CHAPTER 7

Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab

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ABSTRACT

Purpose: In a recent randomized phase III clinical trial in metastatic colorectal cancer patients, the addition of the anti-epidermal growth factor receptor (EGFR) monoclonal antibody (MAb) cetuximab to bevacizumab and chemotherapy resulted in decreased progression-free survival, in particular for patients with the high-affinity FcγRIIIA.

Experimental Design: The presence of natural killer (NK) cells and type 2 (M2) macrophages in colorectal cancer was determined by immunohistochemistry using antibodies to lineage-specific markers, respectively, NKp46 and CD68 with CD163. Influence of tumor-bound cetuximab on M2 macrophages was performed *in vitro* with EGFR-expressing tumor cells and short-term differentiated monocytes from blood donors, who were typed for the FcγRIIIA polymorphism (CD16).

Results: ADCC by NK cells is generally proposed as one of the anti-tumor mechanisms of MAbs. We found that CD163-positive, M2 macrophages are much more abundant in colorectal carcinomas. *In vitro* analysis of M2 macrophages revealed high levels of Fc-gamma receptors (FcγRs) and PD-L1, and production of IL-10 and VEGF, but not IL-12. These anti-inflammatory and tumor-promoting mediators were released upon co-culture with EGFR-positive tumor cells loaded with low concentrations of cetuximab. Macrophage activation depended on EGFR expression on the tumor cells, FcγRs, target specificity of the MAb and mobility of antibody complexes. Cetuximab-induced macrophage responses were more pronounced for FCGR3A 158-valine (high affinity) carriers.

Conclusion: These results suggest that tumor-promoting M2 macrophages are activated by the therapeutic MAb cetuximab in the local tumor microenvironment and argue that this immune mechanism should be taken into account for the application of therapeutic antibodies.

Translational Relevance: In a recent clinical phase III study, the addition of the therapeutic MAb cetuximab was evaluated in colorectal carcinoma patients who received chemotherapy and bevacizumab. Surprisingly, patients with cetuximab inclusion in the protocol had a worse progression-free survival than those treated with standard treatment. This detrimental effect was more pronounced in patients with the high affinity Fc-binding receptor FcγRIIIA, implying a role for immune cells. In our consecutive study, we now show that macrophages with M2 differentiation profile are abundantly present in colorectal carcinomas, much more than ADCC-mediating natural killer cells. M2 macrophages are efficiently activated by low dose cetuximab, resulting in the release of immune suppressive and tumor-promoting mediators. Macrophages with the high affinity valine-encoding FcγRIIIA displayed an enhanced activation. We conclude that therapeutic monoclonal antibodies, such as cetuximab, can support tumor growth via tumor associated macrophages in the tumor microenvironment, in addition to their direct cytostatic activity.

INTRODUCTION

Monoclonal antibodies (MAbs) have become important agents for the treatment of many types of malignancies. Generally, their principal mechanism of action is blocking growth factor pathways that are essential for tumor growth and progression. So far, all clinically applied MAbs contain the Fc region of human IgG, which efficiently mediates activation through Fcγ receptors (FcγRs) on several types of immune cells. These IgG-binding receptors actually contribute to the clinical effect of MAbs, in addition to their direct inhibition on tumor growth (1). The role of immune activation is corroborated by several studies describing an association between the rs396991 polymorphism (*FCGR3A* 158Phe→Val) in the gene encoding FcγRIIIA (also known as CD16) and clinical outcome after treatment with the therapeutic MAbs rituximab (anti-CD20), trastuzumab (anti-HER2/*neu*) and cetuximab (anti-epidermal growth factor receptor, EGFR) (2-4). The high affinity valine allele has been associated with increased clinical response and survival in these studies, which is in line with *in vitro* studies indicating that antibody-dependent cellular cytotoxicity (ADCC) is more extensive for this allele (5).

Recently, the addition of cetuximab to bevacizumab plus chemotherapy resulted in decreased progression-free survival in a large clinical trial in metastatic colorectal cancer (CAIRO2 study) (6). To explain this unexpected result, we subsequently analyzed which gene polymorphisms were related to poor outcome in this cohort. This study revealed that patients expressing the high affinity allele for the FcγRIIIA (158Val) had a shorter progression-free time span, but only when cetuximab was added (7). Possibly, the detrimental effect of cetuximab is a consequence of activation of tumor promoting immune cells, rather than triggering tumoricidal ADCC by NK cells. FcγRs are also expressed by macrophages, which have been demonstrated in colorectal carcinomas (8, 9)Tumor-associated macrophages are characterized as M2-type cells and are known to possess anti-inflammatory, pro-angiogenic and tumor-promoting properties (10). Recent literature even implies an active involvement of FcγRs and myeloid cells in carcinoma development (11). We therefore hypothesized that therapeutic antibodies, such as cetuximab, might locally cross-link FcγRs on intratumoral M2-type macrophages by EGFR-binding on tumor cells, resulting in activation and release of tumor-promoting mediators.

These molecular interactions might explain the negative effect of the addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial.

MATERIALS AND METHODS

Immunohistochemical stainings

Formalin fixed and paraffin embedded tumors from ten untreated stage III colorectal cancer patients, of whom the majority was moderately differentiated, 1 well and 2 were poorly differentiated, were selected from the pathology archive of the Leiden University Medical Center. All patients showed recurrent disease at follow-up. Staining for NK cells and M2 macrophages was described before (12).

Cell-cultures

Colorectal adenocarcinoma cell lines LoVo and HCT-15 were kindly provided by Dr. van Wezel (Leiden University Medical Center, the Netherlands) and epidermoid skin cancer line A431 was obtained from ATCC (Manassas, VA, USA). M2-type macrophages and dendritic cells (DCs) were differentiated from purified $CD14^+$ monocytes (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated as previously described (12), using M-CSF (R&D, Minneapolis, USA) or GM-CSF (Invitrogen) with IL-4 (Invitrogen). Cells were activated by 250 ng/ml LPS (Sigma-Aldrich, St. Louis, USA) or tumor cells with MAbs cetuximab (Erbitux®, Merck, Darmstadt, Germany), rituximab or bevacizumab (Mabthera® and Avastin®, respectively; Roche, Welwyn Garden City, UK).

Experimental conditions

At day 6 of the monocyte differentiation cultures, tumor cell lines were plated in a density of 50.000 cells per well in 48-wells plates. After 2 h, 250 ng/ml LPS or MAbs were added together with M2 macrophages in a density of 100.000 cells per well. After 24 h, supernatants were collected and analyzed for IL-10 (Sanquin, Amsterdam, Netherlands), IL-8, VEGF (eBioscience, Vienna, Austria) and IL-12p70 (BD-Biosciences, Minneapolis, MN, USA) production. Macrophages were removed from the culture plates by scraping and stained with monoclonal antibodies (all purchased from BD-Biosciences, except for anti- PD1-L from eBioscience). Samples were recorded using a FACS Calibur with Cellquest software (BD-Biosciences). Data were analyzed with FlowJo software (Tree star, Ashland, USA). Macrophages were separated from tumor cell-lines by gating for HLA-DR.

Genotyping

Genomic DNA was isolated from monocytes with MagnaPure Compact (Roche, Almere, Netherlands) and genotyping for *FCGR3A* c.818A>C (C_25815666_10; rs396991) was performed as previously described (7).

RESULTS

Colon carcinomas are heavily infiltrated with type 2 macrophages, but not with NK cells.

To investigate immune cell infiltration of colorectal cancers, we stained ten tumors for the common macrophage marker CD68, and the scavenger receptor CD163, which is typically expressed by M2-type macrophages. All colorectal tumors were extensively infiltrated with this type of macrophages (figure 1a). By contrast, hardly any NK cells were observed using the NK-lineage specific receptor NKp46. We thus envisage that cetuximab treatment might impact on these infiltrating macrophages, and that local ADCC via NK cells plays a minor role.

The influence of cetuximab on macrophages was studied on freshly isolated monocytes that were differentiated *in vitro* into $CD1a^{-}CD14^{+}CD163^{+}$ macrophages (12). The expression of Fcγ receptors FcγRI (CD64), FcγRII (CD32) and FcγRIIIA (CD16) and release of cytokines after activation by the strong TLR stimulus LPS was examined (figure 1b, c). M2 macrophages strongly displayed all three Fc-binding receptors and produced high amounts of the anti-inflammatory IL-10, as well as IL-8 and the pro-angiogenic VEGF, but not the immunostimulatory IL-12. Control monocyte-derived DCs displayed an opposite profile, which is in line with their T-cell stimulating function. These data strongly suggested that M2-type macrophages could potentially be stimulated by MAbs to exert an anti-inflammatory and pro-angiogenic role in the tumor microenvironment.

Cetuximab induces activation of type 2 macrophages

M2 macrophages were then activated by cetuximab in the presence of tumor cells. Three tumor lines were used (A431, LoVo and HCT-15) and flow cytometry analysis showed that A431

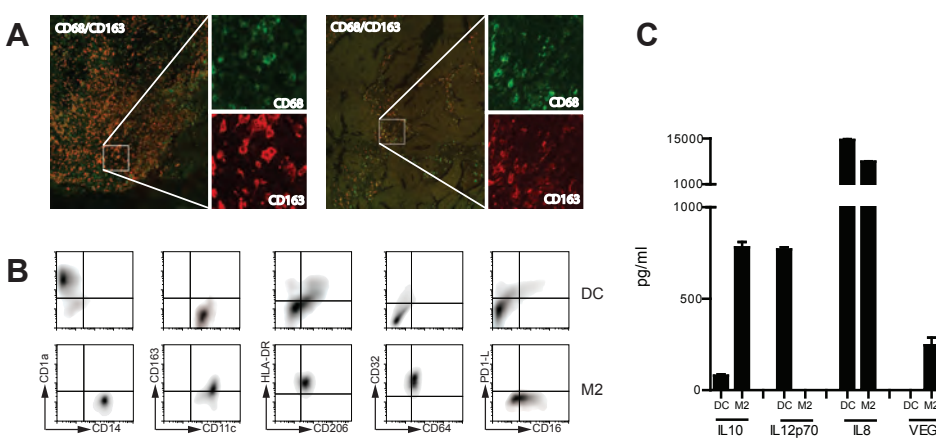

Figure 1. Detection and characterization of M2 macrophages in colorectal cancer. **A**, Two representative examples of two color immunofluorescent staining of stage III colorectal cancers with high (left panel) and low (right panel) infiltration of $CD68^{+}$ (green) macrophages expressing the typical type 2 marker $CD163^{+}$ (red). **B**, Flow cytometry analysis of M2 macrophages and monocyte-derived dendritic cells (mDC). Fcγ receptors are indicated by the CD nomenclature: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). **C**, Production of IL-10, IL-12p70, IL-8 and VEGF by mDCs and M2 macrophages after overnight stimulation with LPS.

highly expressed EGFR, whereas EGFR staining of LoVo and HCT-15 was much lower (figure 2a). Importantly, co-culture of macrophages with cetuximab-opsonized A431 tumor cells resulted in production of IL-10 and IL-8, whereas EGFR-low tumors LoVo and HCT-15 did not activate macrophages (figure 2b, suppl figure 1). Notably, the release of IL-8 upon cetuximab treatment exceeded that of the positive control LPS (suppl figure 1). The A431 tumor cells spontaneously produced VEGF, so this mediator could not be used in succeeding experiments to determine macrophage activation. To corroborate the notion that macrophage activation was the result of cross-linking Fcγ receptors, we incubated tumor cells with MAbs specific for the non-expressed CD20 (rituximab), or the soluble VEGF (bevacizumab), both containing the same IgG_1 isotype. This did not lead to IL-10 production (figure 2c), suggesting that only membrane-bound proteins as EGFR can efficiently trigger macrophages via FcγR. Furthermore, competition of Fc-binding by high concentrations of rituximab resulted in a dose-dependent decrease of IL-10 production (suppl figure 2). Interestingly, IL-10 was also not detected when cetuximab was coated on culture plates (figure 2c), suggesting that the molecular interaction of EGFR-cetuximab-FcγR required the flexibility of fluid membranes for proper cross-linking.

The cetuximab mediated activation of M2 macrophages was dose-dependent (figure 3) and concentrations as low as 10 ng/ml were sufficient to down-regulate cell surface levels of CD16 and to up-regulate the inhibitory molecule PD1-L (figure 3a) and to release IL-10 and IL-8 (figure 3b, suppl figure 1). These data showed that very low concentrations cetuximab induced the release of anti-inflammatory mediators from M2 macrophages through cross-linking of Fcγ receptors.

Effect of FCGRIIIA polymorphism

Addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial decreased the progression-free survival of metastatic colorectal cancer patients, especially for those with

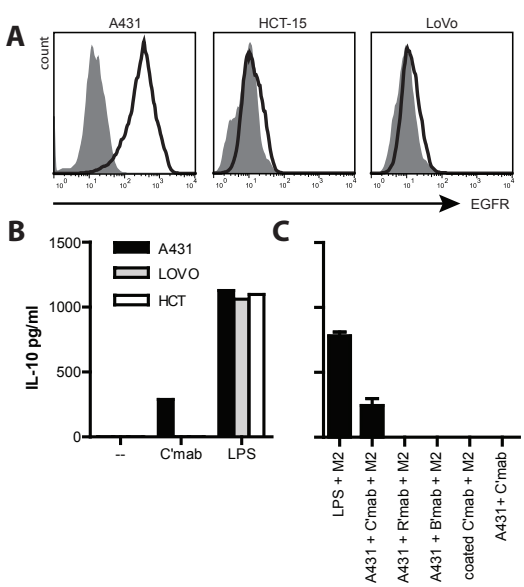

Figure 2. FcγR cross-linking by cetuximab activates M2 macrophages. **A**, Flow cytometry analysis of EGFR expression on tumor cell lines A431, HCT-15 and LoVo. Filled histograms represent isotype control antibody, solid lines indicate staining with anti-EGFR antibody. **B**, IL-10 production by M2 macrophages upon co-culture with tumor cells and 1 µg/ml cetuximab (c'mab). Addition of LPS (250 ng/ml) served as positive control. **C**, IL-10 production by macrophages depends on interaction of macrophages and cetuximab-pulsed A431 tumor cells. Plate-bound cetuximab (10 µg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibodies rituximab (r'mab, 1 µg/ml) or bevacizumab (b'mab, 1 µg/ml) could not replace cetuximab.

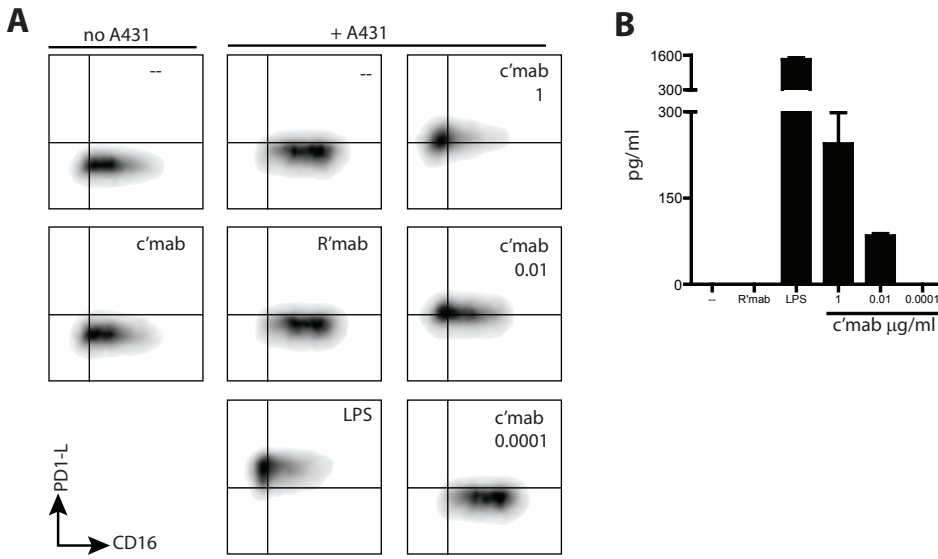

Figure 3. Degree of M2 macrophage activation depends on cetuximab concentration. **A**, Flow cytometry analysis of PD1-L up-regulation and CD16 down-regulation on M2 macrophages upon co-culture with EGFR-positive A431 tumor cells and different cetuximab (c'mab) concentrations. Rituximab (r'mab) served as negative control, LPS as positive control. **B**, Macrophage-derived IL-10 was measured in overnight supernatants.

high affinity FcγRIIIA genotype encoding the valine residue (6, 7). We examined the influence of this polymorphism on the degree of M2 macrophage activation by cetuximab using 22 healthy donors, consisting of 12 homozygous 158-Phe and ten 158-Val carriers (figure 4). Analysis of IL-10 release and CD16 down-regulation on M2 macrophages showed an apparent stronger activation of cells with the high affinity valine allele (figure 4). These differences did not reach statistical significance for cytokine release, most likely due to high variation within the groups and very high production (figure 4a and suppl figure 1, respectively). Notably, macrophage activation in this system is presumably also mediated by other Fcγ receptors, such as FcγRI, resulting in less pronounced differences between 158-Phe and 158-Val carriers. In conclusion, our data show that cetuximab can induce the release of anti-inflammatory mediators from M2 macrophages and that this effect might explain the negative clinical effect of this MAb in the recent CAIRO2 study.

DISCUSSION

Our data show that type 2 macrophages are abundantly present in colon carcinoma and are activated by cetuximab-opsonized tumor cells, resulting in anti-inflammatory and tumor promoting mediators, including IL-10 and VEGF. M2 macrophages are known to actively contribute to tumor growth via angiogenesis and immune suppression (10). Previous research on the immune mechanisms of therapeutic MAbs has focused on anti-tumor effects such as ADCC or phagocytosis. ADCC mediated by NK cells or PBMCs has been described for cetuximab

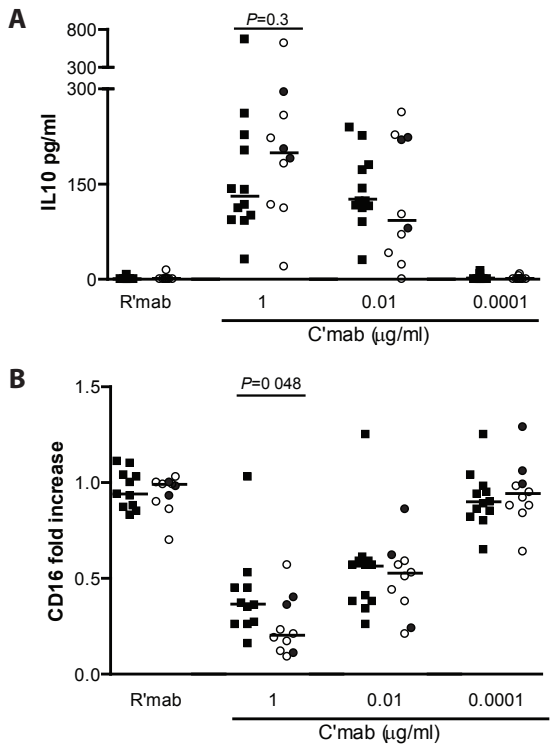

Figure 4. Activation difference of *FCGR3A* (CD16)-typed M2 macrophages. M2 macrophages of twelve homozygous 158-Phe (solid squares) and ten 158-Valine allele carriers (open circles are heterozygous and solid circles are homozygous) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. **A**, CD16 down-regulation and **B**, IL-10 release were measured. Lines represent median values and differences between the two groups at 1 µg/ml cetuximab were calculated by Mann Whitney test

(5, 13), however, staining for NK cells in colorectal carcinoma revealed that these cells are rare in colorectal cancers. Fcγ receptors are also expressed by macrophages and these cells were abundantly present in this tumor type (figure 1). Previous studies have shown that macrophages are present in all stages of colon tumors and that higher numbers of macrophages are found in more advanced stages of disease (8, 9). M2 macrophages are efficient in phagocytosis of rituximab-opsonized B cells (14), but we question the relevance of this FcγR-mediated process for solid tumors like colorectal carcinoma. Based on our findings, we rather suggest that activation of intratumoral M2 macrophages leads to release of tumor-promoting mediators.

The detrimental effect of cetuximab addition in the CAIRO2 trial was unanticipated (6), since the combination of cetuximab and anti-VEGF therapy appeared effective in mouse models (15-17) However, the Fcγ receptor-mediated effects by cetuximab could not be evaluated in these models, as the human Fc-region of cetuximab does not interact with the murine FcγRs. Future studies in mice expressing human FcγR might elucidate immune mechanisms of therapeutic MAbs and, importantly, better predict the outcome of combination studies. Our results indicate that the release of multiple anti-inflammatory and pro-angiogenic mediators by M2 macrophages could account for the decreased therapy efficacy for those patients that were treated with the combination of cetuximab, the anti-VEGF MAb bevacizumab and chemotherapy (6). The finding that M2 macrophages encoding the high affinity FcγRIIIA (valine-carriers) displayed a more pronounced activation (figure 4) corroborated our previous

observation that patients with this high affinity receptor had an even worse progression-free survival than those with 158-Phe homozygosity (7). Strikingly, removal of the high-affinity valine-carriers from the CAIRO2 cohort revealed that the addition of cetuximab did not lead to worse clinical outcome, compared with the trial arm of conventional therapy. On the contrary, the homozygous 158-Phe FcγRIIIA patients seemed to benefit from the addition of cetuximab. However, this analysis was performed on the *KRAS* wild type patients and groups sizes were too small to draw firm conclusions. Notably, bevacizumab binds soluble VEGF and therefore does not cross-link FcγRs and activate intratumoral macrophages (figure 2).

One intriguing question still remains: why does cetuximab mediate anti-tumor effects as a single agent (18, 19), whereas its addition to bevacizumab plus chemo-therapy leads to worse survival? Based on our findings, we speculate that cetuximab induces local release of pro-tumor mediators, amongst which VEGF, and thereby neutralizes the beneficial therapeutic effect of bevacizumab. Interestingly, a clinical study with cetuximab as monotherapy for metastatic colorectal cancers also revealed an increased progression-free survival for low-affinity carriers of FcγRIIIA, especially in combination with certain FCGR2A alleles (20). Combination with chemotherapy might tip the balance further towards macrophage activation by up-regulation of EGFR expression, as shown for fluoropyrimidines and irinotecan (21) and, importantly, also by recruiting immune-suppressive macrophages to the tumor site (22). These indirect immune effects might then overrule the direct growth-inhibiting effect of EGFR blockade.

In conclusion, therapeutic antibodies mediate a plethora of *in vivo* effector arms that reach beyond their on-target function or immediate complement-mediated cytotoxicity. These FcR-dependent mechanisms are diverse *in vivo* and several factors determine the outcome and employed effector arm, including the type of tumor (solid or circulating), type of immune infiltrate (macrophages or NK cells) and combinations with other therapeutics. We now propose an adverse mechanism by which therapeutic MAbs might promote tumor growth via activation of infiltrated macrophages, which are known for their pro-angiogenic and immune-suppressive functions. Clinical testing of engineered MAbs with Fc-regions with increased affinity to FcγRs should be performed very carefully (1, 23), because tumor-promoting effects by intratumoral M2 macrophages could lead to tumor promotion instead of tumor repression.

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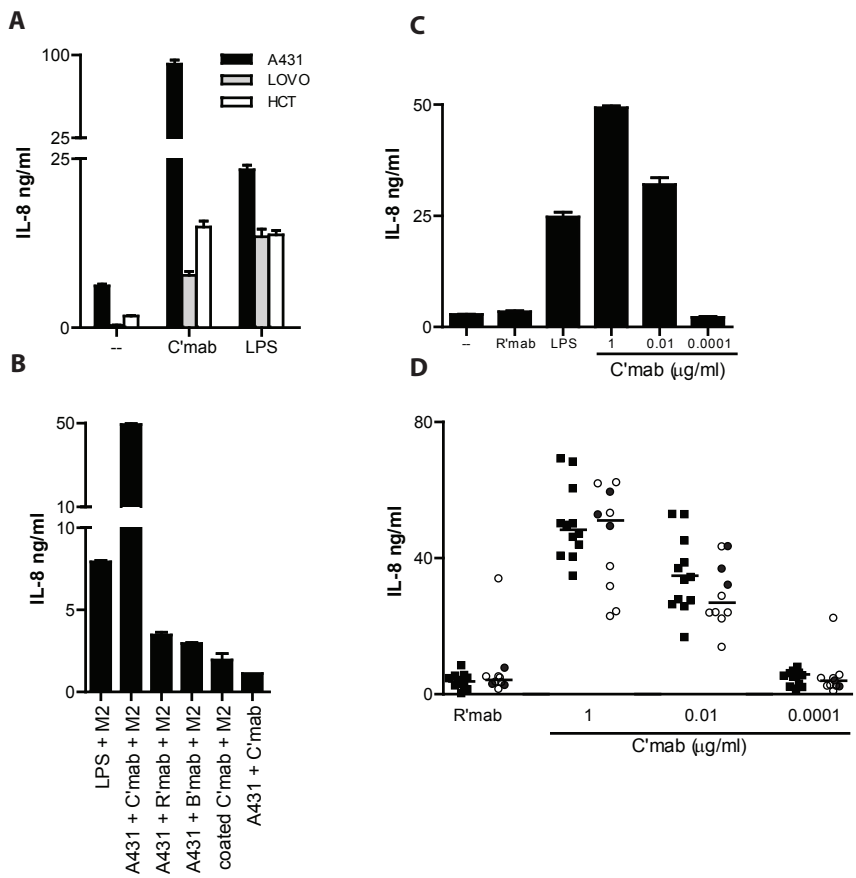
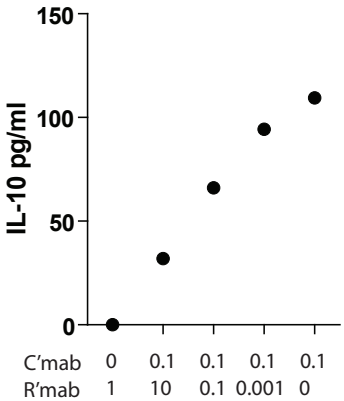


Supplemental figure 1. IL-8 production upon Fc-mediated M2 macrophage activation. **A**, IL-8 production after 24 h co-culture of M2 macrophages with cetuximab (c'mab, 1 μg/ml) opsonized A431 tumor cells (white bars), LoVo cells (light grey bars) and HCT-15 cells (dark grey bars). Addition of LPS (250 ng/ml) served as positive control. **B,** IL-8 production by macrophages depends on interaction of macrophages and cetuximab opsonized tumor cells. Plate-bound cetuximab (10 μg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibody rituximab (r'mab, 1 μg/ml) or bevacizumab (b'mab 1 μg/ml) could not replace cetuximab. **C,** Overnight IL-8 production by M2 macrophages is dependent on the concentration of cetuximab. **D,** Both the phenylalanine homozygote donors (solid squares, n = 12) and valine allele carriers (Phe/Val [open circles] and Val/Val [solid circles] donors combined; n = 10) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. IL-8 release was measured and lines represent median values.



Supplemental figure 2. Competition for Fc-binding by rituximab. A431 tumor cells were loaded without cetuximab (c'mab 0) or with a sub-optimal dose of 0.1 μg/ml cetuximab (c'mab 0.1). Rituximab (r'mab) was added in higher (10 μg/ml), equal (0.1 μg/ml) or lower concentration and M2 macorphages were added. IL10 release was measured after 24 hr by ELISA.