Pharmacogenetics of advanced colorectal cancer treatment

Pander, J.

4

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

Jan Pander[#] • Moniek Heusinkveld[#] • Tahar van der Straaten • Ekaterina Jordanova
Renée Baak-Pablo • Hans Gelderblom • Hans Morreau • Sjoerd van der Burg
Henk-Jan Guchelaar • Thorbald van Hall

[#] These authors contributed equally

Submitted

Abstract

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 efficacy. In order to explain this unexpected clinical observation, we undertook the current hypothesis driven study. Antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells is generally proposed as one of the anti-tumor mechanisms of MAbs. However, we found that CD163-positive, type 2 macrophages (M2s) 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 that were pulsed 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-mediated macrophage responses seemed more pronounced for *FCGR3A* 158-valine carriers, which is the high affinity variant. These results suggest that tumor-promoting type 2 macrophages are activated by this therapeutic MAb in the local tumor microenvironment and argue that this immune mechanism should be taken into account for the application of therapeutic antibodies.

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 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], which was especially significant for patients carrying the high affinity valine allele.[7] Possibly, the detrimental effect of cetuximab is a consequence of activation of tumor promoting immune cells, rather than triggering tumoricidal ADCC. Tumor-associated macrophages are characterized as M2-type cells and are known to possess anti-inflammatory, pro-angiogenic and tumor-promoting properties.[8] Recent literature even implies an active involvement of FcγRs and myeloid cells in carcinoma development.[9] 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 were selected form the pathology archive of the Leiden University Medical Center. Staining for NK cells and M2 macrophages was described before.[10]

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[10], 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

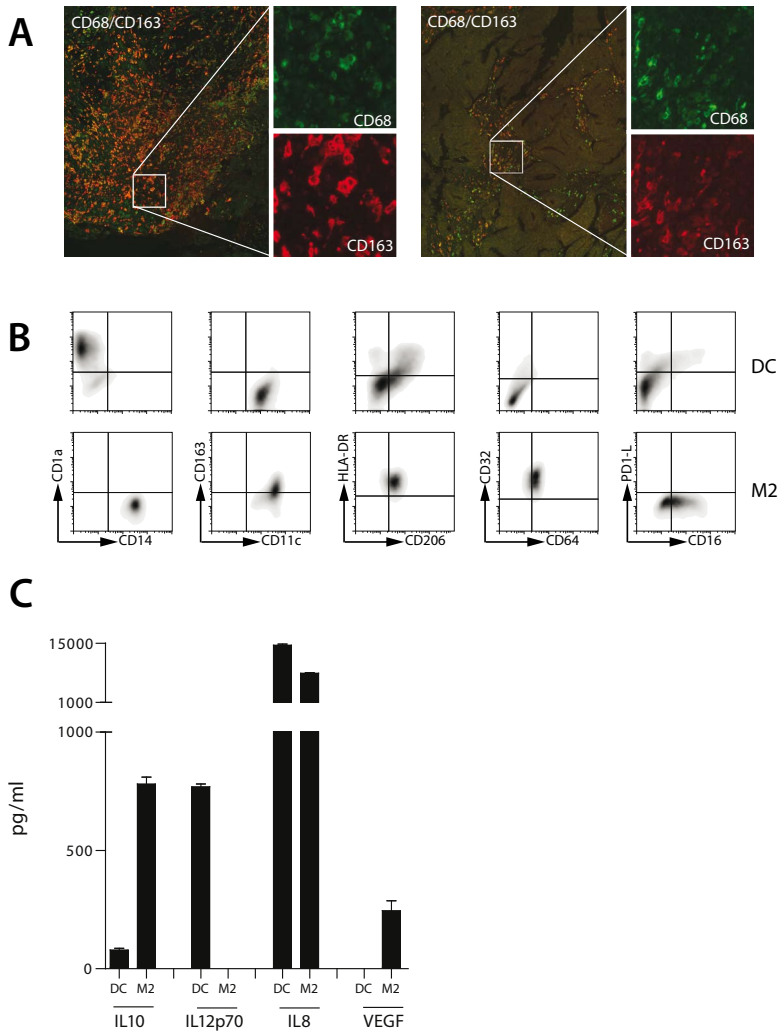
Genomic DNA was isolated from monocytes with MagnaPure Compact (Roche, Almere, Netherlands) and genotyping for *FCGR3A* c.818A>C (C_25815666_10; rs396991) was performed as previously described.[7]

Results

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

To investigate immune cell infiltration of primary stage III colorectal cancers, we stained ten tumors for the common macrophage marker CD68, and the scavenger receptor CD163, which is typically expressed by M2-type cells. 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.

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

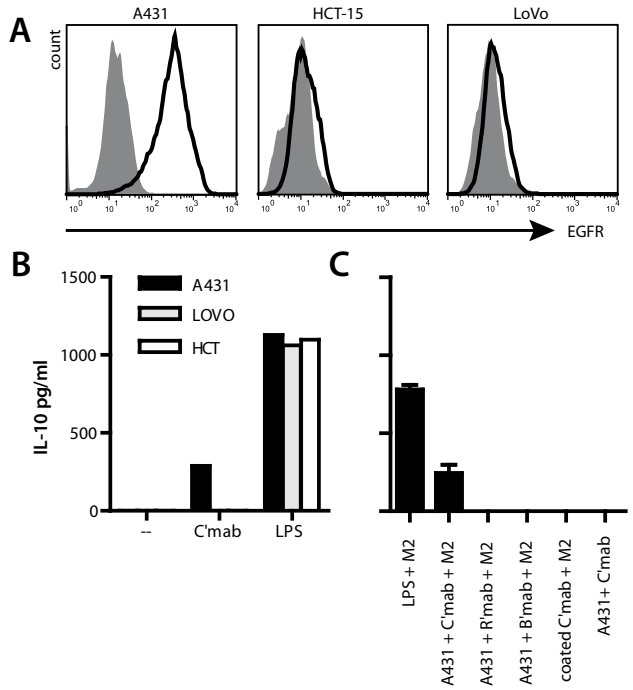
The influence of cetuximab on macrophages was studied on freshly isolated monocytes that were differentiated *in vitro* into $CD1a^{-}CD14^{+}CD163^{+}$ macrophages.[10] The expression of Fcγ receptors FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) and release of cytokines after activation by the strong 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 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, figure S1). Notably, the release of IL-8 upon cetuximab treatment exceeded that of the positive control LPS (figure S1). 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). Furthermore, competition of Fc-binding by high concentrations of rituximab resulted in a dose-dependent decrease of IL-10 production (figure S2). 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, figure S1). These data showed that very low concentrations cetuximab induced the release of anti-inflammatory mediators from M2 macrophages through cross-linking of Fcγ receptors.

Figure 2 FcγR cross-linking by cetuximab activates M2 macrophages

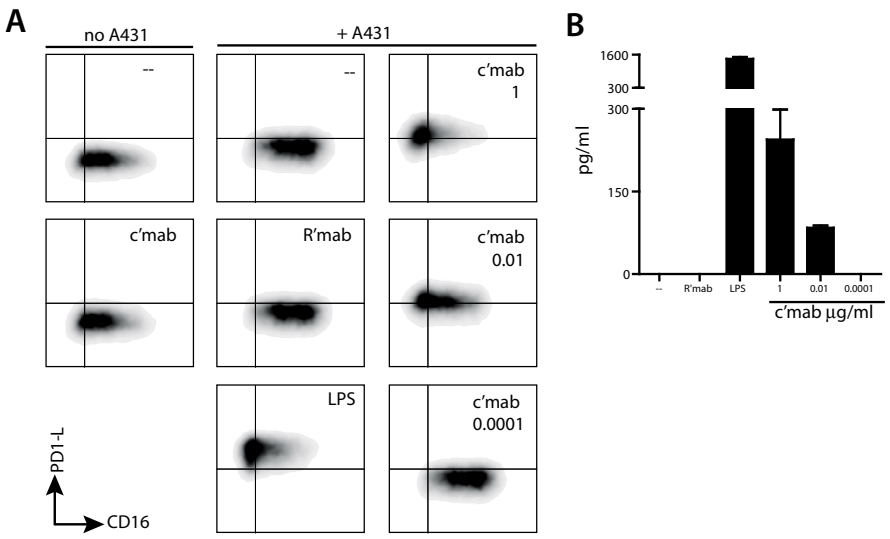


A, Flow cytometry analysis of EGFR expression on tumor cell lines A431, HCT-15 and LoVo. Filled histograms represent isotype control antibody, solid lines indicate staining with anti-EGFR antibody. **B**, IL-10 production by M2 macrophages upon co-culture with tumor cells and 1 μg/ml cetuximab (c'mab). 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.

Effect of *FCGR3A* 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 high affinity FcγRIII 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-

Figure 3 Degree of M2 macrophage activation depends on cetuximab concentration



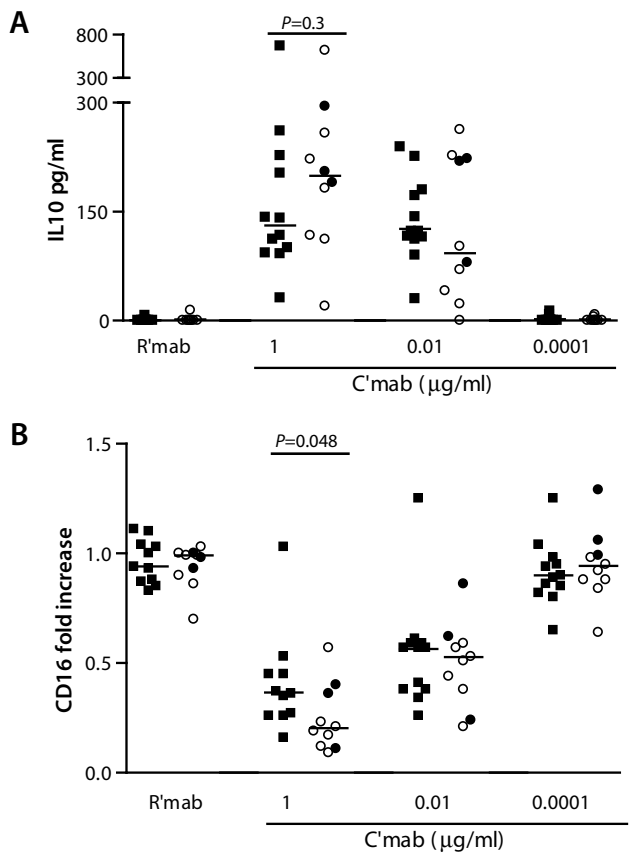
A, Flow cytometry analysis of PD1-L up-regulation and CD16 down-regulation on M2 macrophages upon co-culture with EGFR-positive A431 tumor cells and different cetuximab (c'mab) concentrations. Rituximab (r'mab) served as negative control, LPS as positive control. **B**, Macrophage-derived IL-10 was measured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

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 4b and figure S1, respectively). 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

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.

known to actively contribute to tumor growth via angiogenesis and immune suppression.[8] 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[5], however, staining for NK cells in

stage III 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). M2 macrophages are efficient in phagocytosis of rituximab-opsonized B cells[11], but we question the relevance of this FcγR-mediated process for solid tumors like colorectal carcinoma. We here report that cetuximab-mediated cross-linking of Fcγ receptors leads to release of tumor-promoting mediators.

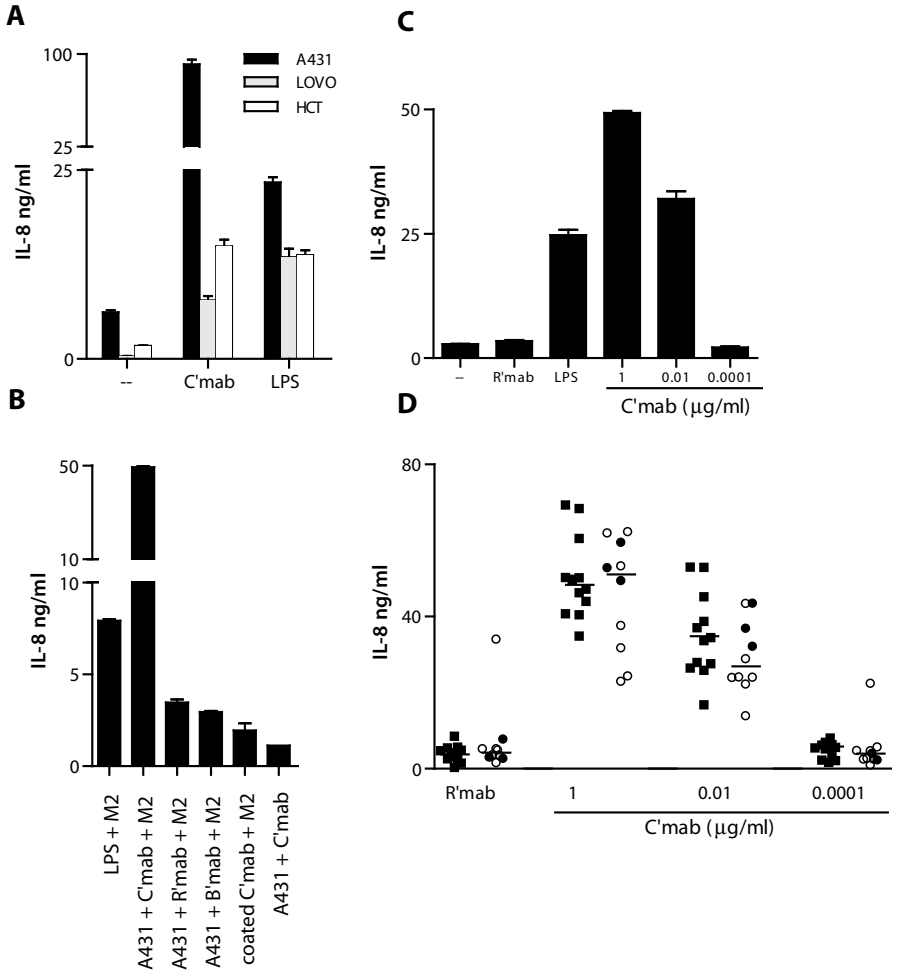
The detrimental effect of cetuximab addition in the CAIRO2 trial was unanticipated[6], since the combination of cetuximab and anti-VEGF therapy appeared effective in mouse models.[12-14] However, the Fcγ receptor-mediated effects by cetuximab could not be evaluated in these models, as the human Fc-region of cetuximab does not interact with the murine FcγRs. 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 fact that M2 macrophages encoding the high affinity FcγRIIIA (valine-carriers) displayed a more pronounced activation (figure 4) corroborated the finding that patients with this high affinity receptor had an even worse progression-free survival than those with 158-Phe homozygosity.[7] Notably, bevacizumab binds soluble VEGF and therefore does not cross-link FcγRs. Moreover, the use of previous or concomitant chemotherapy could influence the infiltration of myeloid cells because of bone marrow suppression.[15] Also, expression of EGFR on tumor cells could be up-regulated by chemotherapy such as fluoropyrimidines and irinotecan.[16]

In conclusion, clinical testing of engineered MAbs with Fc-regions with increased affinity to FcγRs should be performed very carefully[1,17], because tumor-promoting effects by intratumoral M2 macrophages could lead to tumor promotion instead of tumor repression.

Acknowledgements

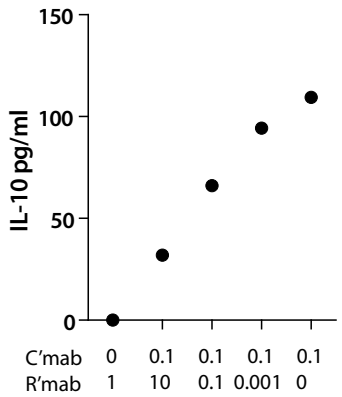
We thank R. Goedemans for technical support and M.H. Lampen and M. Meyering for providing CD14-monocytes.

Figure S1 IL-8 production upon Fc-mediated M2 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.

Figure S2 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 ug/ml), equal (0.1 ug/ml) or lower concentration and M2 macrophages were added. IL10 release was measured after 24 hr by ELISA.

References

1. Weiner LM, Dhodapkar MV, Ferrone S. Monoclonal antibodies for cancer immunotherapy. Lancet 2009;373:1033-40.
2. Bibeau F, Lopez-Crapez E, Di FF, *et al.* Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122-9.
3. Cartron G, Dacheux L, Salles G, *et al.* Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002;99:754-8.
4. Musolino A, Naldi N, Bortesi B, *et al.* Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/*neu*-positive metastatic breast cancer. J Clin Oncol 2008;26:1789-96.
5. López-Albaitero A, Lee SC, Morgan S, *et al.* Role of polymorphic Fc gamma receptor IIIa and EGFR expression level in cetuximab mediated, NK cell dependent in vitro cytotoxicity of head and neck squamous cell carcinoma cells. Cancer Immunol Immunother 2009;58:1853-64.
6. Tol J, Koopman M, Cats A, *et al.* Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 2009;360:563-72.
7. Pander J, Gelderblom H, Antonini NF, *et al.* Correlation of FCGR3A and EGFR germline polymorphisms with the efficacy of cetuximab in KRAS wild-type metastatic colorectal cancer. Eur J Cancer 2010;46:1829-34.
8. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010;11:889-96.
9. Andreu P, Johansson M, Affara NI, *et al.* FcRγ activation regulates inflammation-associated squamous carcinogenesis. Cancer Cell 2010;17:121-34.
10. van Dongen M, Savage ND, Jordanova ES, *et al.* Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. Int J Cancer 2010;127:899-909.
11. Leidi M, Gotti E, Bologna L, *et al.* M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol 2009;182:4415-22.
12. Tonra JR, Deevi DS, Corcoran E, *et al.* Synergistic antitumor effects of combined epidermal growth factor receptor and vascular endothelial growth factor receptor-2 targeted therapy. Clin Cancer Res 2006;12:2197-207.
13. Shaheen RM, Ahmad SA, Liu W, *et al.* Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. Br J Cancer 2001;85:584-9.
14. Jung YD, Mansfield PF, Akagi M, *et al.* Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer 2002;38:1133-40.
15. Douillard JY, Cunningham D, Roth AD, *et al.* Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet 2000;355:1041-7.
16. Correale P, Marra M, Remondo C, *et al.* Cytotoxic drugs up-regulate epidermal growth factor receptor (EGFR) expression in colon cancer cells and enhance their susceptibility to EGFR-targeted antibody-dependent cell-mediated-cytotoxicity (ADCC). Eur J Cancer 2010;46:1703-11.
17. Griggs J, Zinkewich-Peotti K. The state of the art: immune-mediated mechanisms of monoclonal antibodies in cancer therapy. Br J Cancer 2009;101:1807-12.