CHAPTER 6

EXPERIMENTALLY INDUCED LIVER METASTASES FROM COLORECTAL CANCER CAN BE PREVENTED BY MONONUCLEAR PHAGOCYTE-MEDIATED MONOCLONAL ANTIBODY THERAPY

CHAPTER 6
Abstract

Background & Aims
Development of liver metastases is a frequent complication in patients with colorectal cancer (CRC), even after successful resection of the primary tumor. As such, post-operative adjuvant therapies that aim to eliminate residual disease after surgery may improve patient outcome.

Methods
We used a colon carcinoma liver metastases model, in which CC531s colon carcinoma cells are injected into the portal circulation by a surgical procedure. As injected tumor cells are arrested in the liver, this model is suitable for investigating the interaction of tumor cells with the liver microenvironment. By administering tumor specific monoclonal antibodies (mAb) directly post-operatively, we were able to determine the effect of antibody therapy on eradication of arrested tumor cells and subsequent liver metastases outgrowth.

Results
We showed that post-operative treatment with tumor specific monoclonal antibodies (mAb) prevents liver metastases outgrowth. Antibody-dependent phagocytosis (ADPh) was the main mechanism involved, as enhanced uptake of tumor cells by innate mononuclear phagocytes in the liver was observed after mAb therapy. Furthermore, Kupffer cells (KC) were identified as the most prominent effector cells, as depletion of KC abolished therapeutic efficacy. This was partly compensated by monocytes when animals were treated with a high mAb dose, but monocytes were unable to phagocytose tumor cells when rats were treated with low mAb doses.

Conclusions
The finding that KC and monocytes can eliminate tumor cells through ADPh has important and promising clinical implications for designing new adjuvant therapies for patients undergoing CRC resection.


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Part II

Introduction

The high morbidity and mortality associated with patients with colorectal carcinoma (CRC) is frequently caused by metastases development, which most commonly presents in the liver. A substantial fraction (10-25%) of the patients with CRC has already developed liver metastases at the time of diagnosis. Although surgical resection of the tumor is obligatory in the vast majority of patients and provides a good chance for cure, increasing evidence emerges that indicates that surgery paradoxically enhances metastases development 1-3. We previously defined a novel route of metastasis that is associated with surgical trauma in addition to the classical route of metastases development. This concept implies that surgery induces an inflammatory response, which creates permissive circumstances for circulating tumor cells to adhere in target organs and develop into metastases. Circulating tumor cells are present in ~ 65% of patients with CRC prior to surgery 4. Moreover, this number is increased following tumor resection due to shedding of tumor cells 5. Thus, the presence of viable tumor cells in the circulation combined with the augmented potential of these cells to adhere enhances the risk for metastases development after surgical resection of tumors. Because 20-50% of patients who do not have visible metastases at the time of surgery will develop secondary disease even after successful resection of the primary tumor, enhanced tumor cell adhesion after surgery is strongly supported 6.

In spite of the increased risk for inducing metastases, surgical resection will however eliminate the bulk of the tumor. Consequently, only minimal residual disease will be present in CRC patients post-surgically, which renders them exceptionally suitable for adjuvant therapy. It was shown that peri-operative chemotherapy after resection of CRC resulted in decreased metastases development and increased survival in patients with stage III colorectal cancer 7, 8. However, stage II patients did not benefit from additional chemotherapy, indicating that improvement of therapeutic strategies is warranted. Because mAb therapy is currently successfully being used to treat several forms of cancer, including lymphoma and breast cancer 9, anti-tumor mAb might represent promising new peri-operative treatment modalities. Several mAb that target CRC are currently available. Although not in the context of surgical resection, mAb that targeted the epidermal growth factor receptor yielded promising results in phase II trials 10. Additionally, one study reported that post-operative treatment (within 42 days after surgery) with mAb directed against epithelial cell adhesion molecule resulted in a 23% decrease of tumor recurrence and a 32% improve in survival of patients that suffered from CRC 11. However, discrepant results have been obtained in similar clinical trials, indicating that more research is needed to address this question 12.

Despite the overwhelming interest in using mAb for the treatment of cancer, the in vivo mode of action of this therapy is not elucidated. The mAb may act directly on tumor cells by influencing signaling via tumor antigens, potentially leading to apoptosis or inhibition of proliferation 13. Additionally, by recruiting the complement cascade, mAb might induce complement dependent cytotoxicity 14. Through binding to immunoglobulin (Ig) G Fc receptors (FcγR), mAb furthermore provides a link between immune cells and tumor cells, which may lead to ADCC 9. Although ADCC has been extensively shown in vitro to be an important effector mechanism of tumor cell killing, the occurrence of ADCC in vivo has not yet been directly demonstrated. However, as anti-tumor mAb therapy is ineffective in mice lacking Fc receptors, the role of ADCC as an important effector mechanism is
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supported\textsuperscript{15}. Additionally, polymorphisms in FcγRII and FcγRIII influenced clinical outcome in lymphoma patients after anti-CD20 mAb treatment, further supporting that FcγR-mediated effector functions by immune cells may play an important role in determining the success of anti-tumor mAb therapy\textsuperscript{16}. It was demonstrated that monocytes, macrophages, polymorphonuclear cells and natural killer (NK) cells are capable of performing ADCC in vitro\textsuperscript{9,17}, but their relative contribution to in vivo therapeutic efficacy is not yet determined. Previously we showed in a melanoma mouse model that antibody therapy for liver metastases was dependent on mouse FcγRI (mFcγRI) and mFcγRIV, suggesting a role for cells of the myeloid lineage\textsuperscript{18}. In the current study, we investigated the role of these cells in mAb therapy in more detail, and identified KC as the main effector cells. Additionally, provided that high mAb dose was given, monocytes were able to contribute to antibody-dependent phagocytosis and capable of partly compensating the absence of KC.
Methods

Antibodies
Mouse anti-ED1 (pan monocyte/macrophage marker) and mouse anti-ED2 (mature tissue macrophage marker) mAb were purchased at Serotec, Oxford, UK. As second antibodies AlexaFluor-labeled goat anti-mouse mAb (Molecular Probes Inc., Eugene, OR) were used. Mouse anti-CC531s mAb were produced from hybridomas MG4-γ1 (mIgG1), MG4-γ2a (mIgG2a) and MG4-γ2b (mIgG2b)19,20. The MG4-γ2a and MG4-γ2b hybridoma’s were produced by isotype switching of the MG4-γ1 hybridoma as described by Boot et al21. As such, the Fc parts of these mAb have been modified but the characteristics (specificity, avidity and affinity) of the F(ab)2 fragments remain identical. Binding of mAb isotypes to CC531s cells was investigated with flow cytometry (FACS Calibur, Becton Dickinson, San Diego, CA). To exclude aspecific effects of IgG1 mAb on liver metastases development, mouse anti-luciferase (IgG1), THB4 (IgG2a) or OKT8 (IgG2b) mAb were used as isotype control for in vivo experiments.

Flow cytometry
CC531s were incubated with primary MG4 isotype mAbs (10 µg/ml) for 45 min at 4°C. After washing, visualization was achieved by using PE-conjugated rabbit-anti-mouse IgG mAb (1:200). Subsequently, binding of MG4 isotype mAb to CC531s cells was analyzed with flow cytometry (BD).

Cell culture
The CC531s tumor cell line is a moderately differentiated, weakly immunogenic colonic adenocarcinoma, which was originally induced in Wag/Rij rats by 1,2-dimethylhydrazine. It is transplantable in syngeneic rats, and does not spontaneously metastasize22. Tumor cells were cultured under standard incubator conditions in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (200 µM) (further referred to as complete RPMI). Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution, and contained single tumor cells as well as small clusters (2-8 cells). Viability was assessed by trypan blue exclusion and always exceeded 95%. For fluorescent labeling, CC531s cells (5x10^6 cells/ml) were incubated in complete RPMI containing 0.05 µg/ml 3,3'-dioctadecyloxacarbocyanine perchlorate (Molecular Probes Inc.) for 30 minutes at 37°C and subsequently washed with Hanks’ balanced salt solution, according to the manufacture’s instructions. For in vitro experiments bone marrow derived macrophages were obtained by incubating freshly harvested bone marrow from a Wag/Rij rat with L929 conditioned medium as previously described23. After 6 days macrophages were harvested by 20 minute incubation with 0,4 % lidocaine solution and subsequent scraping. After washing macrophages were stained with PKH 67 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s description.

In vitro cytotoxicity assay
Cytotoxicity assays were performed by co-culturing CC531s tumor cells and PKH67-labeled macrophages in a ratio of 1:15 in the presence of 10 µg/ml MG4-γ1, MG4-γ2a, and MG4-γ2b. The percentage of eradicated tumor cells was calculated by determining the ratio of
macrophages to tumor cells after 24 h. As the number of macrophages remains constant throughout the experiment, but the number of tumor cells diminishes due to tumor cell killing, the ratio reflects cytotoxicity. Next, these ratios were compared to the ratio of macrophages to tumor cells of separately cultured macrophages and tumor cells that were mixed just before flow cytometry, representing 0% cytotoxicity. Chamber slides (Ibitreat μ slide eight well chamber slides, Ibidi, Munchen, Germany) were used for video microscopy using the Olympus CellR real-time live-imaging station (type IX81, UPLFLN 40 × O/1.3 lens, Münster, Germany). Pictures were taken every 3 min with an Olympus ColorView II camera for 3 h, followed by a 6-min interval for 7 h. Additionally, random pictures were taken after 24 h.

**Animals**

Male inbred Wag/Rij rats, weighing 180-220 g, were obtained from Charles River, Maastricht, The Netherlands. Animals were kept under standard laboratory conditions and had access to food and water ad libitum. The Committee for Animal Research of the VUmc approved the experiments, according to the national guidelines.

**Animal models**

To induce liver metastases, a laparotomy (2-cm midline incision) was performed under anesthesia. Next, a mesenteric vein was exposed and tumor cells were injected. Animals received intraperitoneal (i.p.) injections (0.5 ml) containing low dose (50 μg) or high dose (200 μg) mAb or PBS directly after inoculation of tumor cells (day 0) as well as on day 2 and 4. Rats (n≥8 per group) were sacrificed after 14 days and tumor score was determined. Tumor score (in mm) was defined by adding the maximum diameter of each individual tumor nodule, as previously described. KC were depleted by injecting 0.5 ml liposome-encapsulated clodronate in the tail vein at days -4 and -2. We previously described that KC depletion prior to tumor cell administration dramatically enhanced liver metastases outgrowth. As such, CC531s cell dose was lowered to 2 * 10^5 in KC depleted animals for ethical reasons. Moreover, in this way development of total tumor score was comparable in control versus KC depleted animals after 14 days, enabling appropriate determination of mAb therapy efficacy in both groups. Alternatively, animals received one i.p. injection containing 50 or 200 μg mAb or PBS following injection of tumor cells. After 24 hours, animals (n=4 per group) were sacrificed and livers were snap frozen for microscopical analyses.

**Fluorescence microscopy**

Cryostat tissue sections of 5 μm were fixed for 10 minutes in acetone and air-dried. Slides were incubated for 1 hour with primary mAb (10 μg/ml) at 4°C in a humidified tissue chamber. After washing, visualization was achieved by incubating with Alexafluor®-labeled goat anti-mouse mAb (1:400). Nuclei were stained with Hoechst (10 μg/ml, Molecular Probes). Sections were washed, mounted and examined with a Leica DM6000B fluorescence microscope (Leica Microsystems, Heidelberg, Germany). The number of phagocytosed tumor cells 24 hours after administration was determined by analyzing 6 liver samples per animal, of which 5 randomly selected fields were analyzed. On average, ~150 tumor cells

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were counted per sample. The quantity of ED1⁺ and ED2⁺ cells was also determined by analyzing 6 liver samples per animal, of which 5 randomly selected fields were examined. However, due to the large number of ED1⁺ or ED2⁺ cells, which rendered individual counting not feasible, quantity of positive pixels per field of view was digitally determined instead, using the digital image analysis program AnalySIS (Soft Imaging System GmbH, Münster, Germany). By means of a constant predefined threshold for color components, positively stained areas were used for quantification.

Statistical analysis
Data was analyzed with Mann-Whitney U tests, preceded by Kruskal-Wallis tests for comparison of multiple groups if necessary. Significance was accepted at $p < 0.05$. 

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Results

Treatment with IgG1 anti-tumor mAb successfully prevents development of liver metastases. It was demonstrated that circulating tumor cells are present in the majority of patients undergoing surgical resection of CRC. Previously we showed that circulating tumor cells have an increased potential to adhere in the liver and develop into metastases after surgical trauma. As such, postoperative adjuvant therapies that target these circulating tumor cells, like anti-tumor mAb therapy might improve clinical outcome after surgery. Therefore we studied the potential of post-operative mAb treatment after a surgical procedure (laparotomy and injection in a mesenteric vein) using a rat colon carcinoma liver metastasis model. It was previously demonstrated that subclasses of IgG exhibit considerable differences in their capacity to mediate effector functions, which is dependent on their differential affinities for activating FcγR compared to inhibitory FcγR and the capacity to activate complement. Because detailed knowledge of the rat FcγR system is not yet available, we first investigated the IgG isotype that displays the highest therapeutic efficacy (Fig. 1A-B). After inoculation of liver metastases by a surgical procedure, rats were treated i.p. on day 0, 2 and 4 with PBS or 200 μg anti-CC531s mAb (MG4) with identical F(ab)2 region but with different IgG Fc tails (IgG1, IgG2a or IgG2b). All MG4 isotypes bound equally to CC531s cells (Fig 1C). On day 14, animals were sacrificed, livers were excised and tumor scores were determined by adding the maximum diameter of each individual tumor nodule per rat. Eight out of nine PBS treated rats developed liver metastases, and overall tumor score was 92 +/- 90 mm. Treatment with either MG4-γ2a (IgG2a) or MG4-γ2b (IgG2b) mAb resulted in liver metastases development in all animals except one with an average tumor score of respectively 113 +/- 91 or 35 +/- 27 mm. Although treatment with MG4-γ2b mAb appeared to reduce development of tumor score in the liver, these differences were not statistically different. By contrast, only one rat treated with MG4-γ1 (IgG1) mAb developed a small amount of tumors whereas all other animals were tumor-free (Fig 1B). Treatment with an anti-luciferase IgG1 (as isotype control) did not diminish the number of metastases (data not shown), which ruled out aspecific effects of IgG1 mAb treatment on metastases formation. Additionally, metastases were neither observed in other organs, nor in the liver 3 months after mAb treatment (data not shown). Thus, we show that MG4-γ1 mAb specifically prevented development of liver metastases. Antibody-dependent phagocytosis of tumor cells by the mononuclear phagocyte network is responsible for therapeutic efficacy of mAb.

We previously demonstrated that mFcγRI and mFcγRIV, which are exclusively expressed on mononuclear phagocytes, are essential for the therapeutic effect of mAb therapy in a mouse model. As such, we first investigated the capacity of macrophages to mediate cytotoxicity towards CC531s tumor cells in vitro. Incubation with MG4-γ1, MG4-γ2a or MG4-γ2b mAb alone did not result in diminished tumor growth, excluding direct effects of MG4 mAb on tumor development (data not shown). However, whereas a co-culture of macrophages and CC531s tumor cells in the presence of MG4-γ2a mAb did not result in diminished outgrowth of tumor cells compared to isotype control, the addition of MG4-γ1 or MG4-γ2b mAb led to increased tumor cytotoxicity and decreased tumor cell outgrowth after 24 h (Fig. 2A-E). As MG4-γ1 mAb was most effective in diminishing tumor cell outgrowth, video microscopy was used to gain more insight in the mechanism of antibody-dependent macrophage-mediated cytotoxicity. Although close
contact between macrophages and tumor cells was observed in the presence of irrelevant mIgG1 mAb, interaction did not lead to tumor cell killing. In contrast, the addition of MG4-γ1 mAb resulted in rapid antibody-dependent phagocytosis (ADPh) of tumor cells by macrophages (Fig. 2F–M and Supplementary data).

To investigate whether liver macrophages (Kupffer cells, KC) were involved in the observed therapeutic effect of tumor specific mAb, we next performed in vivo experiments in which KC had been depleted prior to injection of MG4-γ1 mAb. KC were depleted by i.v. administration of liposome-encapsulated clodronate on days −4 and −2. On day 0, animals received tumor cells and were treated either with PBS or with 200 μg MG4-γ1 mAb on days 0, 2, and 4. Treatment with 200 μg MG4-γ1 mAb was highly efficient in control animals, since liver metastases development was prevented in six out of eight animals (Fig 3A and B). Additionally, the tumor score in the two animals that did develop tumors was minimal (tumor score of respectively, 10 and 15 mm). By contrast, all untreated animals developed liver metastases with an average tumor score of 213 ± 194 mm. Interestingly, after KC depletion, seven out of nine animals developed liver metastases in both MG4-γ1-treated and PBS-treated groups, indicating that successfullness of therapy depends on the presence of KC. However, albeit not statistically different, KC-depleted animals that were treated with MG4-γ1 mAb had a tumor score of 115 ± 146 mm, compared to 344 ± 217 mm (p = 0.069) in untreated KC-depleted animals, which suggested that MG4-γ1 mAb treatment still exhibited some remaining effector functions (Fig 3A and B).
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To gain more insight in the mechanisms by which mAb exerted their therapeutic effect, events that occurred shortly after tumor cell arrest were studied next. Livers from rats were analyzed 24 hours after administration of fluorescently labeled CC531s cells. On average 150 CC531s were counted per sample. The anti-ED2 mAb, which selectively stains KC in the liver, was used to investigate interaction between KC and CC531s tumor cells (Fig 4A,C,E). In the absence of MG4-γ1 mAb, KC had already phagocytosed respectively 78 +/- 7 % of tumor cells present in the liver, confirming the potent role of KC in tumor cell killing. However, tumor cell phagocytosis by KC increased to respectively 92 +/- 2 % after administration of high dose MG4-γ1 (Fig 4G). This indicates that KC mediate antibody-dependent phagocytosis (ADPh) and subsequent cytotoxicity in the presence of MG4-γ1 mAb, resulting in more efficient tumor cell killing. Because mAb therapy seemed to have a modest therapeutic effect in the absence of KC and as it has been previously reported that monocytes are involved in therapeutic efficacy of mAb 27, the pan macrophage/monocyte marker ED1 was used to investigate interaction of newly recruited monocytes and tumor

Figure 2 Macrophages decrease tumor cell outgrowth in vitro by antibody-dependent phagocytosis (A) Percentage of CC531s tumor cells that are eradicated, after 24 h, by macrophages in the presence of MG4-γ1, MG4-γ2a, and MG4-γ2b. Microscopic images of co-cultures of CC531s cells and macrophages in the presence of MG4-γ1 (B) or isotype control (C) mAb after 24 h. (D and E) For clarity reasons we have indicated CC531s cells by red marking which represents the area covered by tumor cells in panel B or C. (F–M) Excerpts from videos showing interaction between macrophages (green) and tumor cells with isotype control (F–I) or MG4-γ4 mAb (J–M) over time.

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Figure 3 Treatment with IgG1 anti-tumor mAb depends on KC
(A and B) The effect of high dose mAb therapy on liver metastases development was evaluated in KC-depleted animals. After intravenous administration of PBS or liposome-encapsulated clodronate on days −4 and −2, animals were administered CC531s tumor cells on day 0. Subsequently, animals were treated with PBS or 200 μg MG4-γ1 mAb on days 0, 2, and 4. After 14 days the livers were excised and the tumor score was determined.

Figure 4 KC and monocytes mediate ADPh after high dose mAb treatment
Twenty-four hours after inoculation of CC531s tumor cells and treatment with 200 μg MG4-γ1 mAb or PBS, control and KC-depleted animals were sacrificed and livers were excised. (A and B) Phagocytosis of DiO-labeled CC531s cells (green) by ED2+ Kupffer cells or ED1+ monocytes (red) was determined by immunofluorescent analysis. After depletion of KC by injection of liposome-encapsulated clodronate on days −4 and −2, CC531s tumor cells in MG4-γ1 treated animals were no longer phagocytosed by ED2+ KC (C), but were phagocytosed by ED1+ monocytes (D). (E) Treatment with PBS did not result in CC531s tumor cell phagocytosis by ED2+ KC. (F) Treatment with PBS resulted in less phagocytosis of CC531s tumor cells by ED1+ monocytes, compared to treatment with 200 μg MG4-γ1 mAb. (G-H) The number of CC531s tumor cells phagocytosed by ED2+ KC and ED1+ monocytes was quantified after 24 h. Six liver samples were taken from every animal, and per sample, five randomly selected fields were chosen for analysis. On average □150 CC531s tumor cells were analyzed per sample (G). The percentage of phagocytosed CC531s cells by ED2+ KC is shown in black bars. (H) The percentage of phagocytosed CC531s cells by ED1+ KC/monocytes in control animals or ED1+ monocytes in KC-depleted animals is respectively shown in grey and white bars.

(Fig 4B,D,F). In control animals, ED1 stains both KC and monocytes (Fig 4H, grey bars). However, after depletion of KC by administration of liposome-encapsulated clodronate, only newly recruited monocytes express ED1 (Fig 4H, white bars). Microscopical analysis showed that in the absence of KC, monocytes had phagocytosed respectively 50.7 ± 8.5% of CC531s cells 24 hours after tumor cell administration. Moreover, treatment with MG4-γ1
mAb in the absence of KC increased tumor cell phagocytosis by monocytes to respectively 61.9 +/- 4.2 %, indicating that monocytes contribute to ADPh.

To investigate whether arrest of tumor cells in liver sinusoids or administration of MG4-γ1 mAb also triggered increased recruitment of effector cells, KC and monocyte numbers were investigated after 24 h. Due to the large number of ED1+ or ED2+ cells, which rendered individual counting not feasible, quantity of positive pixels per field of view was digitally determined. However, neither administration of tumor cells nor injection of MG4-γ1 mAb resulted in enhanced presence of KC or monocytes into the liver (data not shown). As such, MG4-γ1 mAb induced recruitment of effector cells was ruled out as the underlying cause of increased tumor cell phagocytosis. KC are superior in mediating ADPh compared to recruited monocytes.

When rats were treated with a lower dose (50μg) mAb therapy, a modest increase in tumor cell phagocytosis by KC was observed (Fig 5A,C). However, administration of low dose MG4-γ1 mAb did not increase phagocytic activity of monocytes in KC-depleted animals (Fig 5B,D; white bars). Albeit somewhat less efficient than administering high doses (200 μg) of mAb, low dose (50 μg) therapy significantly decreased liver metastases development in control rats. By contrast, all KC depleted animals from untreated and mAb treated groups developed liver metastases and tumor score was comparable between the two groups (Fig 5E-F).

Figure 5 After low dose mAb therapy, ADPh is exclusively mediated by KC (A–D). Twenty-four hours after inoculation of DiO-labeled CC531s tumor cells and treatment with 50 μg MG4-γ1 mAb or PBS, control, and KC-depleted animals were sacrificed and livers were excised. (A) Phagocytosis of DiO-labeled CC531s cells (green) by ED2+ Kupffer cells (red). (B) After low dose mAb therapy, ED1+ monocytes (red) were unable to phagocytose CC531s cells (green). Six liver samples were taken from every animal, and per sample, five randomly selected fields were chosen for analysis. On average 150 CC531s tumor cells were analyzed per sample. (C) The percentage of phagocytosed CC531s cells by ED2+ KC is shown in black bars. (D) The percentage of phagocytosed CC531s cells by ED1+ KC/monocytes in control animals or ED1+ monocytes in KC-depleted animals is shown in grey and white bars respectively. Data are represented as mean ± standard deviation. (E and F) The effect of low dose mAb therapy on liver metastases development was evaluated in KC-depleted animals. After intravenous administration of PBS or liposome-encapsulated clodronate on days −4 and −2, animals were administered CC531s tumor cells on day 0. Subsequently, animals were treated with PBS or 50 μg MG4-γ1 mAb on days 0, 2, and 4. After 14 days the livers were excised and the tumor score was determined. The tumor score was defined by adding the maximum diameter of each individual tumor nodule.
Resection of the primary tumor is the preferred and only treatment modality that can provide long-term disease-free survival of patients with CRC. Unfortunately, even patients who are eligible for intentionally curative surgery are at risk of developing metastases in the course of their disease. We previously showed that surgical trauma paradoxically augments the development of liver metastases. Post-operative treatment that targets minimal residual disease after surgery might therefore improve clinical outcome. As such, we studied the potential of therapeutic mAb to prevent tumor outgrowth in the liver directly after a surgical procedure by performing a laparotomy which was followed by injection of tumor cells in the mesenteric vein. In this model, the interaction of circulating tumor cells with the liver microenvironment is investigated, rather than other steps of metastases development such as detachment of cells from the primary tumor and endothelial penetration. Treatment with tumor specific mIgG1, but not mIgG2a or mIgG2b mAb efficiently prevented liver metastases outgrowth in the vast majority of rats, and significantly reduced tumor score in the few animals that did develop tumors. This could not be explained by differences in affinity, as only the Fc part of MG4 mAb were modified, whereas the F(ab)2 fragment remained unaltered. All MG4 isotype mAb had equal ability to bind to CC531s cells. This is in accordance with earlier experiments showing equal staining patterns and intensities of MG4-γ1 and MG4-γ2a in CC531s tumors, indicating that these mAb have similar homing capacities to CC531s tumors in vivo. Interestingly, whereas mouse effector cells exert optimal cytotoxicity in the presence of mIgG2a mAb, while mIgG1 is ineffective, rat macrophages kill tumor cells most effectively in the presence of mIgG1. This indicates differences in affinity for different IgG isotypes between murine and rat Fc receptors. Hence, caution should be taken to directly translate experiments performed in rodents to the clinical setting.

Knowledge of the mechanisms by which tumor specific mAb exert their anti-tumor effects will help to rationally design improved future clinical strategies to battle cancer. However, the exact mechanisms by which mAb mediate their effects remain a subject of debate. Several clinical mAb were shown to act directly on tumor cells by inducing apoptosis or growth arrest. However, although it is unknown which epitope is targeted by anti-CC531s mAb, no direct effect of the mAb was observed in vitro, supporting that these mAb do not signal directly (data not shown). It was shown in a mouse model of metastatic lymphoma that mAb bound to tumor cells could activate the complement system, which led to complement dependent cytotoxicity. However, MG4-γ1, which in our model efficiently prevented liver metastases outgrowth, neither induced complement mediated lysis in vitro nor complement activation in situ. Additionally, mAb therapy for treatment in a mouse melanoma liver metastases model did not rely on a functional complement system since mAb treatment was still effective in C1q deficient mice. As such, a role for complement in preventing liver metastases outgrowth after mAb therapy seems unlikely.

Monocytes, macrophages, polymorphonuclear cells and natural killer (NK) cells are able to induce ADCC in vitro. However, conclusive data identifying the effector cell population responsible for the in vivo therapeutic effect is lacking. Traditionally, NK cells are considered the main effectors of inducing ADCC following mAb therapy, as NK cells are more potent mediators of ADCC in vitro compared to cells from the myeloid lineage. Additionally, the number and activity of NK cells correlated with clinical response to mAb therapy.
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However, several observations suggest that cells other than NK cells are involved in mAb therapy. Administration of mAb therapy in mice lacking mFcγRIII (the only FcγR on NK cells) still exerts profound anti-tumor effect. Furthermore, the observation that mAb therapy is more efficient in mice lacking the inhibitory mFcγRII, which is not present on NK cells, supports that other FcγR bearing cells are involved. Nimmerjahn et al. respectively showed that mAb therapy for prevention of lung metastases development in mice was dependent on either mFcγRIV or mFcγRI. Both studies suggest that mAb therapy is mediated by cells of the mononuclear phagocyte network, as these are the only cells that express mFcγRI or mFcγRIV. Additionally, mAb therapy for prevention of melanoma liver metastases in mice was mediated by both mFcγRI and mFcγRIV, showing functional redundancy between these receptors. KC were at least partly involved in preventing melanoma outgrowth since depletion reduced therapeutic efficacy. However, because it was shown that tumor cell apoptosis was increased by cross-linking of antigens via mAb binding to FcγR, it cannot be excluded that signaling of target cells is altered after clustering of antigens through binding to FcγR-expressing effector cells. Since both ADCC and cross-linking of tumor antigens depend on FcγR, experiments with FcγR deficient mice cannot provide a definite answer.

Here, we show that the therapeutic effect of mAb therapy for the prevention of liver metastases depends on the innate mononuclear phagocyte network, which eradicates tumor cells via ADPh. Previously, we and others demonstrated that Kupffer cells have innate ability of arresting and eliminating tumor cells through phagocytosis in vitro and in vivo. We now demonstrate that in the presence of MG4-γ1, macrophages efficiently prevent tumor cell outgrowth by antibody-dependent phagocytosis in vitro and in vivo. Additionally, mAb therapy is not effective in the absence of KC and monocytes, indicating that antibody-dependent phagocytosis is not merely the result of enhanced uptake of dead tumor cells. As such, we show for the first time the active involvement of KC in phagocytosis and killing of tumor cells after mAb therapy. The increase of phagocytosis following administration of MG4-γ1 mAb after 24 h seems modest, as phagocytosis of tumor cells was increased with approximately 15%. However, we speculate that this percentage increases over time since mAb therapy successfully prevented the development of liver metastases after 14 days. Alternatively it may be possible that other clodronate liposome sensitive cells account for removal of the remaining small percentage of tumor cells. However this seems less likely due to reasons mentioned above.

In the absence of KC, monocytes were able to partly compensate for lack of KC function as high dose mAb therapy was still moderately effective in KC-depleted animals. Thus the innate mononuclear phagocyte network is responsible for the therapeutic effect of mAb therapy, which is supported by Uchida et al. who previously reported that monocytes were the dominant effector cells for B cell depletion after anti-CD20 mAb treatment. Although we still observe the presence of monocytes in the liver after administration of clodronate liposomes, we cannot formally exclude an effect of clodronate liposomes on monocytes, since they are capable of phagocytosing liposomes. As such our data may underestimate the role of monocytes in the presence of KC. However, phagocytic capacity of available monocytes was abrogated after treatment with lower doses of mAb (50 μg), whereas KC were still able to phagocytose tumor cells. Consequently, low dose mAb therapy was highly
successful in control animals, but was no longer able to prevent liver metastases outgrowth in KC-depleted animals. This indicates that, although both monocytes and KC are able to perform ADPh, KC are a more potent effector cell population in mAb therapy.

In conclusion, we show that treatment with anti-tumor mAb can efficiently prevent liver metastases development in an experimental animal model. Thus, mAb therapy in patients who are at risk of developing liver metastases – such as patients undergoing resection of primary tumors – may benefit in clinical outcome by preventing tumor recurrence. Furthermore, therapeutic efficacy of mAb for the prevention of liver metastases depends on ADPh mediated by the innate mononuclear phagocyte network. Additionally, stimulation of KC and monocyte function and/or numbers, for example by administration of (granulocyte) macrophage colony-stimulating factor, might further enhance efficacy of mAb therapy. Enhanced killing capacity of human Kupffer cells was previously demonstrated after activation with human GM-CSF and interferon gamma. Because all resources are currently available to investigate the therapeutic efficacy of peri-operative anti-tumor mAb, this promising strategy could be explored in the clinic without delay.

Legends
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Experimentally induced liver metastases from colorectal cancer can be prevented by mononuclear phagocyte-mediated monoclonal antibody therapy