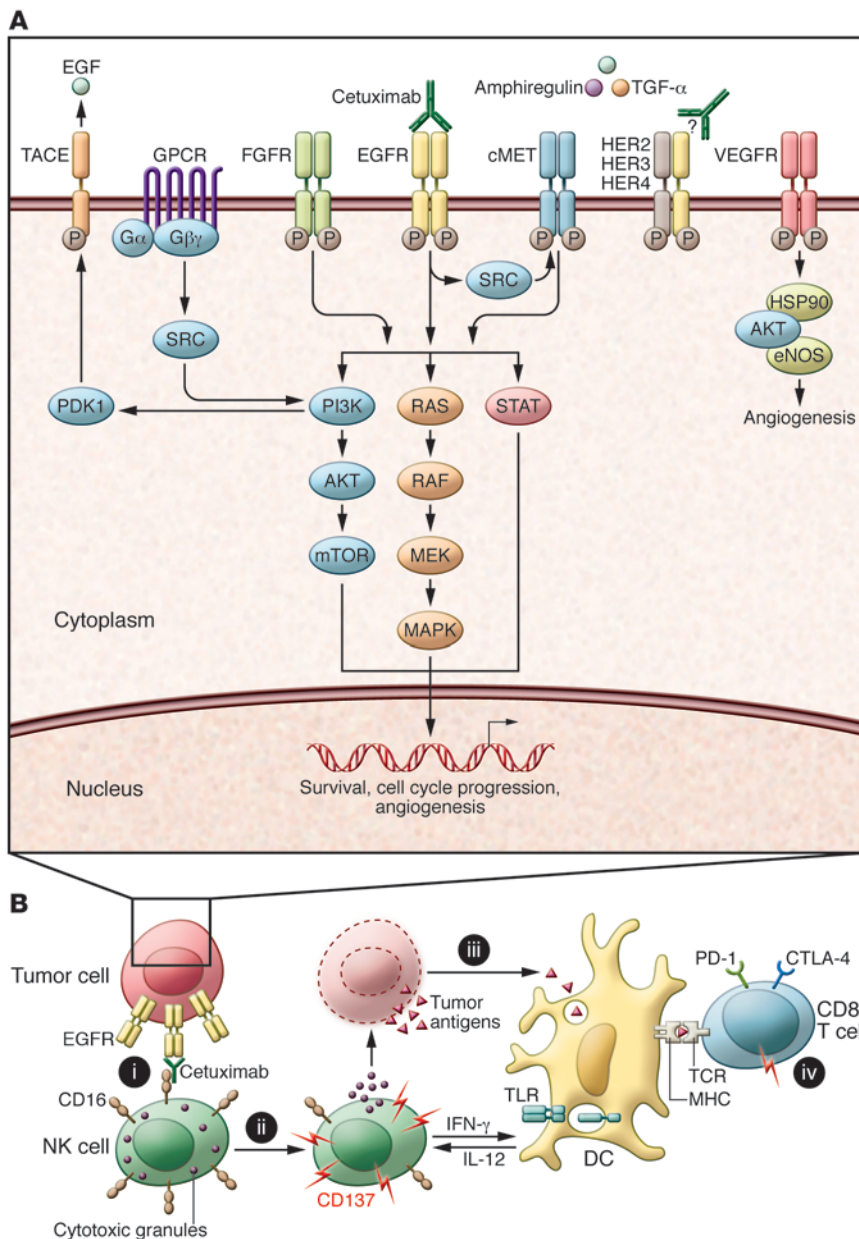
Figure 1
Intracellular and extracellular approaches to increasing cetuximab efficacy. (**A**) The inside story. Cetuximab binds to and inhibits EGFR, preventing binding of EGFR ligands and EGFR-dependent activation of cancer-promoting pathways. Blockade of EGFR signaling can be circumvented by crossactivation of accessory RTKs, such as FGFR, cMET, and VEGFR, GPCR signaling, or EGFR-independent activation of any signaling node downstream of EGFR. Cetuximab is being investigated in combination with agents to block other cancer-associated signaling pathways in order to boost efficacy. (**B**) The outside story. (i) The exposed Fc region of cetuximab bound to EGFR on tumor cells interacts with CD16 on the NK cell surface, promoting NK cell activation. (ii) Once activated, NK cells upregulate CD137 and produce IFN-γ, which promotes DC maturation. Additionally, NK activation results in cytotoxic degranulation, resulting in tumor cell lysis and the release of TAs. (iii) TAs are taken up by DCs, which present the antigens to CD8+ T cells (iv). Cetuximab induces both innate and adaptive immune responses. Strategies aimed to amplify the immunologic efficacy of cetuximab enhance NK cell activation, antigen processing and presentation by DCs, or T cell activation.

An outside perspective on blocking EGFR with cetuximab

Two observations in HNSCC stimulated the search for extracellular immune mechanisms of cetuximab (Figure 1B). First, despite their demonstrated abrogation of EGFR signaling, nonimmunogenic small-molecule inhibitors have not shown clinical efficacy in randomized trials. Second, although both EGFR phosphorylation and tumor proliferation are curtailed in response to cetuximab in vitro, apoptosis or lysis of tumor cells requires coculture with lymphocytes (4). Immune modeling suggests that cetuximab induces sequential innate and adaptive immune responses (5).

These models indicate that EGFR serves as a tumor antigen (TA) that is bound by the variable fragment (Fab) of cetuximab, leaving the exposed IgG1 constant fragment (Fc) on cetuximab-coated cells able to bind FcγR IIIa (CD16) on NK cells. Fc-CD16 binding then triggers antibody-dependent cell-mediated cytotoxicity (ADCC). In vitro, effective cetuximab-mediated ADCC depends upon IgG1 isotype, Fc fragment glycosylation, and CD16 polymorphisms, which influence the strength of the bond between Fc and CD16 (4, 6). Crosslinking of Fc with CD16 activates NK cells and upregulates expression of the costimulatory receptor CD137, production of IFN-γ,

and cytotoxicity. In turn, activated NK cells induce IFN-γ–dependent DC maturation, enhancing antigen presentation and cross-priming of EGFR-specific CD8+ cytotoxic T lymphocytes (7). In theory, strategies to amplify cetuximab-induced NK cell activation would stimulate both innate and adaptive immunity, the latter required for long-lasting immune protection.

A sequential approach to enhancing cetuximab efficacy

Kohrt and colleagues present evidence that sequential administration of cetuximab followed by an agonistic anti-CD137 mAb potentiates NK cell degranulation

Table 1
Cetuximab-combination trials

Cotargeting resistance nodes in the EGFR-signaling network

Target	Drugs	NCT identifier (anatomic site)
PI3-kinase	PX-866	NCT01252628 (HNSCC)
	BYL719	NCT01602315 (HNSCC), NCT01719380 (*BRAF*mut CRC)
	BKM120	NCT01816984 (HNSCC)
cMet	Tivantinib	NCT01602315 (HNSCC)
		NCT01892527 (HNSCC)
		NCT00957853 (CRC)
VEGFR	Bevacizumab	NCT00409565 (HNSCC)
	Pazopanib	NCT01716416 (HNSCC)
	BAY 43-9006	NCT00326495 (CRC)
HER2	Neratinib	NCT01960023 (CRC)
	Afatinib	NCT01540682 (solid tumors)
HER3	RO5479599	NCT01482377 (HER3+ solid tumors)
	REGN1400	NCT01727869 (CRC, HNSCC)
	ABT-700	NCT01472016 (FOLFIRI-cetuximab eligible solid tumors)
HER4	Afatinib	NCT02020577 (solid tumors)
IGF-1R	Cixutumumab	NCT00957853 (HNSCC)
	OSI 906	NCT01427205 (HNSCC)
RAF	BAY 43-9006	NCT00326495 (CRC)
BRAF V600E	LGX818	NCT01719380 (*BRAF*mut CRC)
	Vemurafenib	NCT01524978 (*BRAF*mut CRC)
SRC	Dasatinib	NCT01488318 (HNSCC)
		NCT00882583 (HNSCC)
mTOR	Temsirolimus	NCT00593060 (CRC), NCT01256385 (HNSCC)
	Sirolimus	NCT00940381 (solid tumors)
	Everolimus	NCT01133678 (HNSCC)
HSP90	AUY922	NCT01294826 (CRC)
CDK 4/6	PD 0332991	NCT02101034 (HNSCC)
Aurora kinase	MLN8237	NCT01540682 (HNSCC)

Augmenting ADCC, DC activation, and T cell crosspriming

Target	Drugs	NCT identifier (anatomic site)
CD137 agonist	BMS-663513	Pending (HNSCC, CRC)
	PF-05082566	
TLR agonists	VTX-2337 (TLR8)	NCT01334177 (HNSCC)
		NCT01836029 (HNSCC)
	EMD 1201081 (TLR9)	NCT01040832 (HNSCC)
Cytokine milieu	Recombinant IL-12	NCT01468896 (HNSCC)
Fc-CD16 binding	CetuGEX	NCT02052960 (HNSCC monotherapy)

Overcoming T cell anergy

Target	Drugs	NCT identifier (anatomic site)
CTLA-4	Ipilimumab	NCT01860430 (HNSCC)
PD-1/PD-L1	BMS-936558	
	BMS-936559	
Treg depletion	Cyclosporine	NCT01581970 (HNSCC)

and cytotoxicity against EGFR-expressing HNSCC, mutant *KRAS* CRC, and WT *KRAS* CRC cell lines in vitro and as xenografts in murine models (8). A major limitation of many murine xenograft models (9, 10) is the use of immunosuppressed animals, which limits analysis to the innate immune response; however, Kohrt et al. evaluated the effectiveness of cetuximab/anti-CD137 combination therapy against

syngeneic xenografts in immune-competent BALB/c mice, using an engineered murine cell line (TUBO) transfected with human EGFR (TUBO-EGFR) (6). While NK cells were necessary for initiation of the therapeutic effect of cetuximab against TUBO-EGFR, depletion of CD8+ T cells also abrogated efficacy. Importantly, CD8+ T cells were required to mediate the memory response and epitope spreading that resulted in rejection of TUBO and TUBO-EGFR xenografts both in mice previously cured with combination therapy and in untreated mice that received adoptively transferred CD8+ splenocytes from cured animals. Kohrt and colleagues also determined that CD137 is upregulated on intratumoral and peripheral NK cells from patients with HNSCC treated with cetuximab, highlighting the translational potential of this therapeutic approach (8).

CD137 is a member of the costimulatory family of TNF receptors that is induced upon activation of NK and T cells. Its ligand, CD137L, is expressed on macrophages, activated B cells, and DCs. Endogenous ligation enhances ADCC by NK cells, CD28-dependent activation of the T cell receptor, and expansion/memory differentiation of CD8+ T cells while inhibiting the immunosuppressive function of CD4+ Tregs (11). Additionally, CD137 can be stimulated exogenously by engineered mAbs. Enthusiasm for CD137-based immunotherapy emerged after preclinical studies demonstrated that administration of an anti-CD137 mAb eradicated murine sarcomas and mastocytomas in immune-competent animals and was associated with expansion of CD8+ TA-specific T cells (12). In tumors that were not responsive to CD137, a combination of TA-derived peptide vaccine plus anti-CD137 mAb broke T cell naiveté, promoting tumor regression. These results underscored the possibility that poorly immunogenic tumors, similar to human epithelial malignancies, would require combinatorial approaches in which T cell activation is followed by CD137 agonism. The approach used by Kohrt and colleagues combines cetuximab, which primes NK cell activation by Fc-CD16 crosslinking, and anti-CD137 mAb, which augments both ADCC and the T cell response to generate immunologic memory (8). Results from other preclinical studies targeting HER2-expressing breast cancer with trastuzumab/anti-CD137 and CD20+ lymphoma with rituximab/anti-CD137 suggest that

sequential therapy with an IgG1, TA-targeting mAb and an anti-CD137 mAb may be broadly applicable (9, 10).

Kohrt and colleagues hypothesized that combined TA-targeting and anti-CD137 mAb would be less toxic than alternative NK cell–stimulating strategies, such as exogenous cytokine administration, due to restriction of NK activation to the TA-expressing tumor microenvironment (8); however, clinical experience tempers this conclusion. Two humanized, agonist anti-CD137 mAbs (BMS-663513 and PF-05082566) have entered clinical development. While phase I trials for BMS-663513 suggested clinical activity, 11% of patients exhibited serious, reversible liver inflammation, an on-target toxicity likely due to polyclonal T cell infiltration (13, 14). Furthermore, development of BMS-663513 was temporarily suspended during a follow-up phase II trial, when two hepatic fatalities occurred in cohorts receiving a dose greater than 1 mg/kg. Because the biologic activity of BMS-663513 was evident at doses logarithmically lower that 1 mg/kg, phase I trials to evaluate doses in the 0.03 to 0.3 mg/kg range are now recruiting. Safety data will be necessary before planned combination trials with TA-targeting mAbs proceed.

Limitations and future directions
While a combinatorial therapeutic approach remains promising, certain translational limitations must be acknowledged. First, the work by Kohrt and colleagues appears agnostic to the intracellular and genomic context of EGFR inhibition (8). Cell lines were selected on the basis of EGFR expression, including SCC4 and SCC6 (HNSCC), PC1 (pancreatic cancer), and T84 and HCT116 G13D (WT *KRAS* and mutant *KRAS* CRC), as ADCC activity correlates with TA concentration but not *KRAS* mutational status (15). EGFR expression also correlates with response to EGFR inhibition in vitro, and HNSCC xenografts in athymic nude mice are uniformly sensitive to cetuximab (16); therefore, the rationale for use of these cell lines is somewhat unclear, and the modest effect of cetuximab monotherapy in the SCC6 xenograft model is surprising. Better characterization of altered intracellular signaling would enhance understanding of the overall therapeutic response to cetuximab, as EGFR-signaling blockade has multiple effects on the immune response, including inhibition of TLR3, decreased EGFR-

dependent STAT3 signaling, and enhanced function of $EGFR_{875-889}$-reactive CD4^{+} Th cells, which promote CD8^{+} T cell responses against common ERBB and cMET epitopes (17, 18). Second, while Kohrt et al. describe several mutations in genes other than *KRAS* within the chosen cell lines, this information does not clearly inform the design or interpretation of experiments (8). Unlike in CRC, no link between a particular genetic mutation and clinical cetuximab resistance in HNSCC has been identified. Furthermore, the paucity of genetically engineered HNSCC mouse models limits identification of predictive biomarkers. Given their resemblance to human tumors, patient-derived tumor grafts are promising models for evaluating cetuximab resistance (16). Third, evaluation of the response of tumorigenic human cells in mice is only possible in the context of a compromised host immune system, where only innate immunity persists. Kohrt and colleagues addressed this limitation by using the TUBO-EGFR murine tumor cell line in immune-competent animals (8). Of note, the TUBO progenitor is HER2 dependent, and TUBO-EGFR cells are more responsive to trastuzumab than cetuximab (6). Given the known heterodimerization of EGFR and HER2 and efficacy of dual EGFR/HER2 inhibition in HNSCC models, HER2 expression in TUBO-EGFR cells may influence the signaling response. Additionally, the therapeutic results from an immune-competent mouse model may not translate to human patients. Despite a striking conservation of transcriptional programs during murine and human immune system differentiation, several hundred immune genes diverge and their regulatory mechanisms are less well conserved (19). The ongoing technologic advances in engrafting *Il2rg* mutated, immunodeficient mice with functional human immune systems will likely provide greater translational confidence (20).

Despite acknowledged limitations, this study by Kohrt and colleagues introduces a new therapeutic paradigm. Much like the EGFR signaling network, the immune cascade following TA-targeting mAb treatment contains key nodes that can be targeted to amplify the antitumor immune response. Multiple approaches have entered human clinical trials to enhance cetuximab-dependent responses, including stimulation of ADCC with Fc fragment glyco-engineering, CD137 agonists, or IL-12, activation of DCs with TLR agonists, and

activation of CD8^{+} T cells with CD137 agonists, suppression of Tregs, or blockade of the coinhibitory receptors CTLA-4 and PD-1 (Table 1). Ultimately, evaluation of immunotherapy strategies against genetically defined human cancers in humanized mice will permit synthesis of the inside and outside stories and accelerate bedside translation.

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Sex, drugs, and trial design: sex influences the heart and drug responses

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Preclinical studies indicate that the phosphodiesterase 5 (PDE5) inhibitor sildenafil is protective against hypertrophy-induced cardiac remodeling. Despite an initial clinical study demonstrating sildenafil-dependent amelioration of pathological remodeling, the cardioprotective effect of this drug was not significant in a large placebo-controlled clinical trail. In this issue, Sasaki and colleagues reveal that the efficacy of PDE5 inhibition in female mice requires estrogen. Induction of cardiac stress in male and intact female mice resulted in increased activation of protein kinase G (PKG) signaling, which was further enhanced by sildenafil. PKG activity was not enhanced in ovariectomized (OVX) female mice as a result of cardiac stress, but administration of estrogen restored PKG activation and enhancement by sildenafil. These data highlight the importance of considering sex-specific differences and drug responses in clinical trial design.

Regulation of cGMP levels in the heart

An increase in nitric oxide (NO) or activation of natriuretic peptide triggers guanylyl cyclase (sGC), resulting in the generation of cGMP, which in turn promotes protein kinase G (PKG) signaling to regulate multiple cellular processes, including the cardiac response to stress. In addition to sGC, phosphodiesterases (PDEs) regulate cGMP levels via degradation (see Figure 1). While many PDEs are promiscuous and can degrade either cAMP or cGMP (1), PDE5 is reported to specifically degrade cGMP and is inhibited by the drug sildenafil. Under basal conditions, levels of PDE5 are low in the heart; therefore, PDE5 inhibition with sildenafil has

little effect on cGMP and PKG activation (2). In contrast, conditions that induce or are associated with hypertrophic stress, including overexpression of the G protein Gq, transaortic constriction, or natriuretic peptide signaling, lead to increased sGC activity, subsequent cGMP production, and activation of PKG, which phosphorylates and activates a number of targets. PKG signaling is thought to reduce hypertrophic remodeling, and previous studies (3–5) have shown that PDE5 inhibition by drugs such as sildenafil can ameliorate the pathological cardiac remodeling that is associated with hypertrophy and heart failure (4–6). Conversely, overexpression of PDE5 in mice promotes pathological remodeling in the heart in the absence of cardiac stress (3, 7). Compared with tissue from healthy subjects, PDE5 expression is upregulated in patients with right ventricular failure (8). Furthermore, a small clinical trial demonstrated that silden-

afil reduces pathological remodeling in patients with systolic heart failure (9); importantly, however, a large multicenter, placebo-controlled trial (RELAX) did not show a beneficial effect of sildenafil in the treatment of heart failure with preserved ejection fraction (10).

Sex matters: sex-dependent responses to cardiac stress

Although sex-associated differences are increasingly recognized in nonreproductive physiology, metabolism, and disease, these differences are generally not taken into account during clinical trial design or in drug treatment (11); however, this paradigm is beginning to change. For example, just last year the FDA recommended that women receive a different (lower) dose of zolpidem, a drug used to treat insomnia, than that recommended for men (12). In the FDA drug safety announcement about the new zolpidem dosing recommendations for women, it was noted that "women appear to be more susceptible to this risk (next morning impairment) because they eliminate zolpidem from their bodies more slowly than men." This example highlights that sex-dependent drug effects need to be taken into consideration when developing dosage guidelines.

There have been numerous sex-specific differences reported in cardiovascular physiology and disease (13). The majority of preclinical studies showing a beneficial effect of PDE5 inhibition on adverse remodeling were performed on male ani-