CHAPTER 7

EPIDERMAL GROWTH FACTOR RECEPTOR AS TARGET FOR MONOCLONAL ANTIBODY-MEDIATED PHAGOCYTOSIS OF COLON CARCINOMA CELLS BY MACROPHAGES
Abstract

During resection of primary colorectal tumours malignant cells disseminate from the tumour 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 tumour cells. Although surgical resection of colorectal tumours is the only curative option to ascertain survival, trauma inflicted by this procedure may lead to enhanced outgrowth of distant metastases and decrease both overall and disease free survival in a cohort of patients. Peri-operative treatment with specific monoclonal antibodies (mAb) directed against circulating tumour cells prevented surgically induced liver metastases of colorectal tumours in several animal models. Successful elimination of circulating tumour cells was mediated by liver macrophages (Kupffer cells). We now demonstrate that by using the 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 (ADPh). Efficacy of ADPh was dependent of surface expression of EGFR on malignant cells, but independent of mutations in downstream EGFR kinases KRAS and BRAF. Since 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.

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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. Surgical excision of the primary tumour is the basis of curative therapy and critical for improved survival. 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. Moreover, 10-25% of patients without detectable CLM at time of diagnosis, will subsequently develop distant metastases after removal of the primary tumour. This supports 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 tumour cells into the circulation. Furthermore, methods to isolate circulating malignant cells from the blood of CRC patients have been described since 1960-70s. The percentage of CRC patients, in which free circulating tumour cells have been detected, ranges between 10-70% depending on the method of detection. Nowadays the presence of free circulating tumour cells has been proposed as an independent prognostic factor for survival of CRC patients. Experiments in animal models showed that dissemination of tumour cells into the circulation occurs spontaneously from existing solid tumours, which is enhanced after manipulation of the tumour. The augmentation of free circulating tumour cells after and during surgery of the primary tumour has also been extensively described in human patients. Furthermore, increased numbers of circulating tumour cells in the portal system have been associated with decreased overall survival.

Over the years evidence has accumulated, which paradoxically supports that trauma, inflicted by surgical procedures to excise primary tumours, is associated with risk of developing metastases. By imitating surgery procedures in animal models we previously demonstrated that surgical trauma induced a systemic effect leading to enhanced tumour cell adhesion of circulating tumour cells but not to enhance growth of existing tumour cell clusters. The exact mechanisms of surgery-induced tumour 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 tumour cell adhesion and outgrowth.

Nonetheless, resection of CRC is mandatory as first treatment, and will remove the bulk of the tumour load. The peri-operative period is an attractive window of opportunity in which eliminating the remaining disseminated circulating tumour cells may reduce development of liver metastases and improve overall patient survival. A promising approach to reduce surgically induced metastases formation may be the stimulation of anti-tumour immune responses with the use of monoclonal antibodies (mAb). mAb can have direct effects on tumour 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 therapy on regression of tumours in patients with larger metastatic tumour masses. Studies on the 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 mAb, in whom the primary colorectal tumour 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 tumour. 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 murine anti-EpCam antibodies, but until now the 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 chimeric or humanised anti-EGFR mAb has yielded fluctuating and somewhat disappointing results when treating existing colorectal cancer metastases. The low response rate in CRC patients can be explained by frequently occurring mutations in the EGFR downstream targets KRAS and BRAF, making the tumour unresponsive for EGFR-inhibiting therapies. However, it has been shown that mutations in the EGFR downstream targets are not affecting Fc-mediated effector functions. 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 human anti-EGFR mAb zalutumumab, previously demonstrated to have a dual mechanism of action, namely signalling inhibition and ADCC, represents a suitable candidate for peri-operative treatment of patients undergoing resection for primary CRC.
Materials & 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 29. 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. A human IgG1, λ, against hepatitis C E2 envelope protein was
included in all experiments as control mAb 31. For the human IgG2 variant of zalutumumab
(zalu-IgG2), the variable regions of the immunoglobulin heavy chain of zalutumumab was
expressed recombinantly as human hIgG2. This human heavy chain construct was co-
expressed with the appropriate original human kappa light chain. Secondary goat F(ab’)2
anti human IgG-RPE antibodies were purchased from AbD Sertotec (Oxford, UK).

Cell Culture

Tumour 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 manufacture’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\textsuperscript{hEGFR low}, C26\textsuperscript{hEGFR int/low}, C26\textsuperscript{hEGFR int/high} or C26\textsuperscript{hEGFR high} clones.
Cells were resorted every 2 weeks with a minimum of 10 sortings to obtain stable C26\textsuperscript{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 (4x10⁶/ well) for in vitro cytotoxicity assays or in 8 well ibiTreat µ-Slides (IBIDI, Munich, Germany) for live cell imaging (2x10⁵/ 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 donors 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, Upppsala, 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 (4x10⁶/ well) for in vitro cytotoxicity assays.

**Flow cytometry**
Human carcinoma cell lines HT29, RKO, HCT116 and A431 or mouse C26\textsuperscript{REGFR} 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).
Materials and methods

Fluorescent labeling

For in vitro cytotoxicity assays human or mouse tumour 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 perchlorateperchlorate (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 DiI 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. Tumour cells were labelled with DiO for these experiments.

Viability assays

MTT assay

Cell viability of different tumour cells was measured by MTT assay as previously described. Briefly, after stimulation with a concentration range of Zalutumumab or HEPCγ1 mAb for 4 to 120 h, tumour 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 DiI-labeled human or mouse tumour 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 tumour cells and macrophages that had taken up tumour cells (double-positive) were determined by flow cytometry after 24 hours of co-culture. Percentages of tumour cells and double-positive macrophages after culture without mAb 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. DiI or green DiO-labeled tumour 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.
Results

Inhibition of cell proliferation by zalutumumab is dependent on the level of EGFR expression

Anti-EGFR mAb have been shown to competitively inhibit EGF binding and subsequent activation of downstream receptor tyrosine kinases leading to $G_1$ cell cycle arrest and growth inhibition. Therefore we first analysed whether incubation with zalutumumab led to diminished tumour 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. Cells were grown in the presence of different concentrations of zalutumumab. No effect on cell viability of the colon carcinoma cell lines; KRAS mutated HCT116 cells, BRAF mutated HT29 cells or the low EGFR-expressing RKO cell line with zalutumumab was observed, even in the presence of saturating concentrations (data not shown) of zalutumumab (10 µg/ml) for 4 or 24 hours (Figure 1B and Supplementary Figure 1A). However, incubation with zalutumumab concentrations of 1 µg/ml or higher for at least 24h, significantly reduced proliferation of A431 vulvar carcinoma cells (Figure 1C and Supplementary Figure 1A), which express high levels of EGFR (Figure 1A). No effects were seen on short term cell viability of the A431 cells (Figure 1B).

![Figure 1](image-url)

(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
Results

Macrophages kill tumour 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 tumour cells by murine macrophages. Incubation of A431 tumour cells with macrophages in the presence of increasing concentrations of control mAb did not lead to decreased tumour cell numbers after 24h (Figure 2B/C). However, incubation of macrophage-A431 co-cultures with low concentrations of Zalutumumab, ranging from 100 to 1 ng/ml, at which Zalutumumab had no direct effect on cell viability or proliferation (supplementary 1A), led to significant reduced A431 cell numbers (Figure 2A/C). This effect was abrogated only at concentrations lower than 0.1 ng/ml (Figure 2C). 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 control mAb.

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 and supplementary video 1), whereas addition of a control mAb did not lead to tumour cell uptake (Figure 3C, D). Tumour cells were gradually degraded after ADPh, and virtually no live tumour cells were present after 16 hours (Figure 3A, B), whereas A431 cells grew out in clusters in the presence of a control antibody (Figure 3C, D, and supplementary video 2).
Results

Figure 2
FACS analysis of A431/macrophage co-cultures after 24 h. treatment with different concentrations of (A) zalutumumab or (B) control mAb. DiI 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 (D) double positive macrophages, lowest concentration of control mAb is set as 100%. **p=<0.01, ***p=<0.001

Antibody dependent phagocytosis of tumour cells by macrophages leads to slow lysosomal degradation

Within 60 minutes of co-culturing macrophages and human A431 tumour cells with zalutumumab, macrophages efficiently phagocytosed one to three whole tumour cells (Figure 4a, and supplementary video 3). However, subsequent killing and degradation of phagocytosed tumour cells took up to >20 hours, depending on the number of tumour cells which had been taken up. To visualize activated lysosomes, macrophages were labelled with the acidic organelle-specific dye Lyso-ID. Within 30 minutes after phagocytosis, lysosomes were recruited towards a tumour cell-containing phagosome in macrophages, which led to acidification of the phagosome in the next 1-2 hours, and degradation of tumour cells (Figure 4a).
Results

Figure 3 Live cell video microscopy
(A) Phagocytosis of Dil (Red) labelled A431 cells opsonised with Zalutumumab antibodies by DiO (green) labelled macrophages also (B) depicted as bright field images. Treatment of macrophage-A431 co-cultures with irrelevant control mAb antibodies depicted as (C) fluorescent or (D) bright field images.

Addition of the isotype anti-HEPCγ1 mAb to co-cultures of macrophages and A431 tumour cells neither led to phagocytosis of tumour cells nor lysosomal activity (Figure 4b). After 24 hours all A431 tumour cells in co-cultures in the presence of Zalutumumab were degraded and tumour cell debris was observed within macrophages (Figure 4a; right upper panels). By contrast, large tumour cell clusters were present in co-cultures in which the isotype mAb had been added, indicative of outgrowth. (Figure 4b; right lower panels). Macrophages proved very effective in phagocytosing tumour cells and often quickly ingested two or even three whole tumour cells successively (Supplementary Figure 2 and supplementary video 4). Degradation was, however, far less efficient. Interestingly,
Results

Figure 4 hEGFR antibody dependent phagocytosis leads to activation and fusion of lysosomes with tumour cell containing phagosomes in macrophages
Time laps live cell imaging microscope of co-cultures of red acidic organelle-specific dye stained macrophages and green labelled A431 tumour cells. (A) After treatment with Zalutumumab, macrophages (left panel) phagocytose green labelled A431 tumour cells (arrowheads first & second row). After phagocytosis of tumour cells, activated lysosomes (red staining) appear in macrophages around tumour cell containing phagosomes (second & third row). Lysosomes fuse with tumour cell containing phagosomes, acidifying phagosomes (arrows in third & fourth row). After approximately 4 hours first tumour cell is degraded (* in last row). After 24 hours of treatment all tumour cells are phagocytosed and lysosomes contain degraded tumour material (> most right panel). (B) Treatment with isotypic control mAb does not lead to lysosome activation or lysosomal fusion with phagosomes. After 24 hours, A431 tumour cells adhere and clusters of live tumour cells are seen (> most right panel).
the lysosomal machinery in macrophages was not capable of simultaneous lysis and degradation of multiple tumour cells at the same time. This led to sequential lysosomal killing of tumour cells, in which lysosomes likely fused with the first phagosome they encountered. After degradation of one tumour cell, lysosomes were recruited to the next phagosome that contained a tumour cell (Supplementary Figure 2 and supplementary video 4).

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

Mutations in genes encoding KRAS and BRAF kinases downstream of EGFR in tumour cells have been demonstrated to hamper the responsiveness of colorectal tumour cells to anti-EGFR mAb treatment \(^{26}\). We now investigated whether mutations in the EGFR signalling pathway would affect the capacity of macrophages to eliminate tumour cells. FACS analyses of KRAS- mutated HCT116 and macrophage co-cultures after 24 hours showed a dose dependent decrease of tumour 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 an irrelevant isotype control antibody did not induce tumour cell killing by macrophages (Figure 5B-D). Macrophages were similarly able to eradicate BRAF mutated HT29 tumour cells (Figure 5E and F). Live cell imaging data confirmed ADPh- dependent HT29 cell eradication by macrophages in zalkutumumab treated co-cultures after 24 hours (Supplemental Figure 3). RKO cells, which have lower membrane EGFR expression (Figure 1A) were not phagocytised by macrophages, even in the presence of the highest zalkutumumab concentration (Figure 5G-H).

Human macrophages were equally effective in performing ADPh, as treatment of co-cultures with zalkutumumab for 24 hours led to a significant decrease in either HT29 or HCT116 tumour cells (Figure 5I) and enhanced uptake of tumour 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 tumour 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 zalkutumumab had no effect on viability of C26\(^{\text{EGFR low}}\), C26\(^{\text{EGFR int/low}}\), C26\(^{\text{EGFR int/high}}\) or C26\(^{\text{EGFR high}}\) cells (Figure 6B). Furthermore, prolonged culture with 1µg/ml zalutumumab for up to 6 days showed no effect on cell proliferation of all transfectants (Figure 6C).

Culturing mouse macrophages and mouse C26 cells that expressed different levels of human EGFR with irrelevant anti-HEPC antibodies did not lead to phagocytosis of tumour cells by macrophages (Figure 6D-F). By contrast, C26\(^{\text{EGFR int/high}}\) or C26\(^{\text{EGFR high}}\) 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\(^{\text{EGFR int/low}}\) or C26\(^{\text{EGFR low}}\) cells, even in the presence of a 10x higher zalutumumab concentration.
Results

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).

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, a human IgG2 variant was used (zalu-IgG2). Zalutumumab, zalu-N297Q or zalu-IgG2 showed comparable binding to EGFR on either C26hEGFRhigh or A431 cells (data not shown). Co-culturing macrophages and C26hEGFRlow cells in the presence of zalutumumab, zalu-N297Q or zalu-IgG2 did not lead to enhanced tumour cell killing or phagocytosis compared to control mAb (Figure 7A-C). C26hEGFRlow cells were efficiently
**Part II**

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 isotype control mAb

(A/B) FACS analysis of HCT116/macrophage co-cultures after 24 hour treatment with (A) Zalutumumab or (B) irrelevant control mAb. 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 control mAb. (I-J) Treatment of co-cultures of human macrophages with human colorectal tumour cells HCT116 and HT29 with hEGFR-γ1 leads to (I) decreased number of HCT116 and HT29 tumour cells and to (J) enhanced uptake of HCT116 and HT29 tumour cells by human macrophages after 24 hours. *p=<0.05, **p=<0.01, ***p=<0.001

Phagocytosed in the presence of zalutumumab, but its opsonic capacity was significantly decreased when its Fc tail was either changed into an IgG2 isotype or when its asparagine at position 297 was mutated into glutamine (Figure 7A-C). Similar results were observed when human A431 cells were used as target (Figure 7D-E). Incubation of co-cultures with lower concentrations of 0.1 µg/ml or 0.01 mg/ml completely abrogated the opsonic ability effect of zalu-N297Q or zalu-IgG2, respectively.
Results

Supplementary Figure 3
Live cell microscopy after 24 hours treatment of co-cultures of DiO labelled macrophages with DiI labelled colorectal HT29 cells with (A) low (0.1 µg/ml) or (B) high (10 µg/ml) concentrations of Zalutumumab or with (C) high (10 µg/ml) concentrations of irrelevant control mAb. Depicted are fluorescent images (left panel), bright field images (middle panel) or overlay (right panel).

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) Fagocytosis 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 control mAb. (E) Remaining tumour cells and (F) percentage macrophages, which have taken C26hEGFR tumour cells after 24 hours of co-culturing with Zalutumumab or irrelevant control mAb. **p<0.01, ***p<0.001
Results

Part II
Results

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 C26hEGFR high or low (FL2) and macrophage (FL1) co-cultures with 1µg/ml Zalutumumab (left panel), low affinity FCyR1 binding Zalu-IgG2 (second panel), mutated Zalu-N297Q without FCyR1 binding (third panel) or a-specific control mAb (right panel). (B) Viable C26hEGFR high or C26hEGFR 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 control mAb is set as 100%. *p<0.05, **p<0.01, ***p<0.001
Results
Anti-EGFR mAb are currently used to treat (metastatic) colorectal cancer and squamous cell carcinoma of the head and neck (SCCHN) in patients \(^{25, 36}\). Clinical responses of monotherapy with anti-EGFR mAb of patients with existing metastatic colorectal cancer are however limited to \(\sim 10\%\) and this is enhanced to \(\sim 20\%\) when anti-EGFR mAb therapy is used in combination with chemotherapy \(^{25}\). This disappointing response rate is likely due to mutations in K-RAS and B-RAF proteins, leading to an exuberant activation of the pathways downstream of EGFR, independent of binding of EGFR to its natural ligand EGF \(^{21, 26, 37}\). Here we show that blocking EGFR with mAb has neither effect on cell viability nor proliferation of human colorectal tumour cells, which have a mutation in K-RAS or B-RAF as previously demonstrated by Schlaeth et al \(^{27}\). However, killing of human colorectal tumour cells by macrophages via antibody dependent phagocytosis in the presence of human anti-EGFR mAb Zalutumumab was independent on KRAS or BRAF mutational status of tumour cells. As such, we propose a novel mAb-based therapeutic strategy that may potentially benefit a significant population of cancer patients.

Approximately one million patients worldwide are diagnosed yearly with colorectal cancer. Almost all patients require surgery, as resection of the primary tumour is the cornerstone of treatment and the best chance to provide cure \(^4\). However, up to 70\% of the patients with colorectal cancer have disseminated circulating tumour cells in their blood \(^8\), which can be increased by resection or handling of the primary tumour \(^{14, 15}\). Furthermore, the presence of circulating tumour cells is correlated with decreased patient prognosis \(^{13}\). We previously demonstrated that surgery paradoxically promotes adherence of circulating tumour cells, and concomitant outgrowth of liver metastases \(^{19, 38}\). Importantly, anti-tumour 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 \(^{22}\).

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 tumour cells by the myeloid mononuclear network in the liver. Therapeutic efficacy is solely dependent on surface expression of the target antigen. Because more than 80\% of all colorectal cancer tumours show up-regulated EGFR on their surface and mutations in EGFR itself are an infrequent event \(^{39}\) 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 tumour 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 \(^{28}\). Altering the isotype into human IgG2 severely impaired efficient phagocytosis and tumour cell killing by macrophages, presumably due to lower affinity of murine Fcγ receptors for human IgG2 as demonstrated by Overdijk et al. (manuscript in preparation).
Discussion

Two different anti-EGFR mAb are currently used in the clinic to treat (metastasised) colorectal cancer. The prototypic mAb is cetuximab, which is a human/murine chimeric molecule. To minimize the chance of development of human anti-mouse antibodies, Panitumumab was developed, which is a fully human mAb 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 human IgG1 that is in clinical evaluation for the treatment of patients with squamous cell carcinoma of the head and neck (SCCHN) \(^{36}\). 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 undergoing resection of colorectal cancer with Zalutumumab may greatly improve long term patient outcome.
22. van der Blij, G.J. et al. Experimentally induced liver metastases from colorectal cancer can be prevented by mononuclear phagocyte-mediated monoclonal antibody therapy. J. Hepatol. (2010).