Activation of Tumor-Promoting Type 2 Macrophages by EGFR-Targeting Antibody Cetuximab

Jan Pander1, Moniek Heusinkveld2, Tahar van der Straaten1, Ekaterina S. Jordanova3, Renée Baak-Pablo1, Hans Gelderblom2, Hans Morreau3, Sjoerd H. van der Burg2, Henk-Jan Guchelaar1, and Thorbald van Hall2

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 NKp46 and CD68 with CD163, respectively. Influence of tumor-bound cetuximab on M2 macrophages was carried out in vitro with EGFR-expressing tumor cells and short-term differentiated monocytes from blood donors, who were typed for the FcγRIIIA polymorphism (CD16).

Results: Antibody-dependent cellular cytotoxicity by NK cells is generally proposed as one of the antitumor 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 (FcγR) and PD-L1 and production of IL-10 and VEGF but not IL-12. These anti-inflammatory and tumor-promoting mediators were released upon coculture 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-Val (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.

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Introduction

Monoclonal antibodies (mAb) 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 (FcγR) 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 158-Phe→Val) in the gene encoding FcγRIIIA (also known as CD16) and clinical outcome after treatment with the therapeutic mAb 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; ref. 6). To explain this unexpected result, we subsequently analyzed which gene polymorphisms were related to poor outcome in this cohort. This study...
Staining for NK cells and M2 macrophages was described in the pathology archive of the Leiden University Medical Center. The majority were moderately differentiated, 1 well differentiated, 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).

### Materials and Methods

#### Immunohistochemical stainings

Formalin-fixed and paraffin-embedded tumors from 10 untreated stage III colorectal cancer patients, of whom the majority were moderately differentiated, 1 well differentiated, 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).

#### Translational Relevance

In a recent clinical phase III study, the addition of the therapeutic monoclonal antibody (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 type 2 (M2) differentiation profile are abundantly present in colorectal carcinomas, much more than antibody-dependent cellular cytotoxicity–mediating natural killer cells. M2 macrophages are efficiently activated by low-dose cetuximab, resulting in the release of immunosuppressive and tumor-promoting mediators. Macrophages with the high-affinity valine-encoding FcγRIIIA displayed an enhanced activation. We conclude that therapeutic mAbs, such as cetuximab, can support tumor growth via tumor-associated macrophages in the tumor microenvironment, in addition to their direct cytostatic activity.

#### 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 American Type Culture Collection. M2 macrophages and dendritic cells (DC) were differentiated from purified CD14+ monocytes (MACS; Miltenyi Biotec) and differentiated as previously described (12), using M-CSF (R&D System) or GM-CSF (Invitrogen) with IL-4 (Invitrogen). Cells were activated by 250 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich) or tumor cells with mAbs cetuximab (Erbitux; Merck), rituximab, or bevacizumab (Mabthera and Avastin, respectively; Roche).

#### Experimental conditions

At day 6 of the monocyte differentiation cultures, tumor cell lines were plated at a density of 50,000 cells per well in 48-well plates. After 2 hours, 250 ng/mL LPS or mAbs were added together with M2 macrophages at a density of 100,000 cells per well. After 24 hours, supernatants were collected and analyzed for IL-10 (Sanquin), IL-8, VEGF (eBioscience), and IL-12p70 (BD-Biosciences) production. Macrophages were removed from the culture plates by scraping and stained with mAbs (all purchased from BD-Biosciences, except for anti-PD1-L, which was from eBioscience). Samples were recorded using a FACS Calibur flow cytometer with CellQuest software (BD-Biosciences). Data were analyzed with FlowJo software (Tree star). Macrophages were separated from tumor cell lines by gating for HLA-DR.

#### Genotyping

Genomic DNA was isolated from monocytes with MagNA Pure Compact (Roche), and genotyping for FCGR3A c.818A>C (C_25815666_10; rs396991) was carried out as previously described (7).

#### Results

**Colon carcinomas are heavily infiltrated with M2 macrophages but not with NK cells**

To investigate immune cell infiltration of colorectal cancers, we stained 10 tumors for the common macrophage marker CD68, and the scavenger receptor CD163, which is typically expressed by M2 macrophages. All colorectal tumors were extensively infiltrated with this type of macrophages (Fig. 1A). In 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 CD14+CD163- macrophages (12). The expression of FcγRs FcγRI (CD64), FcγRII (CD32), and FcγRIIIA (CD16) and release of cytokines after activation by the strong TLR stimulus LPS was examined (Fig. 1B...
and C). M2 macrophages strongly displayed all 3 Fc-binding receptors and produced high amounts of the anti-inflammatory IL-10, as well as IL-8 and the proangiogenic VEGF, but not the immunostimulatory IL-12. Control monocyte-derived DCs (mDC) displayed an opposite profile, which is in line with their T-cell stimulating function. These data strongly suggested that M2 macrophages could potentially be stimulated by mAbs to exert an anti-inflammatory and proangiogenic role in the tumor microenvironment.

Cetuximab induces activation of M2 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 cytometric analysis showed that A431 highly expressed EGFR whereas EGFR staining of LoVo and HCT-15 was much lower (Fig. 2A). Importantly, coculture of macrophages with cetuximab-opsonized A431 tumor cells resulted in the production of IL-10 and IL-8 whereas EGFR-low tumors LoVo and HCT-15 did not activate macrophages (Fig. 2B, Supplementary Fig. S1). Notably, the release of IL-8 upon cetuximab treatment exceeded that of the positive control LPS (Supplementary Fig. S2). The cetuximab-mediated activation of M2 macrophages was dose dependent (Fig. 3) and concentrations as low as 10 ng/mL were sufficient to downregulate cell surface levels of CD16, to upregulate the inhibitory molecule PD1-L (Fig. 3A), and to release IL-10 and IL-8 (Fig. 3B, Supplementary Fig. S1). These data showed that very low concentrations of cetuximab induced the release of anti-inflammatory mediators from M2 macrophages through cross-linking of FcRs.

Effect of FcγRIIIA 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 FCGRA3 genotype encoding the valine residue (6, 7). We examined the influence of this polymorphism on the degree of M2 macrophage activation by cetuximab on 22 healthy donors, consisting of 12 homozygous 158-Phe and 10 158-Val carriers (Fig. 4). Analysis of IL-10 release and CD16 downregulation on M2 macrophages showed an apparent stronger activation of cells with the high-affinity valine allele (Fig. 4). These differences did not reach statistical significance for cytokine...
release, most likely due to high variation within the groups and very high production (Fig. 4A and Supplementary Fig. S1, respectively). Notably, macrophage activation in this system is presumably also mediated by other FcγRs, 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 M2 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 peripheral blood mononuclear cells has been described for cetuximab (5, 13); however, staining for NK cells in colorectal carcinoma revealed that these cells are rare in colorectal cancers. FcγRs are also expressed by macrophages and these cells were abundantly present in this tumor type (Fig. 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 such as colorectal carcinoma. On the basis of 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), because the combination of cetuximab and anti-VEGF therapy seemed effective in mouse models (15–17). However, the FcγR-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 proangiogenic mediators by M2 macrophages could account for the decreased therapy efficacy for those patients who were treated with the combination of cetuximab, the anti-VEGF mAb bevacizumab, and chemotherapy (6). The finding that M2 macrophages encoding the high-affinity FcγRIIA (valine carriers) displayed a more pronounced activation (Fig. 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 FcγRIIA patients seemed to benefit from the addition of cetuximab. However, this analysis was carried out 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 (Fig. 2).

One intriguing question still remains: Why does cetuximab mediate antitumor effects as a single agent (18, 19), whereas its addition to bevacizumab plus chemotherapy leads to worse survival? On the basis of our findings, we speculate that cetuximab induces local release of protumor mediators, including 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 FcγRIIA, especially in combination with certain FcγRIIa alleles (20). Combination with chemotherapy might tip the balance further toward macrophage activation by upregulation of EGFR expression, as shown for fluoropyrimidines and irinotecan (21), and, importantly, also by recruiting immunosuppressive 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 proangiogenic and immunosuppressive functions. Clinical testing of engineered mAbs with Fc regions with increased affinity to FcγRs and therefore does not cross-link FcγRs and activate intratumoral macrophages (Fig. 2).

**Disclosure of Potential Conflicts of Interest**

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References


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