Epidermal Growth Factor Receptor is a suitable target for pre-operative elimination of circulating tumour cells

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Abstract
The primary treatment of patients with colorectal cancer is surgical resection of the tumour. However, we previously demonstrated that surgery promotes the adherence of circulating tumour cells in the liver with subsequent outgrowth as metastases. Importantly, pre-operative treatment with specific monoclonal antibodies (mAbs) targeting tumour cells prevented surgery-induced liver metastasis development in several animal models. In this study we investigated whether the epidermal growth factor receptor (EGFR) is a suitable target for pre-operative mAb treatment of patients undergoing resection of colorectal tumours. Cetuximab, zalutumumab and panitumumab, three anti-EGFR mAbs, were equally efficient in opsonizing tumour cells. Moreover, all three antibodies induced in vitro antibody dependent phagocytosis (ADCP) of tumour cells by human macrophages at low concentrations, which was independent of mutations in the EGFR signalling pathway. Importantly, plasma of patients who received cetuximab efficiently opsonized tumour cells ex vivo when diluted 40 times. Furthermore, low anti-EGFR concentrations did neither affect proliferation nor migration, which are two important processes during wound healing, while ADCP was effectively induced. These data support that patients can be safely treated with a low dose of anti-EGFR antibodies, prior to resection of colorectal cancer, to eliminate circulating tumour cells without hampering healing of the anastomosis. Ultimately this may significantly improve long term patient outcome.

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**INTRODUCTION**

Colorectal cancer is one of the primary types of cancer cases worldwide. Approximately 1.36 million patients are annually diagnosed with colorectal cancer (CRC), and almost 700,000 patients die worldwide of this disease per year. Most patients require surgery, as resection of the primary tumour is the cornerstone of treatment and still the best curative option. Development of liver metastases is however a frequent complication, and associated with high morbidity and mortality. Prognosis of patients with metastasis is poor, with 5 and 10 year disease-free survival rates of only 27% and 22%, and overall survival rates of 47% and 28%, respectively.

Patients that develop metastatic colorectal cancer, which is positive for the epidermal growth factor receptor (EGFR) can nowadays be treated with a combination therapy of chemotherapy and anti-EGFR monoclonal antibodies (mAbs). These mAbs block the binding-site of the receptor preventing binding of epidermal growth factor (EGF). Therefore, it is only useful to use these mAbs as treatment when the tumour has non-mutated signalling pathways. Frequently observed mutations in especially the kinases KRAS and BRAF result in continuous signalling - irrespective of the binding of EGF -, which renders therapy with anti-EGFR mAbs ineffective. A mouse/human chimeric Immunoglobulin G1 (IgG1) antibody cetuximab (Erbitux), or a human IgG2 antibody panitumumab (Vectibix) are currently used to treat patients with colorectal cancer. Additionally, a third EGFR blocking antibody, the human IgG1 antibody zalutumumab, was developed.

We and others previously demonstrated that surgery enhances the risk of developing liver metastases in animals models. Conventional open colectomy was reported to result in poorer long term cancer related survival of patients with colorectal cancer, compared to laparoscopy-assisted surgery, although this still has to be confirmed in other trials. In animal models, inflammatory responses due to abdominal surgery and especially colectomy induced changes in the liver vasculature, hereby enhancing adhesion of circulating tumour cells and S. Grewal personal communication. Thus, even though resection of CRC is the mandatory first line of treatment in order to remove the bulk of the tumour load, evidence is accumulating that surgery paradoxically creates a niche for circulating tumour cells to adhere.

Unfortunately, up to 70% of the patients with colorectal cancer have disseminated circulating tumour cells (CTC) in their blood. Moreover, an increased number of CTCs was observed shortly after surgery, suggesting shedding of tumour cells due to manipulation of the tumour during surgery. The presence of CTCs is furthermore correlated with poor survival of both patients with primary colorectal cancer and patients with resectable liver metastases. High postoperative levels of CTCs predicted tumour recurrence. Thus, it is likely that resection of the primary tumour or liver metastases promotes adhesion of CTCs and metastasis development in patients.

Consequently, eliminating disseminated CTCs may reduce the development of liver metastases and improve overall patient survival. We previously showed that pre-operative treatment with mAb directed against tumour cells prevented surgery-induced liver metastases development in animal models. The clearance of CTCs in the liver was primarily mediated by liver macrophages (Kupffer cells) and infiltrating monocytes, which executed potent antibody-dependent phagocytosis. The potential of pre-operative mAb therapy to prevent local recurrence or distant metastases has not been investigated thoroughly. One study showed increased 7
year survival and reduced overall mortality in patients with primary colorectal cancer, who had been treated postoperatively with anti-EpCAM mAbs\textsuperscript{19}. mAb treatment had no effect on local recurrence of the primary tumour, but in approximately 30% of the treated patients occurrence of distant metastases was reduced. Therefore, although promising, improvement of efficacy is warranted. Approximately 80% of colorectal carcinoma have up-regulated EGFR expression\textsuperscript{20–22}. Accordingly, EGFR might represent a good candidate for pre-operative mAb therapy.

In this study we therefore investigated the suitability of the anti-EGFR mAbs cetuximab, zalutumumab and panitumumab as target for pre-operative therapy for elimination of CTCs in patients, which may prevent surgery-induced metastases development. Additionally, we investigated the effect on cell proliferation and migration to determine whether treatment with anti-EGFR mAbs might interfere with wound healing.

**Material and Methods**

**Antibodies & plasma**

The therapeutic anti-EGFR antibodies cetuximab (Erbitux) and panitumumab (Vectibix) were purchased from Merck (Schiphol-Rijk, the Netherlands) and Amgen (Breda, the Netherlands), respectively. Zalutumumab (HuMax-EGFR, clone 2F8) was generated by Genmab (Utrecht, the Netherlands) as described previously\textsuperscript{23}. PE-labelled F(ab')\textsubscript{2} fragments of polyclonal goat-anti-human IgG (abD serotec, Kidlington, UK) were used as secondary antibodies to detect binding of anti-EGFR mAbs. Anti-HLA-DR antibodies (clone L243, Biolegend, San Diego, CA) were used to stain human macrophages in ADCP assays. Pre- and post-cetuximab infusion plasma from patients was obtained during the colocetux trial (NCT01691391)\textsuperscript{24}, which was approved by the medical ethical committee of the VUmc. All patients signed an informed consent according to Dutch, and international law.

**Cell Culture**

Tumour cells. The human colorectal carcinoma cell lines Caco2, HCT116, HT29, RKO, SW620, and SW948 and the human epidermoid carcinoma cell A431 (ATCC, Manassas, VA) were cultured under standard incubator conditions in DMEM (Invitrogen, Paisley, UK), supplemented with 10% heat inactivated foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (200 µM) (hereafter referred to as complete DMEM). Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution (Invitrogen). Viability was assessed by trypan blue exclusion and always exceeded 95%.

Human natural killer (NK) cells and macrophages. Human monocytes were isolated from buffycoats < 24h after blood collection (Sanquin blood supply, Amsterdam, the Netherlands) of healthy listed blood donors. All donors gave informed consent. Whole blood was diluted 1:1 in PBS, loaded on lymfoprep (Nyegaard, Oslo, Norway) and cells were separated by density-centrifugation. Peripheral blood mononuclear cells (PBMCs) were extracted from the interphase of the lymfoprep gradient, and washed three times in PBS supplemented with autologous serum. Either NK cells or CD14\textsuperscript{+} monocytes were isolated from the PBMCs with cell separation beads, according to manufacturer’s protocol (negative selection for NK cells, positive selection for CD14\textsuperscript{+} cells, Miltenyi Biotech, Leiden, the Netherlands). Isolated cells were washed and resuspended in complete RPMI 1640 (containing 10% heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (200 µM)). CD14\textsuperscript{+} isolated blood monocytes were cultured with 50ng/ml M-CSF (macrophage-colony stimulating factor) (eBioscience, San Diego, CA) for 8 days in complete RPMI 1640.
Flow cytometry
Human carcinoma cell lines were incubated with anti-human EGFR antibodies (cetuximab, zalutumumab or panitumumab) or patient plasma at different concentrations for 45 minutes on ice. After washing, primary antibodies were detected by incubation with PE-conjugated F(ab')\(_2\) fragments of goat-anti-human IgG. Cells were analysed with flow cytometry (LSRFortessa X20, BD, San Jose, CA).
QiFi kit quantification. To determine the quantitative expression of EGFR on tumour cells the QiFi kit (Dako, Heverlee, Belgium) was used according to manufacturer’s protocol. Briefly, tumour cells were harvested, counted and stained with mouse anti-human EGFR antibodies (clone AY13, Biolegend). Afterwards, excess of antibodies was washed away and a second incubation with Goat F(ab')\(_2\) anti-mouse FITC - provided with the kit - was performed. Cells were analysed with flow cytometry. Calibration beads were used to make a titration curve to allow calculation of absolute numbers of receptors.

Antibody dependent killing
Antibody dependent phagocytosis (ADCP) was performed as described before\(^{25}\). Briefly, tumour cells were harvested and stained with DiI (Sigma-Aldrich, St. Louis, MO,) in complete DMEM according to manufacturer’s protocol. Macrophages were stained with DiO (Molecular Probes Inc, Paisley, UK) in complete RPMI 1640 according to same protocol. Alternatively, macrophages were labelled with the Lyso-ID Red lysosomal staining (Enzo Biochem Inc, Farmingdale, NY), according to the manufacturer's instructions. Tumour cells were labelled with DiO for these experiments. Labelled tumour cells and macrophages in different effector to target (E:T) ratios were co-cultured in the presence of anti-EGFR mAbs or human anti-HEPC IgG1 antibodies as isotype control. Imaging over time was performed on an Olympus IX81-ZDC live cell imager with climate chamber. For flow cytometric analysis, tumour cells were harvested and labelled with cell proliferation dye eF450 (eBioscience) according to manufacturer’s protocol. Co-culture with unlabelled macrophages were performed in the presence of the indicated anti-EGFR antibodies or plasma concentrations. After 24 hours of co-culture, macrophages were stained with anti-HLA-DR antibodies. Percentages of remaining tumour cells were determined by flow cytometry.
Alternatively, for antibody dependent cellular cytotoxicity (ADCC), isolated NK cells were incubated with tumour cells in an E:T ratio of 10:1 in the presence of indicated anti-EGFR mAbs. After 24hrs plates were carefully washed and a 3-hour cell titre blue assay (PR omega, Leiden, the Netherlands) was performed according to manufacturer’s protocol. Readout was performed on a Bio-rad Model 680 Microplate Reader (Bio-rad, Hercules, CA).

Cell proliferation and migration assay
Tumour cells were seeded in 96 wells plates and let to adhere overnight. At day 0 medium was replaced with complete DMEM containing the indicated concentrations of anti-EGFR mAbs and cells were cultured for 72hrs. Cell viability was analysed by cell titre blue assay as described above. For migration experiments, tumour cells were harvested and seeded at optimal concentrations (approximately 7x10\(^4\) cells/well) into culture-inserts designed for in vitro migration/wound healing experiments (Cat no.: 80209, Ibid, Martinsried, Germany). Culture inserts were placed in 24 wells μ-plate.
(Ibidi) and let to adhere overnight. At day 0 inserts were removed and 1ml complete
DMEM with indicated concentrations of anti-EGFR mAbs was added. Gap closure was
analysed over time with the Olympus IX81-ZDC live cell imager.

Statistical analysis
Graphs were produced and statistics was performed in GraphPad Prism 6. Bars
depicted in the graphs represent mean ± SEM. Differences in data were analysed with
2-way ANOVA tests, followed by Tukey’s multiple comparison tests.

Results
Similar opsonisation of EGFR expressing tumour cells by cetuximab,
panitumumab or Zalutumumab
Although approximately 80% of the patients have a tumour, which expresses
EGFR, the level of expression differs between tumours. To investigate which level
of EGFR expression is minimally required to induce ADCP, we first determined EGFR
expression on different epithelial tumour cell lines. The colorectal cell lines HCT116,
HT29, SW948 and Caco2 have similar expression of EGFR, ranging between 20.000
to 40.000 molecules per cell (Table 1). The colorectal cell line RKO expresses ~10
times less EGFR on its cell membrane, whereas the colorectal cell line SW620 does
hardly express EGFR. The epidermoid carcinoma cell A431 has high over-expression
of EGFR (>300.000 molecules per cell). Opsonisation of tumour cells with different
concentrations of anti-EGFR mAbs showed binding in a dose-dependent manner,
and 0,05µg/ml anti-EGFR antibody was in most cases sufficient for >90% saturation
(Figure 1), except for the SW620 cell line, which has very low EGFR expression. No
major differences were observed between binding of the three different antibodies.

Macrophages efficiently induced ADCP at low anti-EGFR mAb concentrations
We previously demonstrated that efficient mAb therapy to prevent surgery-induced
liver metastases was mediated through ADCP by Kupffer cells and to a lesser extent
by monocytes. Monocyte derived macrophages were co-cultured with tumour
cells in the presence of different concentrations anti-EGFR mAbs. For high throughput
quantification, flow cytometry was used for detection of remaining tumour cells.
Tumour cells were not eliminated by macrophages in the presence of an isotype
antibody. When HT29 cells were co-cultured with macrophages in the presence of
cetuximab, concentrations as low as 0,1µg/ml were sufficient to induce ~50% tumour
cell killing (Figure 2A). Moreover, increasing the dose barely improved tumour cell
clearance, and only in the presence of 30µg/ml anti-EGFR mAbs somewhat improved
tumour cell killing was observed. No major differences were observed after incubation
with zalutumumab or panitumumab. Similar results were obtained when the SW948
cell line was used as target (Figure 2B). Macrophages were not able to efficiently clear
CaCo2 or HTC116 cells in the presence of anti-EGFR mAbs in spite of ample EGFR
expression (Figure 2C and D). The RKO cell line, which has low EGFR expression,
was not cleared neither (Figure 2E). A431 cells, with very high EGFR expression,
were extremely efficiently eliminated by macrophages in the presence of anti-EGFR
mAbs (Figure 2F). To investigate the mode of killing, live cell imaging experiments
were performed. In the presence of anti-EGFR mAbs efficient ADCP is observed,
whereas tumour cells grow out in the presence of an isotype control (Figure 3 and
data not shown). Uptake of tumour cells was a fast process, and the majority of
tumour cells were phagocytosed within the first 4 hours. Degradation of tumour
cells by macrophages was however very slow, and even after 20 hours, tumour cell
debris was still observed within macrophages. Breakdown of tumour cells involved lysosomal recruitment, and the formation and acidification of large phagolysosomes that contained the tumour cells (Supplementary figure 1).

Overall, we did not observe any overt differences in ADCP in the presence of the different anti-EGFR mAbs. This was unexpected, since panitumumab is of the IgG2 isotype, which has lower affinity for IgG Fc receptors (Fcγ receptors), and was unable to induce antibody-dependent cellular cytotoxicity (ADCC) via NK cells. Therefore, we performed ADCC assays with NK cells and HT29 or HCT116 in the presence of cetuximab, zalutumumab or panitumumab. Both cetuximab and zalutumumab efficiently induced ADCC of HT29 and HCT116 (Supplementary figure 2 and data not shown). However, as previously shown panitumumab was unable to induce ADCC by NK cells. Thus, all 3 anti-EGFR mAbs effectively induced ADCP by macrophages, but only mAbs of the IgG1 isotype induced ADCC by NK cells.

**FIGURE 1.** A concentration of 0.05-0.1µg/ml anti-EGFR mAbs is sufficient to saturate tumour cells for >90%.

The therapeutic mAbs cetuximab (black bars), zalutumumab (gray bars) and panitumumab (white bars) were for opsonization of tumour cell lines. (A) HCT116, (B) SW948, (C) HT29, (D) Caco2, (E) RKO, (F) SW620 and (G) A431. Concentrations are in µg/ml: 30-10-5-2.5-1-0.5-0.1-0.05-0.01-0.005-0.001.
Proliferation and migration was not affected in the presence of low doses of anti-EGFR mAbs

Healthy colon cells also express EGFR, albeit at lower levels as most colorectal tumours. Nonetheless, treatment with anti-EGFR antibodies may hamper wound healing, which would preclude their use around surgery. We therefore investigated the effect of anti-EGFR mAbs on cell proliferation and migration, as both processes are essential for effective wound healing. Neither A431 nor Caco2 cells have mutations in EGFR signalling pathways (referred to as wildtype). When A431 cells that have very high EGFR expression were cultured for 72 hours proliferation was reduced in the presence of 1µg/ml or higher anti-EGFR mAbs (~30-40% reduction, Figure 4A), whereas Caco2 cells were only affected (~20% inhibition) when more than 5µg/ml anti-EGFR mAbs were added (Figure 4B). No effect on proliferation was observed when HCT116, HT29 and RKO cells, which have mutations in downstream EGFR signalling pathways, were cultured in the presence of anti-EGFR antibodies (Figure 4C-E).

We next used culture inserts to mimic wound healing of an artificial gap (Figure 5). HCT116 cells were able to completely close the gap in approximately 27 hours. No significant differences were observed in the presence of cetuximab, zalutumumab or panitumumab at concentrations up to 30µg/ml (Figure 5B). Moreover, neither Caco2 nor A431 cells - that were affected in their capacity to proliferate - were inhibited in their ability to close the gap in the presence of high amounts of anti-EGFR mAbs (Figure 5C and D).
Current treatment concentrations can be significantly lowered
Patients with metastatic wildtype KRAS colorectal carcinoma are treated with high amounts of cetuximab (400mg/m$^2$) or panitumumab (6mg/kg). When we used plasma of patients that was obtained four hours post infusion to opsonize the tumour cell lines, we observed that 2.5% diluted plasma is sufficient for maximal saturation of EGFR (Figure 6).

FIGURE 3. A431 tumour cells are eliminated via ADCP by macrophages. (A and C) Fluorescent images of phagocytosis of DiI (Red) labelled A431 cells by DiO (green) labelled macrophages in the (A) presence or (C) absence (irrelevant isotype control) of zalutumumab. (B and D) Overlay of fluorescence and bright field images of experiments shown in A and C.
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Discussion

The anti-EGFR mAbs cetuximab and panitumumab are currently used to treat metastatic colorectal cancer. These mAbs have direct effects on tumour cells as antagonists for EGFR, thereby inhibiting proliferation. Furthermore, mAbs recruit immune cells and induce ADCC or ADCP, and can activate the complement pathway, which results in complement-dependent lysis of the tumour cells. Disappointingly, only 10% of patients with metastatic colorectal cancer had clinical responses, after monotherapy with anti-EGFR mAbs. By combining anti-EGFR mAbs with chemotherapy response rates could be enhanced to approximately 20%-30%. These poor response rates were likely due to mutations in KRAS and BRAF proteins, which are signalling proteins downstream of EGFR. As a consequence of these mutations the EGFR signalling pathway is continuously active, independent of binding of EGF to its receptor. When metastatic colorectal carcinoma patients without mutated KRAS were treated with anti-EGFR mAbs, clinically significant response rates and reduced risk of disease progression increased to approximately 60%. Therefore, currently anti-EGFR mAb treatment is only indicated when patients have no KRAS or BRAF mutations. However, as elimination of human colorectal tumour cells by macrophages via ADCP in the presence of human anti-EGFR mAb was independent of mutations in these pathways, that is not a limitation for pre-operative anti-EGFR mAb therapy.

By contrast, expression of EGFR is likely limiting. Tumour cells with high EGFR expression were phagocytosed effectively by macrophages, even in the presence of low antibody concentrations. Tumour cells with low EGFR expression were not taken up, even when high anti-EGFR mAbs were added. Furthermore, ADCP of Caco2 and HCT116 cell lines was ineffective, in spite of similar EGFR expression levels of other cell lines that were phagocytosed. Several studies demonstrated immune evasion by tumour cells via expression of receptors such as CD47 and PD-L1 (programmed cell death ligand 1).

**FIGURE 4. Reduced doses of anti-EGFR antibodies do not affect cell proliferation.**

The effect of therapeutic antibodies on cell proliferation. The effect of therapeutic antibodies on cell proliferation of (A) A431, (B) Caco2, (C) HCT116, (D) HT29 and (E) RKO tumour cells were measure after 72hr. Cetuximab (black bars), zalutumumab (grey bars) and panitumumab (white bars) were diluted to concentrations of 30-10-5-2.5-1-0.5- 0.1μg/ml. Bars represent mean ±SEM. * P<0.05; # P<0.01
death-1 Ligand). Overexpression of CD47 is observed on tumour cells, which acts as ‘don’t eat me signal’, by binding to Sirp-a on macrophages, which is an inhibitory receptor\textsuperscript{36–38}. Tumour cells can also express PD-L1 that was originally described as a signalling molecule to prevent killing by T-cells by interacting with PD-1 or CTLA-4 (cytotoxic T-lymphocyte associated protein 4)\textsuperscript{39}. More recently also an interaction of PD-L2 and repulsive guidance molecule b (RGMb) was observed that also prevented tumour cell kill by macrophages\textsuperscript{40}. By inhibiting cytotoxic effector functions of T cells and macrophages, tumour cells can evade adaptive and innate immune responses. Thus, a co-therapy in which inhibitory receptors or ligands are blocked in combination with tumour-targeting mAbs may improve mAb therapy.

We did not observe major differences between induction of ADCP by macrophages in the presence of cetuximab, panitumumab or zalutumumab, whereas NK cells were
not able to kill tumour cells in the presence of panitumumab. This is likely due to differences in expression of IgG Fc receptors by macrophages and NK cells. NK cells only express the FcγRIIIa that binds with high affinity to IgG1 complexes, including cetuximab and zalutumumab. However, IgG2, such as panitumumab, binds only with low affinity to a specific isotype of the FcγRIIIa and chapter 2. In order to recruit NK cells for mAb therapy, it is therefore beneficial to use antibodies of the IgG1 isotype. By contrast macrophages express FcγRI and FcγRIIa, in addition to FcγRIIIa. It is therefore likely that ADCP in the presence of IgG2 mAbs, e.g. panitumumab, is mediated via either FcγRI or FcγRIIa.

![Graphs showing the effects of plasma dilution on different tumor cell lines](image)

**FIGURE 6. Forty times diluted plasma of patients who received cetuximab is sufficient to saturate tumor cells.**

Plasma from patients, who were infused with cetuximab, was titrated and (A) A431, (B) HCT116, (C) HT29, and (D) SW948 tumor cells were opsonized. Binding of cetuximab to cells was analysed by flow cytometry. Titration curves of plasma from 5 different patients are shown.

We previously showed that Kupffer cells, and to a lesser extend infiltrating monocytes are potent inducers of ADCP of tumor cells that are present in the bloodstream. Therefore, we propose a novel mAb-based therapeutic strategy in which anti-EGFR mAbs are administered shortly before surgery to eliminate circulating tumor cells. It is however essential that wound healing is not affected by pre-operative anti-EGFR mAb therapy, as leakage of the anastomosis can result in major infectious complications, due to colonization of the colon by an abundant microflora. Patients are currently treated with an initiation dose of 400mg/m² cetuximab or 6mg/kg panitumumab followed by repeated doses of 250mg/m² or 6mg/kg respectively,
which is aimed to block EGF binding to EGFR. These high amounts are not needed to induce elimination of single cells by the immune system. We aim to perform one infusion to obtain a concentration of cetuximab of ~1-10µg/ml in the bloodstream around surgery, which was sufficient for in vitro ADCP. In an in vivo xenograft model it was demonstrated that low antibody concentrations were sufficient to eliminate circulating tumour cells, which was independent of KRAS mutation\textsuperscript{45}. Moreover, in the presence of a low concentration anti-EGFR antibodies only minor inhibition of proliferation was observed in cell lines with a wildtype signalling pathway, whereas no effect was observed at all on the migration capacity of the cell lines used. These two processes are critical in wound regeneration but were only marginally affected, we anticipate that anastomosis healing will be minimally influenced by pre-operative infusion of a low dose anti-EGFR mAbs.

In conclusion, colorectal cancer patients may benefit greatly from pre-operative treatment with tumour targeting mAbs, as this may lead to elimination of tumour cells in the circulation by effector cells such as NK cells and the myeloid mononuclear network in the liver. By administering anti-EGFR antibodies to all patients with an EGFR expressing tumour prior to surgery, tumour cells that are released during surgery will be opsonized and cleared by the immune system. We demonstrated that the current dosage for treatment can be lowered since 2,5% patient plasma was still sufficient to fully opsonize tumour cells. Additionally, lower doses, while still inducing efficient tumour cell killing, did not influence processes involved in wound healing. Therefore, we propose that colorectal cancer patients can be safely treated with low dose anti-EGFR antibodies pre-operative to eliminate circulating tumour cells, which may ultimately prevent metastasis formation.

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References

Supplementary Figures

A

1° 30° 130° 180° 255°

24h

B

1° 30° 130° 180° 255°

24h

zalutumumab
control mAb
SUPPLEMENTARY FIGURE 1. Lysosomal degradation of tumour cells after ADCP.
Anti-EGFR 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. Left panels: bright field; middle panels: fluorescence; right panels: overlay. (A) After treatment with Zalutumumab, macrophages 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. Lysosomes fuse with tumour cell containing phagosomes, acidifying phagosomes (arrows in third & fourth row). After approximately 4 hours the first tumour cell is degraded. After 24 hours of treatment all tumour cells are phagocytosed and lysosomes contain degraded tumour material ('>' most right panel). (B) Treatment with isotype 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).

SUPPLEMENTARY FIGURE 2. HT29 cells can be killed by NK cells in the presence of IgG1 antibodies.
Remaining viable HT29 after ADCC by NK cells, as determined by a CTB assay, in the presence of various concentrations anti-EGFR antibodies. The human IgG1 base Cetuximab (black bars) and zalutumumab (gray bars) and the human IgG2 based panitumumab (white bars). Concentrations used 30-10-5-2,5-1-0,5- 0,1μg/ml.