Supporting Information

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SI Materials and Methods

Antibodies. ***Flow cytometry and ELISA antibodies.*** Polyclonal rabbit antibody against mouse Ig was obtained from DAKO and diluted to 1:100 in RPMI 1640/1% FCS. Anti–β-galactosidase antibody clone 4C7 (1) was obtained from the Monoclonal Antibody Service, Clare Hall, Cancer Research U.K., London, United Kingdom. FITC-conjugated murine anti-human IgG (polyclonal) and FITC-conjugated rabbit anti-mouse IgG (polyclonal) were obtained from Sigma-Aldrich. PE-conjugated rabbit anti-mouse IgG (polyclonal) was obtained from DAKO. CD3-FITC (Mab), CD11b-PE (Mab), CD14-PE (Mab), CD16-PE (Mab), CD56-APC (Mab), IgG1-FITC (isotype control, Mab), IgG1-PE (isotype control, Mab) and IgG1-APC (isotype control, Mab) were obtained from BD Biosciences Pharmingen. Murine purified anti-CD19 IgG1 (Mab) was obtained from BD Biosciences. Anti-*ERBB1* antibody (clone EGFR1) was obtained from Monoclonal Antibody Services, Clare Hall, Cancer Research U.K., London, United Kingdom.

Western blot antibodies. Rabbit anti-human ERBB1 (polyclonal), anti-human phospho ERBB1 (tyrosine1173) (clone 53A5), mouse anti-human ERBB2 (clone 44E7), rabbit anti-human phospho ERBB2 (tyrosine 1221/1222) (clone 6B12), anti-human ERBB3 (clone 1B2E), anti-human phospho ERBB3 (Y1289) (clone 21D3), anti-human ERK1/2 (polyclonal), anti-human phospho ERK1/2 (threonine 202/tyrosine 204) (polyclonal), and anti-human B-tubulin (polyclonal) were obtained from Cell Signaling. These were used at a 1:1,000 dilution in TBS [0.1% Tween with 5% (wt/vol) milk powder] at 4 °C with overnight incubation. Polyclonal swine anti-rabbit Ig conjugated with horseradish peroxidase (DAKO) was used as the secondary staining antibody.

Antibodies and small-molecule inhibitors used in killing and inhibition assays. Cetuximab (IMC-C225; Erbitux) is a monoclonal antibody targeting *ERBB1*; it was obtained from Merck and latterly purchased from the Churchill Hospital Pharmacy department, Oxford, United Kingdom. Trastuzumab (also known as herceptin) is a humanized monoclonal antibody targeting *ERBB2* (Genentech) and was obtained from the Churchill Hospital Pharmacy department, Oxford, United Kingdom. Pertuzumab is a humanized IgG1 monoclonal antibody that blocks *ERBB2* dimerization and was kindly provided by Roche Research Department. Lapatinib is a dual receptor (ERBB1 and ERBB2) tyrosine kinase inhibitor and was provided by GlaxoSmithKline Research Department.

Cytokines and Growth Factors. Human recombinant γIFN, M-CSF, and GM-CSF (Peprotech) were used to culture human monocytes. Human recombinant EGF (Peprotech) was used for growth stimulation studies on the CRC cell lines.

Mutations. PCR amplicons were sequenced directly using the appropriate PCR primers and Big Dye Sequencing kit (Applied Biosystems) on an ABI 377 (Applied Biosystems) sequencer. The *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12 and 13), *BRAF* (codon 600), *PIK3CA* (codons 542, 545, and 1047) genes were analyzed. Wherever possible, mutations were confirmed from the Wellcome Trust Sanger Institute Cancer Genome Project Web site (www.sanger.ac.uk/genetics/CGP/) and from previous publications.

Gene-Expression Microarrays. mRNA gene-expression microarray analyses were performed using the Affymetrix Human genome U133+2 chips, as per the manufacturer's instructions. Data analysis was carried out using the Partek Genomics Suite software. These data have been previously reported (2). When analyzing mRNA expression for genes represented by more than one probe, the probe that gave the highest overall expression was, in general, used to represent the expression levels of the relevant gene.

ERBB1 ELISA. ERBB1 levels were determined using a β-galactosidase/anti–β-galactosidase ELISA (1). Cells were deposited onto a poly-L-lysine coated 96-well Nunc-Immuno PolySorp plate at a concentration of 5×10^4cells per well. Murine anti-ERBB1 (Cancer Research U.K.) was used as the primary antibody. Rabbit anti-mouse IgG (DAKO) was added as a secondary step at a dilution of 1:100 in RPMI 1640/ 1% FCS. A GAG complex of β-galactosidase with anti–β-galactosidase antibody was made by dissolving β-galactosidase (Sigma-Aldrich) at a concentration of 500 U/mL in 100 mM Tris/100 mM MgCl/100 mM 2-mercaptoethanol with 300 μg/mL of 4C7 anti–β -galactosidase. This complex was incubated at 4 °C overnight and then added to the cells at a dilution of 1:750 in RPMI 1640/1% FCS. The GAG complex binds to free antigen-binding sites on the rabbit anti-mouse IgG antibody bound to the primary antibody attached to ERBB1 on the cells. The substrate 4-methylumbelliferyl-B-D-galactoside (Sigma-Aldrich) was prepared at a concentration of 0.3 mg/mL in a buffer of 1 mM $MgCl_2$/100 mM 2-mercaptoethanol. Filtered substrate solution was added to each well to start the reaction. After incubation for 40 min in the dark at room temperature, fluorescence was measured using an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

Protein Analysis. ***Extraction of whole-protein lysates from cells.*** Cells were grown to 90% confluence on 10-cm tissue culture dishes (Corning) before protein retrieval. Approximately 250 μL of RIPA lysis buffer (50 mM Tris•HCL, pH 8.0 containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS), containing a mixture of protease inhibitors (Complete Mini tablets; Roche Diagnostics) and phosphatase inhibitors (sodium fluoride and sodium orthovanadate), were added to the attached cells. The lysed cells were then scraped off the culture dish and the resulting mixture was aspirated into a 1.7-mL Eppendorf. The lysate was gently agitated for 45 min before centrifuging at 10,000 × *g* for 15 min at 4 °C. The supernatant was aspirated and the cell debris discarded. Protein concentration was determined by the Protein Pierce Assay (Pierce Biotechnology, Thermoscientific), as per the manufacturer's instructions.

SDS/PAGE. SDS/PAGE was used to probe for ERBB1, ERBB2, ERBB3, ERK1/2, B-tubulin, and their phosphorylated derivatives. A 7.5% acrylamide running gel was used for ERBB1, ERBB2 and ERBB3 (molecular weight = 175–185 kDa), and 12% acrylamide was used for ERK1/2 (molecular weight = 42–44kDa). The Mini–PROTEAN3 (Bio-Rad) apparatus was used to set up the glass plates (1.5-mm gap, 10 × 6 cm). Laemmli buffer (6× concentrate) was added to protein containing lysate and the mixture denatured at 95 °C for 10 min. Equal amounts of denatured protein lysate were added to the gels together with rainbow molecular weight markers (Amersham). Electrophoresis was carried out at 120 V for 1.5–2 h in running buffer (composed of 192 mM glycine, 25 Mm Tris•CL and 0.1% SDS). The gels were placed onto PDVF membranes (Amersham) in a Bio-Rad TE77 semidry transfer apparatus and the protein transfer was carried out at 20 V for 1.5 h.

After protein transfer, the PDVF membrane was washed with TBS-Tween (0.1% Tween in TBS) for 5 min before blocking for

an hour in TBS-Tween with 5% milk powder on a plate shaker. The membrane was then washed with TBS-Tween for a further five minutes. Primary rabbit or mouse anti-human antibody (against either ERBB1, pERBB1, ERBB2, pERBB2, ERBB3, pERBB3, ERK1/2, pERK1/2 or β-tubulin) was diluted in 5% milk TBS-Tween solution and incubated with the membrane overnight at 4 °C. The next morning the membrane was washed three times for 5 min in TBS-Tween, following which a polyclonal swine anti–rabbit-Ig antiserum conjugated with HRP (in 5% milk TBS-Tween solution) was added as the second step. The secondary antibody was left for 1 h at room temperature before washing three times for 5 min in TBS-Tween. The ECL plus Western Blotting Detection system (Amersham) was used to identify the protein bands that reacted with the primary antibody. This process involved mixing reagent A with B and then placing the mixture on the membrane for 5 min. Surplus mixture was removed before exposing to a Kodak film (5 s to 10 min) in a dark room.

Growth Inhibition Assay. Inhibitory effects were measured, generally in triplicate, over three doubling times using the sulforhodamine B (SRB) growth-inhibition assay over a wide range of cetuximab concentrations (0.0001–100 μg/mL). This process has been previously described (3). Briefly, cells were seeded into wells at a concentration of 3,000 cells/100 μL per well. After 24 h, the cells were treated with antibody or isotype IgG1 control. A separate plate seeded with the same number of cells was fixed after centrifuging by adding 50% tricarboxylic acid (TCA) and used as the "$t = 0$" control. The control plate was stored at room temperature and processed with the experimental plates as described below. The treated cells were incubated for three doubling times at 37 °C. On the day of harvest, the plates containing the treated cells were centrifuged and fixed with 50% TCA for 1 h at 4 °C. Plates were washed with 0.5× PBSA and then allowed to dry at room temperature. SRB solution (0.04% in 1% acetic acid) was added and left for 30 min at room temperature, resulting in staining of the fixed protein. Free dye was washed with 1% acetic acid and then the plates were air-dried for 1 h. The bound dye was dissolved with Tris-base (10 mM, pH 9.5) solution by placing on a plate-shaker for 20 min before reading at 540 nm using an automated plate reader.

Growth Factor Stimulation Studies. Cells were serum-starved (0.1% FCS medium) overnight. The medium was then removed and replaced with fresh 0.1% FCS medium containing the desired concentration of *EGF* for 15 min. Blocking was achieved by preincubation with 20 μg/mL of cetuximab (1 h at 37 °C) before adding EGF. A blocking concentration of 20 μg/mL of cetuximab was chosen as this resulted in near-maximal growth inhibition in the SRB assays (Fig. 1*C*).

Isolation of Effector Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood from healthy volunteers who had given their informed consent. Fresh whole blood was mixed with an anticoagulant. The PBMC layer was retrieved after Ficoll/hypaque density centrifugation (Lymphoprep). The mononuclear cells were centrifuged twice, first at 800 × *g* to further remove the Ficoll and then at 200 × *g* to remove the platelets. The resulting PBMCs were resuspended in RPMI 1640/1% FCS/2 mM glutamine and used within 6 h of preparation. The Rosettesep Human NK isolation mixture or Human Monocyte isolation mixture (Stemcell Technologies) was used to enrich for a population of either human natural killer cells or monocytes from blood, as has been described previously (4). A mixture of antibody tetramers recognizes "unwanted" leukocytes. The Rosettesep mixture of antibodies was added to fresh blood and the tube was gently mixed at room temperature for 25 min, after which it was over layered on to 15 mL of lymphoprep; this was centrifuged at 1,200 × *g* in a Beckman Coulter 6R Allegra centrifuge with no braking. NK cells were aspirated from the interface and washed with wash buffer (RPMI 2%/ FCS 2%). The falcon tube was centrifuged at 800 × *g* for 10 min. The supernatant was completely removed and the cell pellet resuspended in RBC lysis buffer (BD Pharmingen) for 10 min; this was then washed twice with wash buffer. Finally, the cell pellet was resuspended in RPMI complete medium and a sample was taken for flow cytometry analysis (5).

Lactate Dehydrogenase Cytotoxicity Assay. The Roche lactate dehydrogenase (LDH) cytotoxicity kit was used to measure the level of cell killing, as per the manufacturer's instructions. Antibody-dependant cellular cytotoxicity ADCC leads to the loss of membrane integrity, thus releasing endogenous LDH, which can be measured to give a proportional measure of cell death. Target cells were grown to ∼80% confluence. On the day of the assay, the cells were trypsinized and resuspended in RPMI1640 medium/1% FCS at a concentration of 2×10^5 cells/mL. Fifty μL were added to each well of a 96-well tissue culture Corning plate. Fifteen microliters of either test or isotype control antibody were added and left for 20 min on ice. Unfractionated PBMC were used (effector:target ratio of 40:1) as effectors and were added to the target cells. The plate was then centrifuged at 900 × *g* for 3 min before incubation at 37 °C for 2 h (5% CO_2). Fifteen minutes before the end of the assay period, lysis buffer was added to target cells in control wells to which no antibody or IgG control had been added to identify maximal lysis. The cells were left on a plate shaker for 15 min and then the plate centrifuged at 900 × *g* for 5 min. One-hundred microliters of supernatant were then removed and put into flat-bottom 96-well plate to which 100 μL of reaction mixture was added. This mixture contained a yellow tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazoliumchloride) together with a catalyst that facilitated its reduction. The plate was left for 30 min for a colorimetric reaction to take place before 50 μL of stop mixture (1 N HCL) were added to each well. The plate was placed on a plate shaker for 10 s before the wells were read in a 490-nm plate reader.

Data Analysis. As described below, cell lines were classified into three categories (sensitive, partially sensitive, or resistant) depending on their direct response to cetuximab in SRB growth inhibition studies (6). The cell lines were divided into the following groups: resistant cell lines displayed less than 33% growth inhibition in the presence of cetuximab; partially sensitive cell lines had between 33–66% inhibition; sensitive cell lines had over 66% growth inhibition. GI50 values (defined as 50% growth inhibitions after three doubling times) were calculated using XLFit program (ID Business Solution) in Microsoft Excel. Antibody-dependent (specific) lysis was calculated as (experimental release – antibody independent release)/(maximum release – antibody independent release) × 100. The SEM of triplicate wells was calculated using Prism GraphPad software. Percentage of cell phagocytosis was calculated with the formula: number of dual-stain positive target cells (cells engulfed by macrophages) divided by the total number of target cells. Copy number analysis (CONAN, a data mining tool used to extract copy number information from the SNP 6.0 dataset) of 39 colorectal cancer (CRC) cell lines on the Welcome Sanger Institute database (www.sanger.ac.uk/genetics/CGP/) was performed to identify any cell lines that had high levels of gene amplification (defined as a ratio greater than 7) in ERBB1, ERBB2, or ERBB3. Standard normal distribution tests were performed to test the significance of differences found and a P value of less than 0.05 was used as the significance threshold. Prism GraphPad Software was used to create inhibition/killing curves.

1. Durbin H, Bodmer WF (1987) A sensitive micro-immunoassay using beta-galactosidase/anti-beta-galactosidase complexes. *J Immunol Methods* 97(1): 19–27.
2. Wilding JL, McGowan S, Liu Y, Bodmer WF (2010) Replication error deficient and proficient colorectal cancer gene expression differences caused by 3'UTR polyT sequence deletions. *Proc Natl Acad Sci USA* 107(49):21058–21063.
3. Bracht K, Nicholls AM, Liu Y, Bodmer WF (2010) 5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency. *Br J Cancer* 103(3):340–346.
4. Ashraf SQ, et al. (2009) Humanised IgG1 antibody variants targeting membrane-bound carcinoembryonic antigen by antibody-dependent cellular cytotoxicity and phagocytosis. *Br J Cancer* 101(10):1758–1768.
5. Conaghan P, et al. (2008) Targeted killing of colorectal cancer cell lines by a humanised IgG1 monoclonal antibody that binds to membrane-bound carcinoembryonic antigen. *Br J Cancer* 98(7):1217–1225.
6. Jhawer M, et al. (2008) PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor cetuximab. *Cancer Res* 68(6):1953–1961.

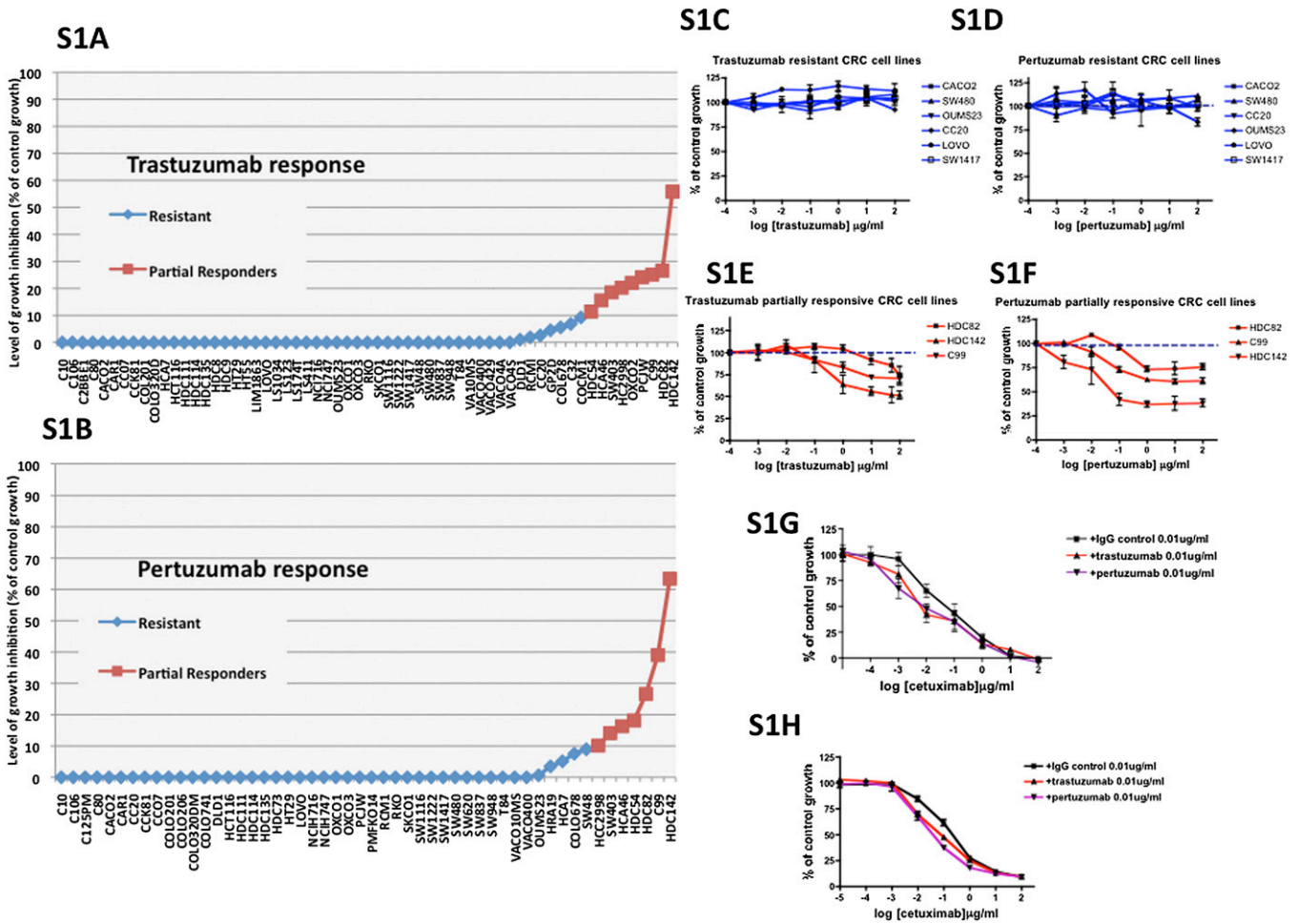

Fig. S1. (*A* and *B*) Rank plot of nonimmune growth inhibition of CRC cell lines by trastuzumab and pertuzumab. Rank plot of the CRC cell-line panel according to sensitivity to (*A*) trastuzumab and (*B*) pertuzumab (SRB assay). Two categories (resistant and partially responsive) are suggested. (*C–H*) Nonimmune growth inhibition of CRC cell lines by trastuzumab and pertuzumab. The SRB assay was used to determine the growth response of CRC cell lines to anti-ERBB2 antibodies ($1 \times 10^{-4} - 1 \times 10^{2}$ µg/mL) over three doubling times. Most CRC cell lines were resistant to trastuzumab and pertuzumab. Representative plots of trastuzumab-resistant (*C*) and pertuzumab-resistant (*D*) cell lines are shown. Only 14.8% (9/61) and 13.5% (7/52), respectively, of the lines displayed some partial responses to trastuzumab (*E*) or pertuzumab (*F*). Combination of trastuzumab or pertuzumab (0.01 µg/mL) with increasing cetuximab concentration resulted in limited additive inhibition in ERBB2-responsive cell lines C99 (*G*) and HDC142 (*H*).

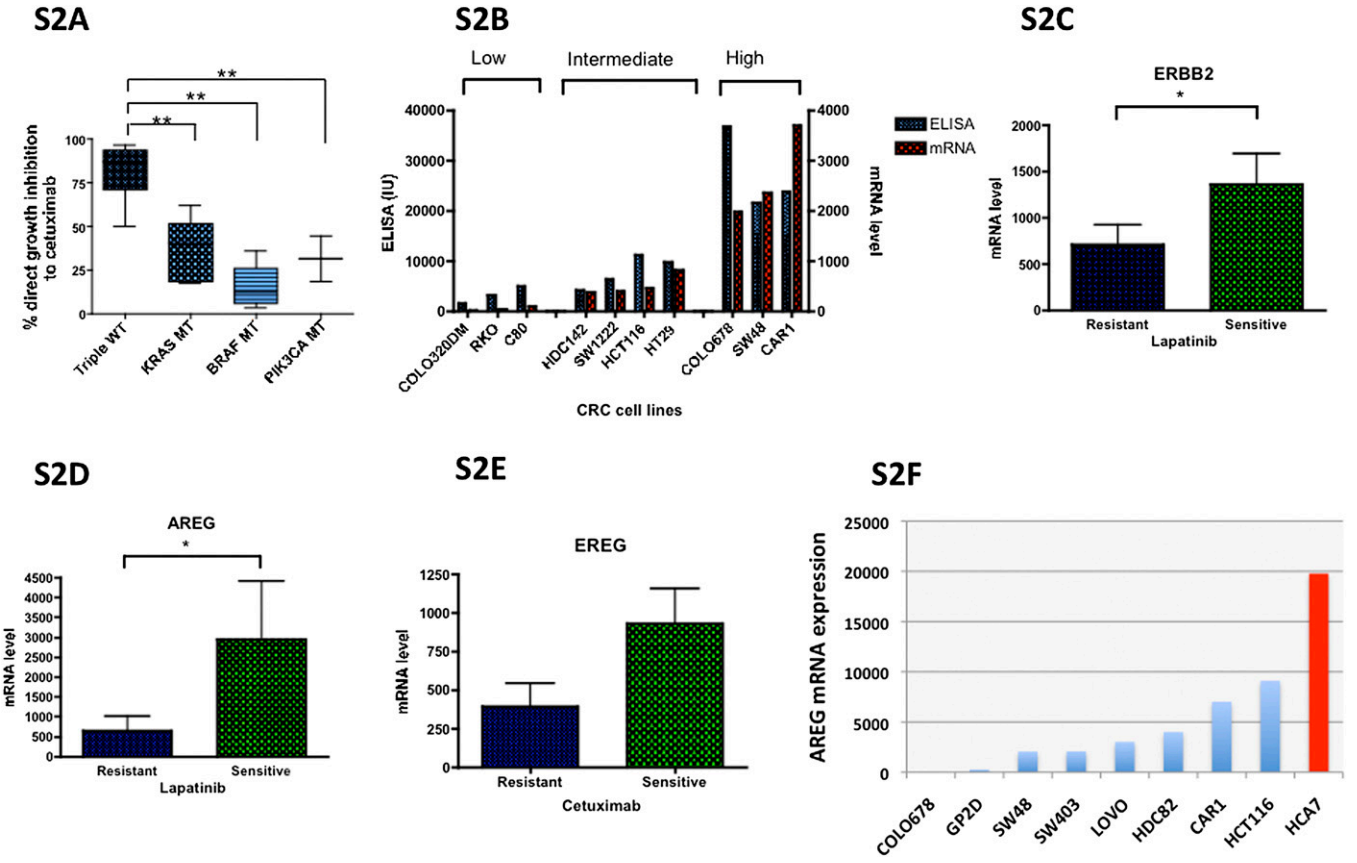

Fig. S2. (*A*) Comparison of the impact of *KRAS*, *BRAF* and *PIK3CA* exon 20 mutations on cetuximab response. The impact of different (*KRAS*, *BRAF*, or *PIK3CA* exon 20) mutations on conferring cetuximab resistance was measured. This process was done by comparing the mean value of growth inhibitory response in cell lines (top tercile of responders for each subset) containing either of these mutations. The *P* value is for *t* test comparison between each cell line group containing mutations with the triple wild-type (WT) cell lines (**$P < 0.003$). (*B*) ERBB receptor expression. Bar graph plot of ERBB1 protein (ELISA) and mRNA expression. Cell lines are plotted in rank order according to mRNA expression (red bars, *Right y* axis). The blue bars represent the corresponding ELISA values (*Left y* axis). (*C*–*E*) ERBB2 and amphiregulin (AREG) expression differences between lapatinib-resistant and -sensitive cell lines. Bar graph plots demonstrating significant differences between the lapatinib-resistant and -sensitive cell lines in (*C*) *ERBB2* and (*D*) *AREG* mRNA levels (Mann–Whitney test, *$P = 0.036$ and 0.026, respectively). There was a near significant difference between cetuximab-resistant and -sensitive cell lines in (*E*) epiregulin (EREG) mRNA levels (Mann–Whitney test, $P = 0.065$). (*F*) AREG levels in cell lines. Rank plot of AREG mRNA expression levels in CAR1, COLO678, GP2D, HCA7, HCT116, HDC82, LOVO, SW403, and SW48. This graph clearly shows the relatively high expression in HCA7 (highlighted by the red rectangle).

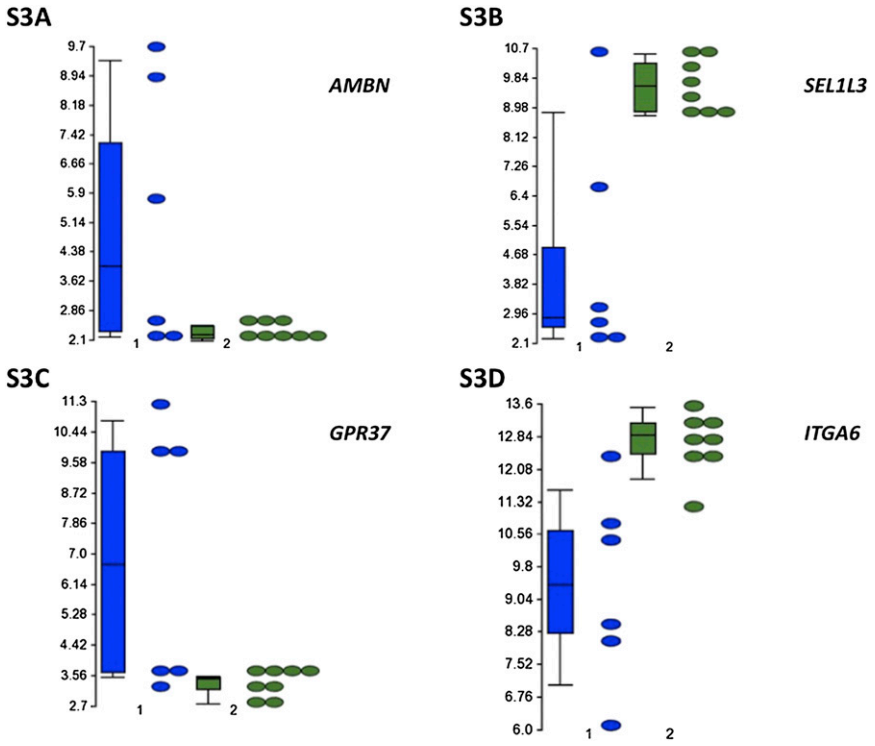

Fig. S3. Differential gene mRNA expression in CRC cell lines resistant and sensitive to the direct effects of cetuximab. (*A*–*D*) Box-and-whiskers plots of AMBN, SEL1L3, GPR37, ITGA6, and mRNA expression in triple WT CRC cells that were resistant (group 1, blue dots) or sensitive (group 2, green dots) to the nonimmune effects of cetuximab. The *y* axis represents the mRNA level with the values plotted on a $\log_2$ scale. The bottom and top of the boxes represent the 25th and 75th percentiles respectively; the whiskers represent the 10th to the 90th percentiles.

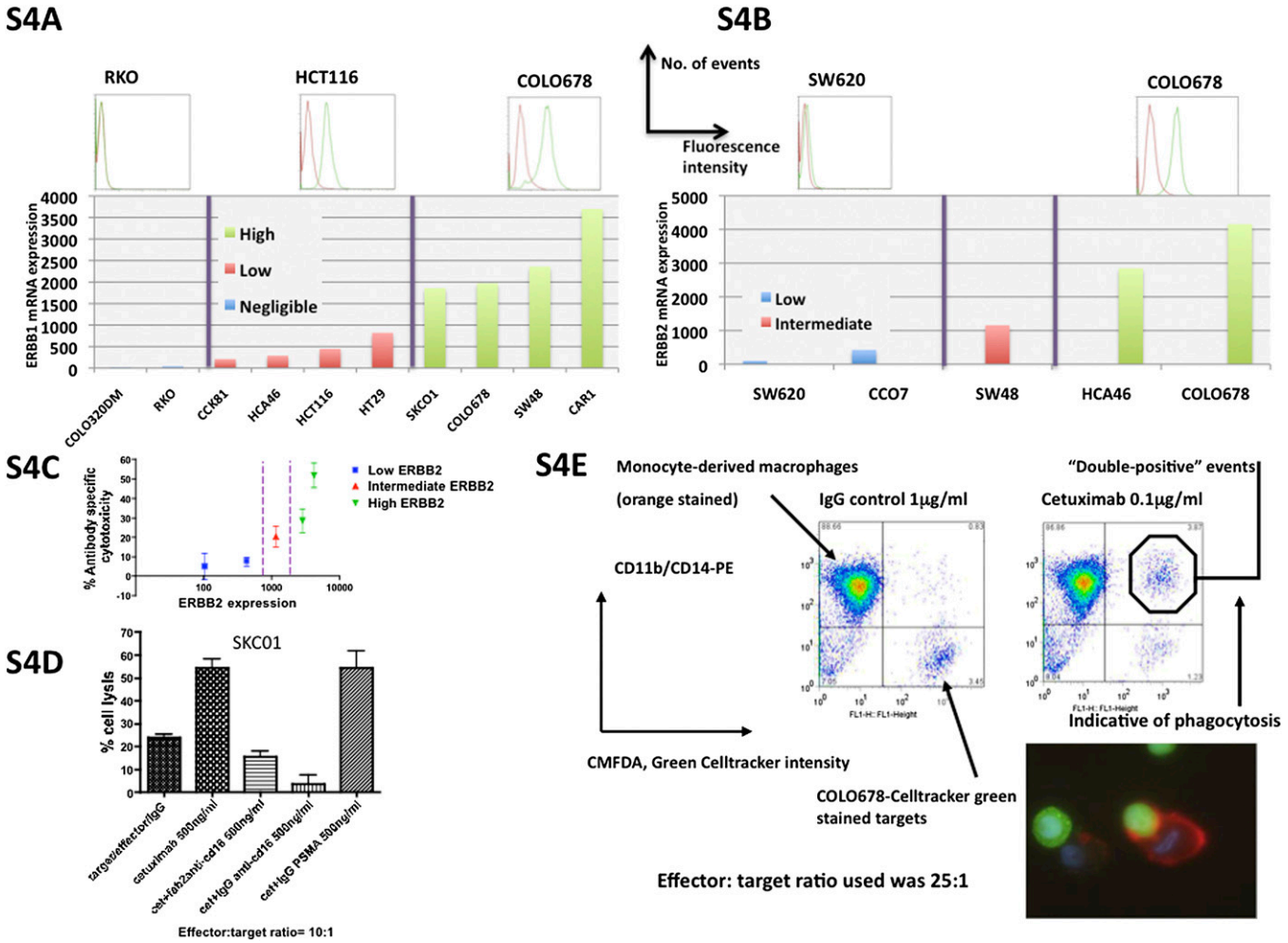

Fig. S4. Immune-mediated responses in ERBB expressing cell lines. (*A*) Rank plot of ERBB1 mRNA expression levels in CAR1, CCK81, COLO320DM, COLO678, HCA46, HCT116, HT29, RKO, SKCO1, and SW48. Flow cytometry histogram plots (*x* and *y* axes indicate FITC intensity and number of events, respectively) using cetuximab (primary anti-ERBB1 antibody) with secondary anti-human IgG-FITC antibodies confirming the range of ERBB1 protein expressions. (*B*) Rank plot of ERBB2 mRNA expression levels in CCO7, COLO678, HCA46, HCA46, and SW620. Flow cytometry histogram plots using trastuzumab (primary anti-ERBB2 antibody) with secondary anti-human IgG-FITC antibodies confirming the range of ERBB2 protein expressions. (*C*) Trastuzumab-mediated ADCC strongly correlated with the ERBB2 expression levels. (*D*) Enriched NK cells mediate ADCC effectively through FcgRIIIa. NK cells were used at an effector:target ratio of 10:1. SKCO1, an ERBB1^{+} cell line, is sensitive to cetuximab-mediated ADCC; this was inhibited in the presence of anti-FcγIIIa (CD16) blocking antibody. An anti-PSMA (prostate specific membrane antigen) was used as an isotype control. (*E*) Cetuximab is able to mediate ADCP of ERBB1^{+} CRC (COLO678) cell lines. Cultured macrophages were used at an effector:target ratio of 25:1. The cultured macrophages were labeled with CD14/CD11b-PE and appear in the *Upper Left* quadrant of the FACS plot (*x* and *y* axes indicate fluorescein and PE intensity, respectively). The targets were labeled with CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes CellTracker) and appear in the *Lower Right* quadrant. On addition of cetuximab (100 ng/mL), there was a shift from the *Lower Right* to the *Upper Right* quadrant. The inset fluorescent picture confirmed these events as red-labeled macrophages taking up green-labeled targets (Magnification: *Inset*, 100×).

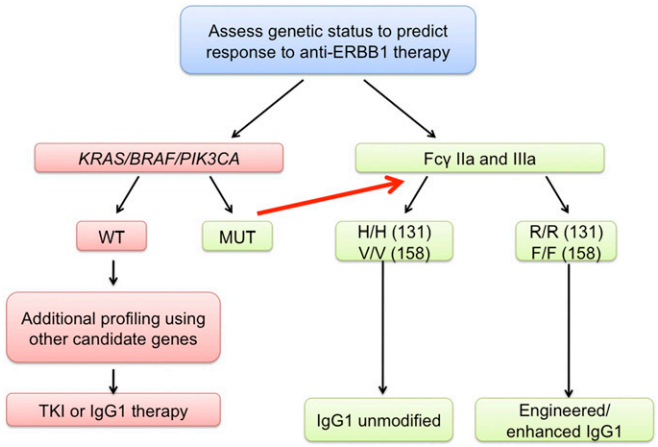

Fig. S5. Algorithm. A potential algorithm that may be used in directing currently available anti-ERBB1 therapy in patients.

Table S1. Correlation between individual mutations in *KRAS*, *BRAF*, or *PIK3CA* exon 20 and cetuximab nonimmune response and correlation between genetic data (*KRAS*, *BRAF*, *PIK3CA* exon 20, and *PTEN* loss) and lapatinib nonimmune response

Gene	Mutation	Triple WT
*KRAS**		
Resistant	24	19
Partial resistance	8	5
Sensitive	0	8
BRAF†		
Resistant	9	34
Partial resistance	1	12
Sensitive	0	8
PIK3CA exon 20‡		
Resistant	5	37
Partial resistance	1	12
Sensitive	0	8
KRAS/BRAF/PIK3CA exon 20/PTEN loss§		
Resistant	35	13
Partial resistance	5	3
Sensitive	0	6

The P value is for a χ^2 test for trend in a 3×2 table.
*$P = 0.0213$, χ^2 for trend.
†$P = 0.0849$, χ^2 for trend.
‡$P = 0.2860$, χ^2 for trend.
§$P < 0.0011$, χ^2 for trend.

Table S2. Association between ERBB receptor and ligand expression levels and cetuximab and lapatinib nonimmune responses: List of KRAS/BRAF/PIK3CA exon 20/PTEN WT cell lines that were defined as resistant and sensitive to cetuximab and lapatinib

Cetuximab-resistant	Cetuximab-sensitive	Lapatinib-resistant	Lapatinib-sensitive
LIM1863	C99	NCIH716	HDC142
CACO2	HDC82	HDC9	OXCO2
HRA19	HDC142	HRA19	C99
COLO320DM	HDC73	HT55	HCA7
NCIH716	CCK81	CACO2	SW48
CC20	HDC54	CC20	HDC73
C10	HCA46	C10	HDC82
HT55	SW48	COLO320DM	

Table S3. Association between ERBB receptor and ligand expression levels and cetuximab and lapatinib nonimmune responses: the mean value of mRNA expression (± SE) and *P* value (Mann–Whitney test) was compared for each gene between the most resistant and sensitive cell lines to cetuximab

Gene	Mean mRNA expression for cetuximab-resistant cell lines	Mean mRNA expression for cetuximab-sensitive cell lines	Mann–Whitney test, *P* value
ERBB1	1814.0 ± 666	1081.0 ± 419	0.505
ERBB2	748.3 ± 239	1272.0 ± 314	0.161
ERBB3	3,633 ± 1,613	7467.0 ± 2,145	0.130
EGF	44.3 ± 19	125.3 ± 61	0.798
TGFα	441.1 ± 248	289.1 ± 67	0.721
AREG	730.4 ± 390	1,192 ± 279	0.130
EREG	394.3 ± 151	932.3 ± 224	0.065
HB-EGF	25.8 ± 7	19.5 ± 2	0.951
BTC	35.8 ± 11	89.4 ± 28	0.105
NRG1	73.3 ± 9	88.9 ± 6	0.574

Table S4. Association between ERBB receptor and ligand expression levels and cetuximab and lapatinib nonimmune responses: the mean value of mRNA expression (± SE) and *P* value (Mann–Whitney test) was compared for each gene between the most resistant and sensitive cell lines to lapatinib

Gene	Mean mRNA expression for lapatinib-resistant cell lines	Mean mRNA expression for lapatinib-sensitive cell lines	Mann–Whitney test, *P* value
ERBB1	1,639.0 ± 613	842.7 ± 364	0.689
ERBB2	705.2 ± 215	1,357.0 ± 336	0.036*
ERBB3	3,425.0 ± 1,438	5,593.0 ± 2,160	0.224
EGF	39.7 ± 17.0	63.8 ± 55	0.456
TGFα	445.4 ± 219	369.0 ± 87	0.607
AREG	653.1 ± 352	2,940.0 ± 1,459	0.026*
EREG	351.2 ± 140	970.2 ± 350	0.088
HB-EGF	24.3 ± 6.0	23.3 ± 4.3	0.776
BTC	49.2 ± 16.6	135.3 ± 46.7	0.066
NRG1	76.4 ± 8.4	84.5 ± 4.9	0.224

*$P < 0.05$.

Table S5. Differential gene expression (Affymetrix Gene Microarray) distinguishing nonimmune cetuximab and lapatinib responses in "triple" WT CRC cell lines

Gene rank	Gene symbol	Gene title	*P* value (resistant vs. sensitive)	Step up (*P* value (resistant vs. sensitive)	Ratio (resistant vs. sensitive)	Fold-change (resistant vs. sensitive)
Cetuximab						
1	*GPR98*	G protein-coupled receptor 98	1.50E-14	8.20E-10	10.851	Resistant up
3	*AMBN*	Ameloblastin	4.87E-09	8.88E-05	7.509	Resistant up
6	*GPR37*	G protein-coupled receptor 37	1.82E-07	0.001658	2.374	Resistant up
9	*ITGA6*	Integrin, α-6	4.29E-07	0.002180	0.0931	Resistant down
10	*SEL1L3*	Sel-1, suppressor of lin-12-like 3	4.39E-07	0.002180	0.0323	Resistant down
12	*PRKAA2*	Protein kinase, AMP-activated, α-82 catalytic subunit	7.12E-07	0.002780	19.099	Resistant up
24	*DOCK4*	Dedicator of cytokinesis	3.42E-06	0.006930	43.498	Resistant up
Lapatinib						
1	*GPR98*	G protein-coupled receptor 98	6.09E-21	3.33E-16	11.416	Resistant up
4	*HAPLN1*	Hyaluronan and proteoglycan link protein 1	3.37E-08	0.000372	11.189	Resistant up
14	*PRKAA2*	Protein kinase, AMP-activated, α-2 catalytic subunit	8.37E-07	0.003053	13.902	Resistant up
10	*SEL1L3*	Sel-1, suppressor of lin-12-like 3	4.39E-07	0.002180	0.0323	Resistant down
22	*DOCK4*	Dedicator of cytokinesis	2.35E-06	0.005060	35.036	Resistant up
25	*ITGA6*	Integrin, α-6	2.64E-06	0.005147	0.1250	Resistant down

Partek Genomic Suite software was used to analyze the Affymetrix Microarray data to find the most differentially expressed genes between resistant and sensitive 'triple WT' cell lines. For cetuximab, six resistant (COLO320DM, CACO2, NCIH716, CC20, C10, and HT55) and eight sensitive (C99, HDC82, HDC142, HDC73, CCK81, HDC54, HCA46, and SW48) cell lines were compared. For lapatinib, seven resistant (CC20, COLO320DM, C10, CACO2, HT55, HDC9, NCIH716) and nine sensitive (HDC142, OXCO2, C99, HCA7, SW48, HDC73, HDC82, CCK81, and HCA46) triple WT cell were analyzed. A list of the most differentially expressed genes likely to be of interest from a functional point of view is shown in each case.

Table S6. GPR98/PRKAA2/DOCK4 gene mRNA expression in CRC cell lines predicts response to the direct effects of cetuximab

Cell line	% Growth inhibition with cetuximab	Sensitivity category	DOCK4	GPR98	PRKAA2
HCA46	72.8	Sensitive	L	L	L
HT55	21.3	Resistant	H	L	H
HDC82	95.5	Sensitive	L	L	L
SW48	69.1	Sensitive	L	L	L
PMFKO14	0	Resistant	H	L	L
HCA7	50	Partial responder	H	L	H
LIM1863	0	Resistant	L	L	L
SW1222	17.7	Resistant	L	L	L
PCJW	33.4	Partial responder	L	L	L
HDC54	74	Sensitive	L	L	L
HDC73	89.1	Sensitive	L	L	L
HDC142	91.6	Sensitive	L	L	L
C99	96.8	Sensitive	L	L	L
OXCO2	47.1	Partial responder	L	L	L
CCK81	87.2	Sensitive	L	L	L
CAR1	39.1	Partial responder	L	L	H
COLO320DM	0	Resistant	H	H	H
CC20	2.8	Resistant	H	H	H
HDC9	35.9	Partial responder	H	H	H
C10	5.9	Resistant	H	H	H
CACO2	0	Resistant	H	H	H
NCIH716	0.6	Resistant	H	H	H

The level of GPR98/PRKAA2/DOCK4 mRNA expression was used to further classify triple WT tumors according to their response to cetuximab. The cell lines were categorized into high (H, defined as being in the top tercile of the rank plot of mRNA expression for each gene) and low (L, defined as the lower two terciles) mRNA expression for each gene. Cell lines were then ranked in the table according to the expression level of each gene. Cell lines with high expression for all three genes are listed toward the bottom of the table and were mainly cetuximab-resistant (five of six, 83.3%). In contrast, 8 of 12 (66.7%) cell lines with low mRNA expression for all three genes were cetuximab-sensitive (listed at the top of the table).

Table S7. Comparing resistant and partial responsive cell lines vs. sensitive with respect to whether all three genes have low expression or at least one gene has high expression gives a 2 × 2 table

Resistant or sensetive	Low expression in all three genes	High expression in at least one gene
Partial responders and resistant	4	10
Sensitive	8	0

Using Fisher's exact test, this association is significant with a *P* value of 0.0017. Odds ratio = 0.025 and Relative Risk = 0.33.