Epidermal growth factor receptor as target for monoclonal antibody-mediated phagocytosis of colon carcinoma cells by macrophages

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Submitted
Abstract

During resection of primary colorectal tumors malignant cells disseminate from the tumor mass and are shed into the portal circulation. Surgical trauma leads to decreased vessel wall integrity and subsequent exposure of underlying extracellular matrix proteins enhances adhesion of disseminated tumor cells. Although surgical resection of colorectal tumors is the only curative option to ascertain survival, trauma inflicted by this procedure may lead to enhanced outgrowth of distant metastases and decreases both overall and disease free survival in a cohort of patients. Peri-operative treatment with specific monoclonal antibodies (mAb) directed against circulating tumor cells alleviated surgically induced liver metastases of colorectal tumors in several animal models. Successful elimination of circulating tumour cells was mediated by liver macrophages (Kupffer cells). We now demonstrate that by using the fully human mAb Zalutumumab directed against the epithelial growth factor receptor (EGFR), macrophages are able to detect and kill colon carcinoma cells via antibody dependent phagocytosis. Efficacy of ADPh was dependent of surface expression of EGFR on malignant cells, but independent of mutations in downstream EGFR kinases KRAS and BRAF. Because EGFR is expressed on ~80% of colorectal tumours, Zalutumumab is therefore an ideal candidate for peri-operative treatment of patients to inhibit surgically induced metastasizing of colorectal cancer, presenting a novel therapeutic strategy use for an established drug.
**Introduction**

With over 700,000 new cases each year, colorectal cancer (CRC) is the second most common malignancy in the western world. Although advances in CRC treatment have increased 5-year relative survival rates from 51% in 1970 to approximately 67% nowadays, CRC still leads to over 300,000 deaths in western society.\(^1\) Surgical excision of the primary tumor is the basis of curative therapy and critical for improved survival.\(^2\)\(^,\)\(^3\) Unfortunately colorectal liver metastases (CLM) are detected in approximately 20-25% of CRC patients at time of diagnosis, and with a median survival of 1 year, prognosis of non-treated patients with CLM is extremely poor.\(^4\) Moreover, 10-25 % of patients without detectable CLM at time of diagnosis, will subsequently develop distant metastases after removal of the primary tumor.\(^5\) This support the presence of undetected minimal residual disease at the time of, or after surgery.

Already in the beginning of the last century William Halsted hypothesized that surgery of mamma carcinomas led to dissemination of free tumor cells into the circulation.\(^6\) Furthermore, methods to isolate circulating malignant cells from the blood of CRC patients have been described since 1960-70s.\(^7\) The percentage of CRC patients, in which free circulating tumor cells have been detected, ranges between 10-70% depending on the method of detection.\(^8\) Nowadays the presence of free circulating tumor cells has been proposed as an independent prognostic factor for survival of CRC patients.\(^9\)\(^,\)\(^10\) Experiments in animal models aptly showed that dissemination of tumor cells into the circulation occurs spontaneously from existing solid tumors, which is enhanced after manipulation of the tumor.\(^11\)\(^,\)\(^12\) The augmentation of free circulating tumor cells after and during surgery of the primary tumor has also been extensively described in human patients. Furthermore, increased numbers of circulating tumor cells in the portal system have been associated with decreased overall survival.\(^13\)\(^-\)\(^15\)

Over the years evidence has accumulated, which paradoxically supports that trauma, inflicted by surgical procedures to excise primary tumors, is associated with risk of developing metastases.\(^5\)\(^,\)\(^16\)\(^,\)\(^17\) By imitating surgery procedures in animal models we previously demonstrated that surgical trauma induced a systemic effect leading to enhanced tumor cell adhesion of circulating tumor cells but not to enhance growth of existing tumor cell clusters.\(^18\) The exact mechanisms of surgery-induced tumor cell adhesion have not yet been completely elucidated, but it was shown that production of reactive oxygen species (ROS) led to damage of the liver vasculature and subsequent enhanced tumor cell adhesion.\(^19\) Nonetheless, resection of the CRC is mandatory as first treatment, and will remove the bulk of the tumor load. The peri-operative period remains an attractive window of opportunity in which expunging the remaining free disseminated tumor cells may reduce formation of liver metastases and improve overall patient survival.\(^3\)\(^,\)\(^16\)\(^,\)\(^17\)
A promising approach to reduce surgically induced metastases formation may be the stimulation of anti-tumor immune responses with the use of monoclonal antibodies (mAb). mAb can have direct effects on tumor cells, like the induction of apoptosis or inhibition of proliferation. Furthermore, mAb can activate the complement pathway, which leads to complement-dependent lysis, and mAb can recruit immune cells for antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADPh). We previously showed that postoperative treatment of rats with mAb directed against rat colon carcinoma cells prevented surgery induced liver metastases development in a rat model. Most clinical trials in which mAb are used as either monotherapy or in addition to chemotherapy focus on direct effects of mAb on regression of tumors in patients with larger metastatic tumor masses. Effects of mAb therapy to prevent new distant recurrences of disease are however sparse. One single study showed increased 7 year survival and reduced overall mortality in patients treated postoperatively with mAbs, in whom the primary colorectal tumor was microscopically completely resected. Treatment with this murine IgG2a mAb directed against human Ep-CAM on colon carcinoma cells had no effect on local recurrence of the primary tumor. However, the occurrence of distant metastases was reduced, albeit only in ~30% of the treated patients. This may be due to the use of xenogenic anti-EpCam antibodies, but until now unprecedented promising outcome of this trial warrants further investigation. Pre- or peri-operative treatment of colorectal patients without any evidence of distant metastases (e.g. stage I/II cancer) with less immunogenic human(ized) IgG1 antibodies that have extended serum half-lives and harness efficient human effector mechanisms may be an elegant way to reduce surgery-induced distant metastases formation.

A potential target for peri-operative immune therapy of colorectal cancer is the human epithelial growth factor receptor, which is up-regulated in 80% of colorectal cancer cases. Monotherapy with humanised anti-EGFR mAb has yielded fluctuating and somewhat disappointing results when treating existing colorectal cancer metastases. This is likely due to the fact that anti-EGFR mAb mostly likely induce direct effects in existing tumors, which are dependent on EGFR signalling. The low response rate in CRC patients can be explained by frequently occurring mutations in the EGFR downstream targets KRAS and BRAF, making the tumor unresponsive for EGFR directed therapies. However, in earlier animal experiments we demonstrated that prevention of surgically-induced metastases development by mAb treatment was crucially dependent on the presence of macrophages and Fcγ Receptors. As such, we investigated whether the fully human anti-EGFR mAb Zalutumumab represents a suitable candidate for peri-operative treatment of patients undergoing resection for primary CRC.
Materials and methods

Antibodies
The human IgG1, κ, EGFR-specific mAb zalutumumab (HuMax-EGFr, clone 2F8) was generated by immunizing HuMAb mice (Medarex, Milpitas, CA) and produced as recombinant proteins as described previously. The N297Q mutation in the Fc part of zalutumumab, referred to as zalu-N297Q was introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was checked by sequencing (LGC Genomics, Berlin, Germany). IgG concentrations were determined by A280 measurements. Human IgG1, κ mAb specific for hepatitis C (HEPC), also generated in HuMAb mice, was included in all experiments as isotype control mAb. For human IgG2 variant of Zalutumumab (Zalu-IgG2) variable heavy chain of Zalutumumab was co-expressed with hlgG2 FC domain. Zalutumumab heavy chain/hlgG2 construct was co-expressed with human κ-light chain. Secondary goat F(ab')2 anti human IgG-RPE antibodies were purchased from AbD Sertotec (Oxford, UK).

Cell Culture

Tumor cells. The human colon carcinoma cell lines HT29, HCT116 and RKO and the human vulvar carcinoma cell A431 (ATCC, Manassas, VA) were cultured under standard incubator conditions in DMEM (Invitrogen, Paisley, UK), supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamin (200 µM) (further referred to as complete DMEM). Murine fibroblast L929 and C26 colon carcinoma cell lines (ATCC) were cultured in RPMI 1640 (Invitrogen), supplemented with 10% heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamin (200 µM) (further referred to as complete RPMI). C26 cells that had been transfected with human EGFR, were cultured in complete RPMI supplemented with 3.5 mg/ml geneticin (G418, Invitrogen). Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution (Invitrogen). Viability was assessed by trypan blue exclusion and always exceeded 95%.

Transfection. Mouse C26 (wildtype; wt) colon carcinoma cells were grown to sub-confluency in 6 well plates and transfected with 1 µg human EGFR construct (hEGFR in pUSE/amp/neo vector, Upstate biotechnology, Lake Placid NY) using the Fugene-6 transfection system (Roche Applied Science, Basel, Switzerland), according to the manufacturer’s instructions. Positive cells were selected 48 hours after transfection, by incubating cells with 3.5 µg/ml G418. C26 expressing hEGFR were sorted using a MoFlo XDP flow cytometry cell sorter (Beckman Coulter Inc, Miami, FL) into C26 hEGFR low, C26 hEGFR int/low, C26 hEGFR int/high or C26 hEGFR high clones. Cells were resorted every 2 weeks with a minimum of 10 sortings to obtain stable C26 hEGFR cell lines.

L929 cell conditioned medium (LCM). Mouse L929 cells secrete macrophage-
colony stimulating factor (M-CSF) and were used to produce L929 cell conditioned medium (LCM) to differentiate macrophages. L929 cells were grown to confluency, after which medium was changed with fresh complete RPMI. Cells were grown for 7 days, after which LCM was harvested, centrifuged at 4750xg for 10 minutes, filtered through 0.2 µm filters and stored at -20°C till further use.

**Murine bone marrow macrophages.** Wild type balb/c mice were bred and maintained at the Central Animal Facility of the VU University Medical Center (Amsterdam, The Netherlands) under standard conditions. The Committee for Animal Research of the VU University Medical Center approved all experiments, according to institutional and national guidelines. Bone marrow was harvested from freshly isolated femur, tibia and humerus. After removal of connective tissues and muscles, bone marrow was flushed and single cell suspensions were made by passing bone marrow through a sterile 70 µm filter (BD Falcon, Bedford, MA). Macrophages were differentiated by incubating bone marrow cells for 7 days with complete DMEM, supplemented with 15% LCM (hereafter referred to as Mø medium). Macrophages were harvested after a 15 minute incubation with trypsin-EDTA and subsequent scraping using a cell scraper. Macrophages were seeded in 24 well plates (4x10^5/ well) for *in vitro* cytotoxicity assays or in 8 well ibiTreat µ-Slides (IBIDI, Munich, Germany) for live cell imaging (2x10^5/ well).

**Human macrophages.** Human monocytes were isolated from human blood buffycoats < 24h after blood collection (Sanquin, Amsterdam, The Netherlands) of healthy listed blood donors. All patients gave informed consent according to the guidelines of the medical ethical committee of the VUMc. Whole blood was diluted 1:1 in PBS, loaded on lymphoprep (Nyegaard, Oslo, Norway) and a gradient was formed by centrifugation (30 minutes, 800 x g without brake). Peripheral blood mononuclear cells (PBMCs) were extracted from the interphase of the lymphoprep gradient, subsequently washed three times in PBS supplemented with autologous serum and reconstituted in complete DMEM. PBMCs were loaded on a Percoll gradient (GE Healthcare, Uppsala, Sweden) (46.1 % Percoll, 0.15 mM NaCl) and centrifuged for 40 minutes at 400xg without brake at 22°C. Human monocytes were isolated from the interphase, washed in complete DMEM, counted and seeded in 10 cm² plastic cell culture petri-dishes (10-15x10⁶ cells/dish) in complete DMEM, supplemented with 10 ng/ml human granulocyte/ macrophage- colony stimulating factor (ImmunoTools, Friesoythe, Germany). Human monocytes were led to differentiate into macrophages for 7 days, after which cells were harvested by incubation with trypsin-EDTA and subsequent scraping using a cell scraper. Macrophages were seeded after fluorescent labelling in 24 well plates (4x10^5/ well) for *in vitro* cytotoxicity assays.

**Flow cytometry**

Human carcinoma cell lines HT29, RKO, HCT116 and A431 or mouse C26EGFR cell lines were incubated with primary anti-human EGFR or anti-HEPC (isotype control) antibodies (20 µg/ml) for 45 minutes at 4°C. After washing, primary antibody was
detected by incubation with PE-conjugated goat-anti-human IgG mAb (1:50). Cells were analyzed with flow cytometry (FacsCalibur, BD, San Jose, CA).

**Fluorescent labeling**

For *in vitro* cytotoxicity assays human or mouse tumor cells were harvested and incubated (1-10x10^6 cells/ml) in complete DMEM supplemented with 2.5 µg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, and subsequently washed three times with complete DMEM. Mouse or human macrophages were incubated (1-10x10^6 cells/ml) in complete Mø medium, supplemented with 2.5 µg/ml 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO, Molecular Probes Inc, Paisley, UK) or DiO for 30 minutes at 37°C and subsequently washed three times with complete Mø medium. Alternatively, macrophages were labeled with the Lyso-ID Red lysosomal staining (Enzo Biochem Inc, Farmingdale, NY), according to the manufacture's instructions. Tumor cells were labelled with DiO for these experiments.

**Viability assays**

**MTT assay.** Cell viability of different tumor cells was measured by MTT assay as previously described 29. Briefly, after stimulation with a concentration range of Zalutumab or HEPCγ1 mAb for 4 to 120 h, tumor cells were incubated with MTT (0.5 mg/ml) for 1-2 h at 37°C. Viable cells converted MTT into insoluble formazan after which excess MTT was washed away. The formed formazan was dissolved in 85%DMSO/14mM glycine and measured at 450 nm in a platereader (BioRad, Hercules, CA).

**In vitro cytotoxicity assays.** Cytotoxicity assays were performed by co-culturing Dil-labeled human or mouse tumor cells and DiO-labeled macrophages in an effector to target (E:T) ratio of 15:1 in the presence of different concentrations human anti-human EGFR IgG1, IgG2 or IgG1 N297Q or human anti-HEPC IgG1 antibodies as isotype control. Percentages of remaining tumor cells and macrophages that had taken up tumor cells (double-positive) were determined by flow cytometry after 24 hours of co-culture. Percentages of tumor cells and double-positive macrophages after culture without mAbs were set at 100%. Real time cytotoxicity assays were performed with an Olympus CellR real-time live-imaging station (type IX81, UPLFLN 40 x O/1.3 lens, Münster, Germany). Macrophages were either labeled with DiO or Lyso-ID Red. Dil or green DiO-labeled tumor cells were added in an E:T ration 15:1 with different concentrations of anti-hEGFR or anti-HEPC antibodies. Pictures were taken every 3 minutes with an Olympus ColorView II camera for 3-6 h, followed by a 6 minutes interval for 18-21 hours. Additionally, random pictures were taken after 24 h.

**Statistical analysis**

Data was analyzed with Bonferroni-Post Hoc tests, preceded by two way ANOVA tests for comparison of multiple groups. Significance was accepted at p < 0.05.
Inhibition of cell proliferation by Zalutumumab is dependent on the level of EGFR expression.

Clinically used anti-EGFR mAbs have been shown to competitively inhibit EGF binding and block activation of downstream receptor tyrosine kinases KRAS and BRAF, subsequently leading to G₁ cell cycle arrest and growth inhibition. Therefore we first analysed whether incubation with zalutumumab— which binds the extracellular domain III of EGFR and locks the receptor in an inactive configuration— led to diminished tumor cell viability or proliferation. EGFR expression was determined on a panel of colon carcinoma cell lines (Figure 1a). HT29 and HCT116 had the highest expression, whereas RKO had minimal EGFR on its surface, and cells were grown in the presence of different concentrations of zalutumumab. No effect on cell viability of all carcinoma cells tested, was observed, even in the presence of saturating concentrations of zalutumumab (10 µg/ml) for 4 or 24 hours (Figure 1b and Suplementary Figure 1a). Incubation with antibody concentrations exceeding 1 µg/ml for over 24h, significantly reduced proliferation of A431 vulvar carcinoma cells.

**Figure 1:** (a) EGFR expression on human carcinoma cell lines, (b) cell viability after 4 hours with Zalutumumab and (c) tumour cell proliferation after EGFR blocking with 1 µg/ml Zalutumumab antibodies in time. Untreated tumour cells set as 100%. **p<0.01, ***p<0.001
cells, which over-express high levels of EGFR (Figure 1a and c), whereas no effects were seen on short term cell viability (Figure 1b). However, long term blocking of EGFR (>24 hours) on KRAS mutated HCT116 cells, BRAF mutated HT29 cells or the low EGFR-expressing RKO cell line with zalutumumab did not lead to any differences in cell proliferation (Figure 1b-c).

**Macrophages kill tumor cells through antibody-dependent phagocytosis.** We previously demonstrated that efficient mAb therapy of surgery-induced liver metastases in rats was mediated by liver macrophages (Kupffer cells; KC) and to a lesser extent by monocytes. As such, we investigated whether addition of zalutumumab would lead to eradication of tumor cells by macrophages. Incubation of A431 tumor cells with macrophages in the presence or absence of increasing concentrations of non-specific anti-HEPC antibodies did not lead to decreased tumor cell outgrowth after 24h (Figure 2a). However, even though blocking EGFR

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**Figure 2:** FACS analysis of A431/macrophage co-cultures after 24 h. treatment with different concentrations of (a) HEPC-γ1 or (b) Zalutumumab. Dil stained A431 measured in FL2, DIO stained macrophages measured in FL1, FL1/FL2 double positive cells represent macrophages which have taken up tumour cells. Analysis of remaining (c) A431 or (e) RKO tumour cells and (d/f) double positive macrophages, lowest concentration of HEPC-γ1 is set as 100%. **p<0.01, ***p<0.001
on A431 cells with 100 ng/ml zalutumumab had no direct effect on cell viability or proliferation (Figure 1b and supplementary 1b), incubation of macrophage-A431 co-cultures with low concentrations of zalutumumab (ranging from 100 to 1 ng/ml) led to significant reduced A431 cell numbers (Figure 2/c). This effect was abrogated only at concentrations lower than 0.1 ng/ml (Figure 2b). Addition of increasing zalutumumab concentrations furthermore led to enhanced numbers of double positive macrophages (Figure 2D), compared to A431/macrophage co-cultures that had been incubated with similar concentrations of isotype control antibodies.

To investigate the mode of killing by macrophages, we performed live cell imaging microscopy, which revealed rapid antibody dependent phagocytosis (ADPh) of whole A431 cells within 90 minutes by macrophages in the presence of zalutumumab (Figure 3a, b), whereas addition of irrelevant isotype antibodies did not lead to tumor cell uptake (Figure 3c, d). Tumor cells were gradually degraded after ADPh, and virtually no live tumor cells were present after 16 hours (Figure 3a, b), whereas A431 cells grew out in clusters in the presence of an isotype antibody (Figure 3c, d, 2).
Antibody dependent phagocytosis of tumor cells by macrophages leads to slow lysosomal degradation

Within 60 minutes after incubation co-cultures of macrophages and human A431 tumor cells with zalutumumab, macrophages efficiently phagocytosed one to three whole tumor cells (Figure 4a). However, subsequent killing and degradation of phagocytosed tumor cells took up to >20 hours, depending on the number of tumor cells which had been taken up. To visualize active lysosomes, macrophages were labelled with the acidic organelle-specific dye Lyso-ID. Within 30 minutes after phagocytosis, lysosomes were recruited towards the tumor cell containing phagosome in macrophages, which lead to acidification of phagosome in the subsequent 1-2 hours, and degradation of tumor cells (Figure 4a). Addition of the isotype anti-HEPCy1 mAb to co-cultures of macrophages and A431 tumor cells neither led to phagocytosis of tumor cells nor subsequent lysosomal activity (Figure 4b). After 24 hours all A431 tumor cells in co-cultures in the presence of zalutumumab were completely degraded and tumor cell debris was observed within macrophages (Figure 4a; right upper panels). By contrast, large cluster of tumor cells were present in co-cultures in which isotype mAb had been added, indicative of outgrowth (Figure 4b; right lower panels).

Macrophages proved very effective in phagocytosing tumor cells and often quickly ingested two or even three whole tumor cells successively (Supplementary Figure 2 4). Degradation was, however, much far less efficient. Interestingly, the lysosomal machinery in macrophages was not capable of simultaneous lysis and degradation of multiple tumor cells at the same time. This led to sequential lysosomal killing of tumor cells, in which lysosomes likely fused with the first phagosome they encountered. After degradation of one tumor cell, lysosomes were recruited to the next phagosome that contained a tumor cell (Supplementary figure 2).

Zalutumumab dependent phagocytosis of tumor cells by macrophages depends on levels of EGFR expression, but is independent on mutations in KRAS or BRAF.

Mutations in genes encoding KRAS and BRAF kinases downstream of EGFR in tumor cells have been demonstrated to hamper the responsiveness of colorectal tumor cells to anti-EGFR mAb treatment. We now investigated whether mutations in the EGFR signalling pathway would affect the capacity of macrophages to eliminate tumor cells. FACS analyses of KRAS- mutated HCT116 and macrophage co-cultures after 24 hours show a dose dependent decrease of tumor cells and an increase in double-positive macrophages after treatment with zalutumumab (Figure 5A), albeit less efficiently than when A431 cells were used as target. Incubating co-cultures with irrelevant isotype antibodies did not induce tumor cell killing by macrophages (Figure 5b). Macrophages were similarly able to eradicate BRAF mutated HT29 tumor cells (Figure 5e and f). Live cell imaging data confirmed ADPh- dependent HT29 cell eradication by macrophages in zalutumumab treated co-cultures after 24 hours (Supplementary figure 3). RKO cells, which have lower membrane EGFR expression (Figure 1a) were not phagocytised by macrophages,
even in the presence of the highest zalutumumab concentration (Figure 5g-h).

Human macrophages were equally effective in performing ADPh, as treatment of co-cultures with zalutumumab for 24 hours led to a significant decrease in either HT29 or HCT116 tumor cells (Figure 5i) and considerably enhanced uptake of tumor cells by human macrophages (Figure 5j). These data suggested that effectiveness of ADPh depended on the level of membrane EGFR expression, but not on mutations in KRAS or BRAF.

To confirm this, we transfected murine C26 tumor cells with human EGFR. Four cell lines were generated, expressing different levels of hEGFR. This ranged from very low (Figure 6a, left panel), reflecting hEGFR levels on human RKO cells (Figure 1a), to higher levels of hEGFR (Figure 6a, right panel) that was comparable to hEGFR levels on human colorectal cell lines HCT116 and HT29 (Figure 1a). Incubation of C26 transfectants with different concentration of zalutumumab had no effect on viability of C26	extsuperscript{hEGFR low}, C26	extsuperscript{hEGFR int/low}, C26	extsuperscript{hEGFR int/high} or C26	extsuperscript{hEGFR high} cells (Figure
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Figure 5: FACS analysis and live cell microscopy HCT116 (KRAS mut) or HT29 (BRAF mut) – macrophage co-cultures with increasing concentrations of Zalutumumab or irrelevant HEPC-γ1 isotype control antibodies. (a/b) FACS analysis of HCT116/macrophage co-cultures after 24 hour treatment with (a) Zalutumumab or (b) irrelevant HEPC-γ1 isotype antibodies. Remaining (c) HCT116, (e) HT29 and (g) RKO tumour cells; percentage macrophages taken up (d) HCT116, (f) HT29 or (h) RKO tumour cells, after 24 hour treatment of co-cultures with increasing concentrations of Zalutumumab or irrelevant HEPC-γ1 isotype antibodies. (i) % remaining HT29 or HCT116 tumor cells by macrophages. (j) % double positive macrophages *p<0.05, **p<0.01, ***p<0.001
6b). Furthermore, prolonged culture with 1 µg/ml zalutumumab for up to 6 days showed no effect on cell proliferation of all transfectants (Figure 6c).

Culturing co-cultures of mouse macrophages and mouse C26 cells that expressed different levels of human EGFR with irrelevant anti-HEPC antibodies did not lead to phagocytosis of tumor cells by macrophages (Figure 6d-f). By contrast, C26<sup>h<sub>EGFR<sup>int/high</sub></sup></sup> or C26<sup>h<sub>EGFR<sup>high</sub></sup></sup> cells were effectively eliminated by macrophages in the presence of low concentrations (0.1 µg/ml) zalutumumab (Figure 6d-f), whereas macrophages were unable to kill C26<sup>h<sub>EGFR<sup>int/low</sub></sup></sup> or C26<sup>h<sub>EGFR<sup>low</sub></sup></sup> cells, even in the presence of a 10x higher zalutumumab concentration.

Additionally, we performed experiment with two modified anti-EGFR mAb. A zalutumumab mutant was generated in which the site for N-linked glycosylation in the Fc domain was eliminated through mutation of the asparagine at position 297 to glutamine (zalu-N297Q). Furthermore, an IgG2 variant was used (zalu-IgG2). Zalutumumab, zalu-N297Q or zalu-IgG2 showed comparable binding to EGFR on either C26<sup>h<sub>EGFR<sup>high</sub></sup></sup> or A431 cells (data not shown). Co-culturing macrophages and C26<sup>h<sub>EGFR<sup>low</sub></sup></sup> cells in the presence of Zalutumumab, zalu-N297Q or zalu-IgG2 did not lead to enhanced tumor cell killing or phagocytosis compared to isotype HEPCγ1 mAb (Figure 7a-c). Opsonising C26<sup>h<sub>EGFR<sup>high</sub></sup></sup> tumor cells with high concentrations (1 µg/ml) of zalu-IgG2 isotype antibodies led to enhanced tumor cell killing compared to isotype HEPCγ1 mAb treatment, whereas this tumor cell cytotoxicity was significantly impaired opposed to Zalutumumab treatment (Figure 7a-b). Although treating C26<sup>h<sub>EGFR<sup>high</sub></sup></sup> macrophage co-cultures with high concentrations (1 µg/ml) of zalu-N297Q almost completely abrogated tumor cell killing compared to Zalutumumab, it still led to a very small increased tumor cell cytotoxicity opposed to isotype HEPCγ1 mAb treatment (Figure 7b). Similarly did zalu-IgG2 and zalu-
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Figure 6: (a) Transfecting mouse C26 colon tumour cells with human EGFR generates 4 C26 variants expressing different levels of hEGFR, ranging from low to high. (b) Cell viability of C26hEGFR variants after 24 hours blocking of hEGFR with increasing concentrations Zalutumumab. (c) Effect of hEGFR blocking with Zalutumumab (1µg/ml) on cell proliferation of different C26hEGFR variants. (d-f) Phagocytosis of C26 variants expressing different levels of hEGFR. (d) FACS analyses of co-cultures of mouse macrophages with C26 cells expressing different levels of hEGFR after 24 hours of treatment with Zalutumumab or irrelevant HEPC-γ1 antibodies. (e) Remaining tumour cells and (f) percentage macrophages, which have taken C26hEGFR tumour cells after 24 hour of co-culturing with Zalutumumab or irrelevant HEPC-γ1 antibodies. **p<0.01, ***p<0.001
**Figure 7:** Treating co-cultures of macrophages and tumour cells with anti-hEGFR antibodies with different FC binding affinities (a) FACS analysis after 24 hour treatment of C26*hEGFR high or low (FL2) and macrophage (FL1) co-cultures with 1µg/ml Zalutumumab (left panel), low affinity FCγR1 binding Zalu-IgG2 (second panel), mutated Zalu-N297Q without FCγR1 binding (third panel) or a-specific α-HEPC (HEPC-γ1) control antibody (right panel). (b) Viable C26*hEGFR high or C26*hEGFR low tumour cells remaining and (c) percentage of tumour cell phagocytosing macrophages in co-cultures after 24 hour treatment. (d) Viable A431 tumour cells remaining and (e) percentage of macrophages which have phagocytosed A431 tumour cells after treatment with different concentrations of different anti-hEGFR antibodies; treatment with a-specific α-HEPC (HEPC-γ1) control antibody is set as 100%. *p<0.05, **p<0.01, ***p<0.001
N297Q treatment with 1 µg/ml mAb enhance tumor cell phagocytosis by mØ compared to isotype HEPCγ1 mAb treatment, however tumor cell phagocytosis was significantly reduced opposed to Zalutumumab treatment (Figure 7c). Repeating phagocytosis experiments with co-cultures of macrophages and human A431 cells confirmed that treating with concentrations of 1 µg/ml of zalu-IgG2 or zalu-N297Q enhanced tumor cell cytotoxicity and phagocytosis by macrophages compared to isotype HEPCγ1 mAb treatment (Figure 7d-e). Both zalu-IgG2 and zalu-N297Q mediated tumor cell killing and phagocytosis by macrophages was however significantly impaired compared to Zalutumumab treatment. Treating with lower concentrations of 0.1 µg/ml completely abrogated the effect of zalu-N297Q treatment on tumor cell phagocytosis and killing by macrophages. Low concentrations (0.1 µg/ml) of zalu-IgG2 still enhanced A431 phagocytosis and killing by macrophages compared to isotype HEPCγ1 mAb treatment (Figure 7d-e), whereas it was significantly reduced compared to Zalutumumab treatment. At concentrations of 0.001 µg/ml (1 ng/ml) only Zalutumumab treatment enhanced tumor cell phagocytosis and killing by macrophages, whereas the effect of both zalu-IgG2 and zalu-N297Q treatment was abrogated. Although the N297Q mutation in FC domain is thought to completely abrogate interactions of IgGs with the human FCγRI,34 its effect on interactions with murine FCγRI or other murine FCγReceptors is not completely known. Because zalu-N297Q still retains its EGFR signal blocking characteristics, effects of treatment with high concentrations of zalu-N297Q on tumor cell proliferation cannot be dismissed. However does this not explain the enhanced antibody dependent tumor cell phagocytosis by mØ after tumor cell opsonisation with zalu-N297Q.

Discussion

Anti-EGFR mAb are currently used to treat (metastatic) colorectal cancer and squamous cell carcinoma of the head and neck (SCCHN) in patients. 25, 35 Monotherapy with anti-hEGFR mAb of patients with existing metastatic colorectal cancer is however limited to ~10% and this is enhanced to ~20% when anti-hEGFR therapy is used in combination with chemotherapy.25 This disappointing response rate is due to mutations in the K-RAS and B-RAF proteins leading to an exuberant activation of the pathways downstream of EGFR, which are independent on binding of EGFR by its natural ligand EGF.21, 26, 36 Here we show that blocking EGFR with mAb has neither effect on cell viability nor proliferation of human colorectal tumor cells, which have a mutation in K-RAS or B-RAF. However, killing of human colorectal tumor cells by macrophages via antibody dependent phagocytosis in the presence of fully human anti-EGFR mAb zalutumumab, was independent on KRAS or BRAF mutational status of tumor cells. As such, we propose a novel mAb-based therapeutic strategy that may potentially benefit a significant population of cancer patients.
Supplementary figure 2: hEGFR-γ1 dependent phagocytosis of multiple carcinoma cells (A431) leads to sequential degradation. Time laps live cell imaging microscope of co-cultures of red acidic organelle-specific dye stained macrophages and green labeled A431 tumour cells. (a) Macrophage with two phagocytosed A431 tumour cells, in first 2 hours after phagocytosis lysosomes fuse and acidify first tumour cell containing phagosome (> in left panel). Only after first tumour cell is killed, lysosomes fuse with second tumour cell containing phagosome (» lower panel). After 5 h first tumour cell is degraded and acidity of phagosome declines, whereas degradation in second tumour cell containing phagosome still persists. (b) Time laps microscopy started 8 hours after phagocytoses of 3 individual A431 tumour cells by one macrophage. First tumour cell (> in upper panel) is already degraded and acidity of phagosome declines, lysosomes have just fused with second phagosome and begin to degrade containing A431 tumour cell (» in upper panel). After approximately 10 hours lysosomes fuse with last tumour cell containing phagosome (” in lower panel).
ANTI-EGFR ANTIBODY AND TUMOR CELL PHAGOCYTOSIS

Supplementary figure 3: Live cell microscopy after 24 hours treatment of co-cultures of DiO labelled macrophages with Dil labelled colorectal HT29 cells with (a) low (0.1 µg/ml) or (b) high (10 µg/ml) concentrations of hEGFR-γ1 antibodies or with (c) high (10 µg/ml) concentrations of irrelevant HepC-γ1 antibodies. Depicted are fluorescent images (left panel), bright field images (middle panel) or overlay (right panel).

Approximately one million patients worldwide are yearly diagnosed with colorectal cancer, and require surgery as resection of the primary tumor is the cornerstone of the treatment and best chance to provide cure. However, up to 70% of patients with colorectal cancer have disseminated circulating tumor cells in their blood, which can be increased by resection or handling of the primary tumor. Furthermore, the presence of circulating tumor cells is correlated with decreased patient prognosis. We previously demonstrated that surgery paradoxically promotes adherence of circulating tumor cell, and concomitant outgrowth of liver metastases as inflammatory mediators induced damage to the liver vasculature. Importantly, anti-tumor mAb therapy prevented liver metastases outgrowth in mice and rats, which was mediated by liver macrophages (Kupffer cells (KC)) and to a lesser extent newly recruited monocytes.

As such, we propose that patients undergoing resection for primary colorectal cancer may greatly benefit from peri-operative mAb immunotherapy, as this will lead to elimination of any remaining circulating tumor cells by the myeloid...
mononuclear network in the liver. Therapeutic efficacy is likely dependent on the expression of the target antigen. Because more than 80% of all colorectal cancer tumors show upregulated EGFR on their surface and mutations in EGFR itself are an infrequent event\(^{38}\) this receptor represents an excellent candidate for mAb prevention of surgically induced metastases formation in patients, especially as ADPh was not impaired by mutations in the signaling pathways of EGFR. By contrast, ADPh is strictly dependent on interactions with Fc receptors on macrophages. Zalutumumab-N297Q - which has a N297Q mutation in its Fc tail that abrogates Fc receptor interactions - is able to specifically bind to EGFR on tumor cells comparable to non-mutated zalutumumab. However, it neither induced phagocytosis nor killing of tumour cells by macrophages. Moreover, we previously demonstrated that successful antibody therapy in mice with specific mouse IgG2a antibodies was dependent on expression of both FcγRI and FcγRIV that are expressed on macrophages.\(^{27}\) Binding of human IgG1 to Fc receptors is comparable to binding of mouse IgG2a, and as such represents the most optimal isotype for induction of ADPh. Altering the isotype into human IgG2 significantly severely impaired efficient phagocytosis and tumor cell killing by macrophages, presumably due to lower affinity of Fcγ receptors for human IgG2.

Two different anti-EGFR mAb are currently used in the clinic to treat (metastasised) colorectal cancer. The prototypic mAb is Cetuximab, which is a humanized molecule. To minimize the chance of development of human anti-mouse antibodies, Panitumumab was developed, which is a fully human mAb. However, Panitumumab is of the IgG2 isotype subclass, and as such is less suitable for prevention of surgery induced metastases, as it will not induce effective ADPh. Zalutumumab is a fully human IgG1 that has been approved by the FDA for the treatment of patients squamous cell carcinoma of the head and neck (SCCHN). We now show that it also represents an excellent candidate for peri-operative treatment to prevent surgery induced liver metastases in patients undergoing resection of primary colorectal cancer.

In conclusion, zalutumumab effectively mediated ADPh by macrophages, which is the most prominent effector mechanism for prevention of surgery-induced liver metastases development. Furthermore, ADPh was not dependent on mutations in K-RAS or B-RAF. As such, we hypothesize that peri-operative treatment of patients suffering form colorectal cancer with zalutumumab may greatly improve long term patient outcome.
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Reference list

22. van der Bij, G.J. et al. Experimentally induced liver metastases from colorectal cancer can be prevented by mononuclear phagocyte-mediated monoclonal antibody therapy. J. Hepatol. (2010).
Reference list


