

Experimental Antibody Therapy of Liver Metastases Reveals Functional Redundancy between Fc γ RI and Fc γ RIV¹

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Many patients with colorectal cancer will develop liver metastases, even after successful surgical removal of the primary tumor at a time at which no visible metastases are present. We previously demonstrated that surgery—although mandatory—paradoxically enhances the risk of developing liver metastases. Because Ab therapy has been acknowledged as a successful strategy to treat malignancies, we studied the potential of postoperative adjuvant Ab therapy to prevent outgrowth of liver metastases. Treatment with murine anti-gp75 (TA99) mAb completely prevented outgrowth of B16F10 liver metastases in over 90% of mice. Therapeutic efficacy was maintained in either C1q- or complement receptor 3-deficient mice but was completely abrogated in FcR γ -chain knockout mice. This indicates that the classical complement pathway was not essential, but interaction with activatory Fc γ R was necessary for successful therapy. TA99-treatment was still effective in Fc γ RI^{-/-}, Fc γ RIII^{-/-}, Fc γ RI/III^{-/-}, and Fc γ RI/II/III^{-/-} mice, suggesting an important role for Fc γ RIV. However, wild-type mice that were treated with TA99 Abs and an Fc γ RIV blocking Ab (mAb 9E9) were protected against development of liver metastases as well. Only when both Fc γ RI and Fc γ RIV functions were simultaneously inhibited, TA99-mediated curative Ab treatment was abrogated, indicating functional redundancy between both IgG receptors in the liver. Furthermore, depletion of liver macrophages (Kupffer cells) reduced the efficacy of Ab therapy, supporting that Kupffer cells are involved as effector cells. Importantly, since Ab treatment almost completely prevented development of liver metastases, postoperative adjuvant Ab therapy may help to improve patient prognosis. *The Journal of Immunology*, 2008, 181: 6829–6836.

Notwithstanding the considerable progress in the treatment of malignancies that has been made over the last years, cancer remains a major problem in our society. For several types of cancer, such as mamma- and colorectal carcinoma, resection of the primary tumor provides the best chance of long-term disease-free survival. However, evidence is accumulating, which supports that surgery paradoxically enhances the risk of metastases development (1). It was demonstrated that surgical peritoneal trauma—which inherently occurs during resection of colorectal cancer—enhances loco-regional tumor development in a rat model (2–4). Moreover, we recently showed that peritoneal surgical trauma dramatically increased outgrowth of liver metastases, which represents the

most common complication in patients with colorectal cancer (5). Ultimately, 20–50% of the patients who do not have evidence of metastatic disease at the time of resection of the primary carcinoma, and who are eligible for intentionally curative surgery, will develop liver metastases in course of the disease (6, 7).

Several mechanisms can be involved in the development of liver metastases after removal of the primary tumor. The number of malignant cells in the peritoneal cavity, circulation and liver was shown to be enhanced after surgery, suggesting that manipulation of the tumor during resection leads to dissemination of tumor cells (1, 8–10). As implantation of circulating tumor cells is a highly inefficient process, and since most tumor cells are rapidly removed by immune cells, inadvertent spillage of tumor cells during surgery can, however, not fully explain the high recurrence rate. Wound healing processes, including release of angiogenic and inflammatory factors, may contribute to enhanced outgrowth of circulating tumor cells as well. Furthermore, surgery was reported to induce immuno-suppression, which temporally may impede immune cell-mediated arrest and elimination of tumor cells (1, 11–13).

Although surgical procedures have been optimized, and patients are treated with additional therapies like chemotherapy and radiotherapy, prognosis remains poor once metastases have developed (14). Therefore, preventative treatment of patients during and after surgery that helps to diminish outgrowth of secondary disease might improve clinical outcome. Anti-tumor Abs have been shown to represent promising drugs against cancer, either as monotherapy or in combination with radio- or chemotherapy (15, 16). As such, Ab therapy may represent an

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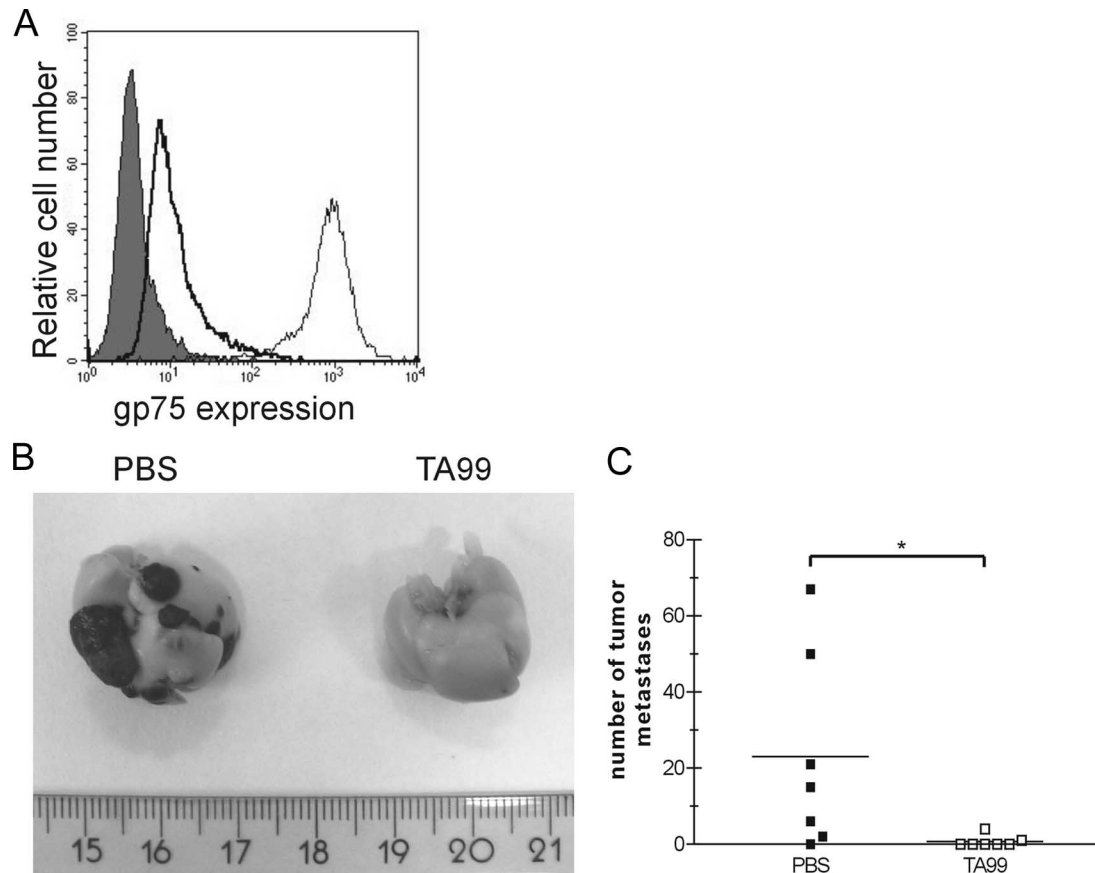


FIGURE 1. TA99 Ab prevents B16F10 outgrowth in liver. *A*, gp75 surface expression (thick line) and total gp75 expression (thin line) levels were determined on B16F10 cells. Filled area represents control mAb. *B*, Livers of PBS- or mAb TA99-treated C57BL/6 mice were analyzed 21 days after tumor inoculation. Black nodules represent metastases. *C*, The number of liver metastases at day 21 per liver with each dot representing one mouse. One representative experiment of eight is shown; *, $p < 0.05$.

attractive peri-operative adjuvant therapy. Although confirmation by other clinical trials is still awaited, in one clinical trial it was reported that 30% of patients with colorectal cancer benefited from postoperative treatment with anti-EpCAM mAb

(17). Several mechanisms by which mAb exert their anti-tumor activity have been proposed. mAb might influence signaling of tumor Ags, leading to apoptosis, or inhibiting proliferation (18). mAb can additionally recruit the classical complement pathway,

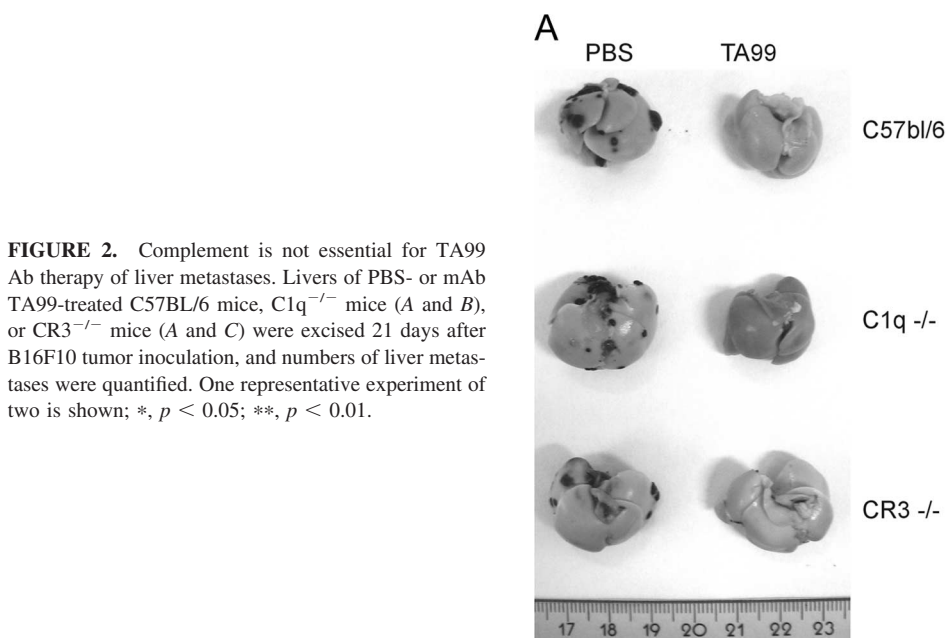


FIGURE 2. Complement is not essential for TA99 Ab therapy of liver metastases. Livers of PBS- or mAb TA99-treated C57BL/6 mice, C1q^{-/-} mice (*A* and *B*), or CR3^{-/-} mice (*A* and *C*) were excised 21 days after B16F10 tumor inoculation, and numbers of liver metastases were quantified. One representative experiment of two is shown; *, $p < 0.05$; **, $p < 0.01$.

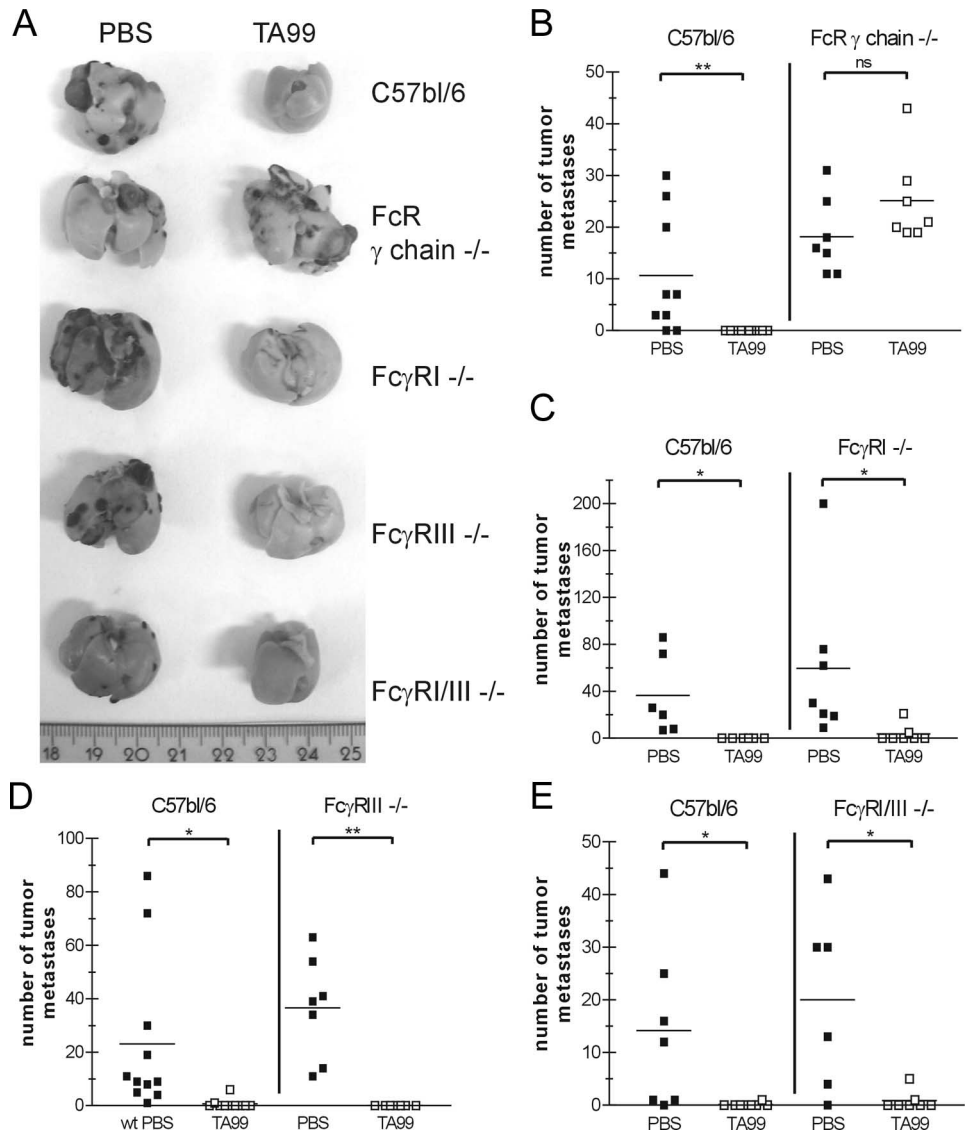


FIGURE 3. Activatory Fc γ R are crucial for TA99 Ab therapy of liver metastases. Mice inoculated with B16F10 cells were treated with either PBS or mAb TA99. After 21 days, livers were excised and tumor load was determined in C57BL/6 mice, FcR γ -chain^{-/-} mice (A and B), Fc γ RI^{-/-} mice (A and C), Fc γ RIII^{-/-} mice (A and D), or Fc γ RI/III^{-/-} mice (A and E). Experiments were repeated at least two times yielding similar results; *, $p < 0.05$; **, $p < 0.01$.

which may result in complement-dependent cytotoxicity (19). Furthermore, IgG Fc receptors on immune cells can bind mAb, which might activate immune effector functions including Ab-dependent cellular cytotoxicity (ADCC)³ (20). In vivo, Fc γ R have been shown to be crucial for therapeutic activity, as protection against tumor growth was abrogated in FcR γ -chain-deficient mice that lack all activatory Fc γ R (21, 22). In human, three classes of Fc γ R have been identified, which all have their own expression pattern on immune cells (20). Recently, a fourth class of Fc γ R, Fc γ RIV, has been identified in mice (23). Fc γ RI, III, and IV are activatory IgG receptors and depend for cell surface expression and effector functions on interaction with the signaling, ITAM containing, FcR γ -chain (24). Fc γ RII, containing an ITIM motif, represents an inhibitory IgG receptor, which activation results in down-regulation of immune responses (20, 24).

To study whether Ab therapy could prevent the outgrowth of tumor cells in the liver, we established a syngeneic metastasis model in immuno-competent mice, in which tumor cells were inoculated during a surgical procedure. Additionally, to evaluate the

underlying mechanisms of therapy, outgrowth of metastases was assessed in wild-type animals as well as in mice deficient for either complement factors or distinct leukocyte Fc γ R. Because we previously demonstrated that Kupffer cells play an essential role in eliminating tumor cells that enter the liver (25), we investigated their involvement in ADCC as well.

Materials and Methods

Mice

C57BL/6 wild-type mice were obtained from Janvier. FcR γ -chain knockout (KO) mice (26), Fc γ RI^{-/-} (CD64 KO) mice (27), Fc γ RIII^{-/-} (CD16 KO) mice (28), and CR3^{-/-} (MAC-1 KO) mice (29) were bred and maintained at the Central Animal Facility of the Utrecht University, The Netherlands. Fc γ RI/III^{-/-} (CD64/CD16 double KO), Fc γ RI/II/III^{-/-} (CD64/CD32/CD16 triple KO), and C1q^{-/-} (C1q KO) mice (30) were bred at the Animal Facility of the LUMC, Leiden, The Netherlands. Fc γ RI^{-/-} mice were backcrossed for six generations to the C57BL/6 background, whereas all other KO mice were backcrossed for more than 10 generations to the C57BL/6 background. Mice between 8 and 16 wk old were used for each experiment. All experiments were approved by the Utrecht University animal ethics committee and performed according to institutional and national guidelines.

Cell culture

The mouse melanoma cell line B16F10, which expresses gp75, was obtained from the American Type Culture Collection. Cells were cultured in

³ Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; KO, knockout.

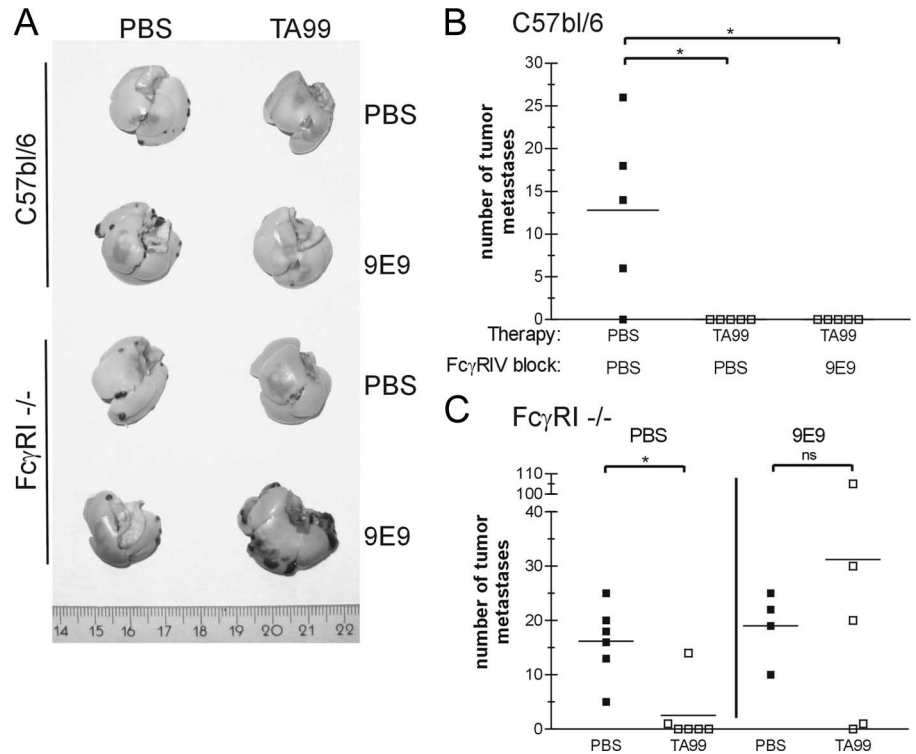


FIGURE 4. Functional redundancy between Fc γ RI and Fc γ RIV in TA99 therapy. C57BL/6 mice (A and B), or Fc γ RI^{-/-} mice (A and C) were inoculated with B16F10 cells and treated with PBS, or mAb TA99, either in combination with or absence of anti-Fc γ RIV mAb 9E9. After 21 days, livers were excised, and numbers of liver metastases were quantified. One representative experiment of two is shown; *, $p < 0.05$.

RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FCS and antibiotics. For experiments, B16F10 cells were harvested using trypsin-EDTA (Invitrogen), washed three times with PBS, and collected in HBSS medium (Invitrogen). In all experiments, cell viability exceeded 95%, as determined by trypan blue exclusion.

Abs and flow cytometry

Supernatant containing mAb TA99 (mIgG2a, anti-gp75) was produced from hybridoma HB-8704 (generously provided by Dr. A. N. Houghton) and purified by protein A-Sepharose chromatography (Amersham Biosciences). Fc γ RIV blocking mAb 9E9 (hamster IgG1) was produced as previously described (23).

B16F10 gp75 surface expression was determined by staining cells with mAb TA99 (10 μ g/ml, 30 min at 4°C), followed by incubation with FITC-conjugated F(ab')₂ of goat anti-mouse IgG Ab (Protos; 30 min at 4°C). To measure total gp75 expression, B16F10 cells were permeabilized with ice-cold methanol/acetone (1:1, 15 min at 4°C) before cells were stained. Cells were analyzed on a FACScan (BD Biosciences).

Immunohistochemistry

Murine liver cryosections (6 μ m) were attached on poly-L-lysine coated slides and fixated in acetone for 10 min. The last 5 min, 0.3% H₂O₂ was added to inhibit endogenous peroxidase activity. Nonspecific binding sites were blocked by incubation with a 5% normal rabbit serum in PBS with 0.5% BSA (15 min, room temperature). Endogenous biotin was blocked using an avidin/biotin blocking kit according to the manufacturer's instructions (Vector Laboratories). After washing in PBS, sections were incubated with a 1/100 dilution of F4/80 hybridoma supernatant (own production) for 45 min followed by incubation with biotinylated rabbit anti-rat Abs (1/150, Vector, 45 min), and incubation with avidin-biotin-HRP complex (Vector Laboratories; 30 min). 3,3'-diaminobenzidine was used as substrate (Sigma-Aldrich), resulting in a brown staining. Sections were counterstained with Mayers' hematoxyline (Klinipath), after which they were embedded in Entellan (Merck).

B16F10 liver metastases

Mice were anesthetized, and a small incision was made in the left flank to reveal the spleen. A total of 2×10^5 B16F10 tumor cells (100 μ l) were injected intrasplenically at a constant rate to allow flow of tumor cells toward the liver. After one minute, the spleen was removed to prevent early death due to a high tumor load in spleen, and the incision was sutured. All steps within this procedure were standardized to minimize variation. Mice

were injected i.p. on days 0, 2, and 4, with 200 μ g TA99 mAb, or as a control with PBS (250 μ l). To block Fc γ RIV, mice were injected i.v. with 200 μ g 9E9 mAb (200 μ l), 30 min before i.p. injection of TA99 mAb, on days 0, 2, and 4. At day 21, mice were sacrificed, and the number of liver metastases was scored in each mouse. Kupffer cells were depleted by injecting 0.2 ml liposome-encapsulated clodronate in the tail vein at days -4 and -2. Clodronate liposomes were prepared as previously described (31).

Statistical analysis

Statistical differences were determined using the two-tailed unpaired Student's *t* test or ANOVA. Significance was accepted when $p < 0.05$.

Results

TA99 mAb treatment prevents B16F10 metastases outgrowth in the liver

We previously demonstrated in a rat model that intraperitoneal surgery, which is required to remove a primary colorectal carcinoma, enhances the risk of development of CC531s colon carcinoma metastases in the liver (5). However, mechanistic research in rats has limitations and we, therefore, established a liver metastasis model in immuno-competent C57BL/6 mice to address the potential and mechanism of anti-tumor Ab to prevent outgrowth of liver metastases. Since, to our knowledge, no syngeneic mouse colon carcinoma cell-line is available to which a mouse anti-tumor mAb is directed, we used the well-studied syngeneic B16F10 melanoma tumor as model system. B16F10 tumor cells express gp75 (also referred to as TYRP1 or TRP-1), and the mouse anti-gp75 IgG2a mAb TA99 is widely used to study the effect of Ab treatment on tumor development (Fig. 1A). For induction of liver metastases, tumor cells can be inoculated into the portal circulation (32). However, due to the small size of both mesenteric and portal veins in mice, direct injection in either vein would lead to a high risk of internal bleeding and unacceptable losses of mice. We, therefore, used an alternative model, in which tumor cells were injected into the spleen, leading to direct flow of tumor cells toward the liver (33). Splenectomy was performed after injection of tumor cells to

prevent early death due to the development of a high tumor load in the spleen.

Following tumor inoculation, metastases developed in the livers of untreated mice (overall mean of 25) within 3 wk. Treatment of mice with TA99 mAb completely prevented tumor outgrowth in 90–95% of mice ($n = 60$) (Fig. 1, *B* and *C*). The remaining 5–10% of TA99 mAb-treated mice developed ~1–4 liver metastases, which were considerably lower numbers compared with untreated animals. In addition, no metastases were observed in other organs of TA99 mAb-treated mice, which was in contrast to untreated mice, in which additional peritoneal metastases (10–20% of mice) and/or lung metastases (less than 5% of mice) were found. To verify whether metastases would develop at later time points, long-term survival of TA99 mAb-treated mice was investigated as well. TA99 mAb-treated mice were sacrificed after 20 wk, at which time mice remained completely tumor free (data not shown). However, when TA99-treated mice were re-challenged i.v. with B16F10 tumor cells; no differences in development of lung metastases were observed compared with naive mice, indicating that TA99 treatment does not induce memory responses (data not shown).

Role of complement in Ab-induced prevention of liver metastases

The underlying mechanisms of Ab-mediated prevention of liver metastases was evaluated by performing experiments in C57BL/6 mouse strains, deficient for specific complement components. First, the role of complement was assessed in $C1q^{-/-}$ mice, in which Ab-mediated activation of the classical complement pathway cannot be initiated (19). Because TA99 mAb treatment effectively prevented tumor outgrowth in the liver of $C1q^{-/-}$ mice (Fig. 2, *A* and *B*), the involvement of the classical pathway of complement was excluded.

Furthermore, CR3—which can enhance Fc receptor-mediated tumor cell killing—has been documented to be important for effective TA99 mAb therapy against development of metastases in the lung (34). However, in contrast to the lung metastasis model, TA99 mAb therapy completely prevented development of metastases in the liver of $CR3^{-/-}$ mice (Fig. 2, *A* and *C*), thereby excluding a role for CR3 in therapy as well.

Role of $Fc\gamma R$ and effector cells in Ab-induced prevention of liver metastases

The role of Fc receptors was assessed using mice that were deficient for the signaling FcR γ -chain, which is required for downstream signaling of $Fc\gamma RI$, $Fc\gamma III$, and $Fc\gamma IV$ (23, 26, 35). No difference in liver metastases development was observed between mAb-treated or untreated FcR γ -chain $^{-/-}$ mice (Fig. 3, *A* and *B*), supporting that effective TA99 mAb therapy of liver metastases is dependent on the presence of activatory $Fc\gamma R$ (21, 22). Therefore, the role of individual activatory $Fc\gamma R$ was evaluated. TA99 mAb treatment effectively prevented tumor outgrowth in $Fc\gamma RI^{-/-}$ mice (Fig. 3*C*). In addition, therapy was not affected in $Fc\gamma RIII^{-/-}$ mice either (Fig. 3*D*). Because TA99 treatment still prevented tumor outgrowth in either $Fc\gamma RI/III^{-/-}$ double or $Fc\gamma RI/II/III^{-/-}$ triple KO mice (Fig. 3*E*, and data not shown), a role for $Fc\gamma RIV$ was implicated. Unexpectedly, however, blocking $Fc\gamma RIV$ did not abrogate therapeutic activity, as wild-type mice that were treated with TA99 mAb in combination with either a blocking anti- $Fc\gamma RIV$ mAb (9E9) or an isotype mAb as a control were still protected against development of metastases (Fig. 4, *A* and *B*, and data not shown). Because $Fc\gamma RI$ and $Fc\gamma RIV$ are both selectively expressed on myeloid cells (23), experiments with blocking $Fc\gamma RIV$ mAb were repeated in $Fc\gamma RI^{-/-}$ mice to investigate redundancy between these two Fc receptors. Only when $Fc\gamma RI$ and

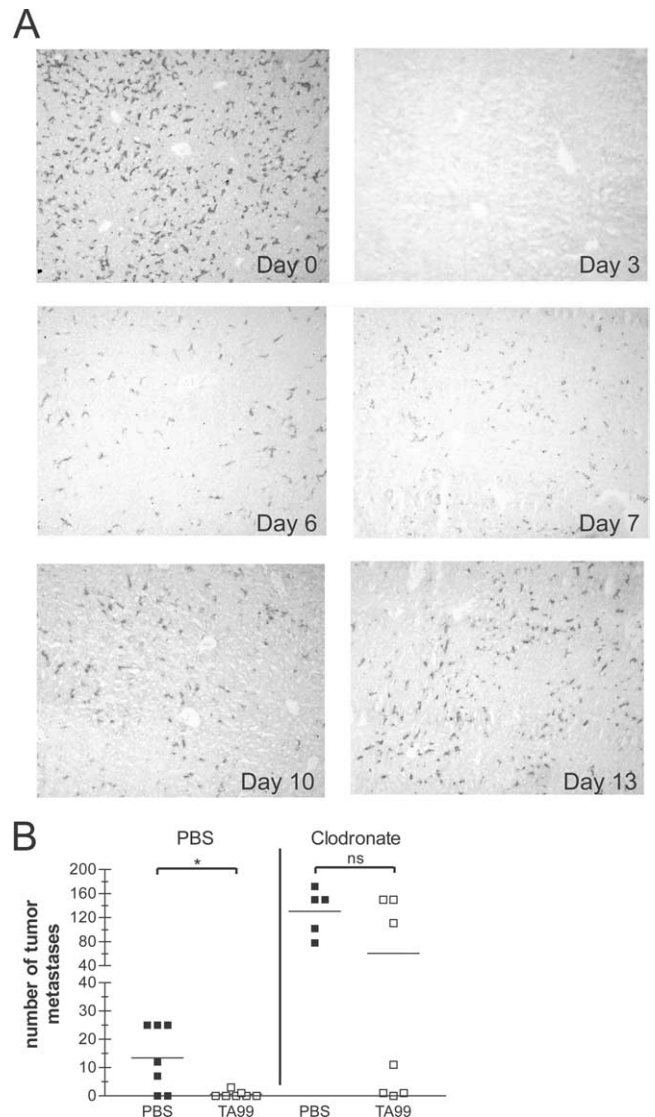


FIGURE 5. Kupffer cells are involved in Ab-dependent killing of tumor cells. *A*, Kupffer cells were depleted by injection with clodronate liposomes. Livers were stained with F4/80 at the indicated time points to investigate the presence of Kupffer cells. *B*, C57BL/6 mice were injected i.v. with PBS (*left panel*) or clodronate-liposomes to deplete Kupffer cells (*right panel*). Thereafter, mice were inoculated with B16F10 cells and treated with PBS or mAb TA99. After 21 days, livers were excised, and numbers of liver metastases were quantified. One representative experiment out of three is shown; *, $p < 0.05$.

$Fc\gamma RIV$ function was inhibited simultaneously, effective TA99 mAb therapy was abrogated (Fig. 4*C*).

Since Kupffer cells express both $Fc\gamma RI$ and $Fc\gamma RIV$ (data not shown), and could, therefore, function as effector cells in this model, the ability of TA99 mAb to prevent liver metastases outgrowth was assessed in the absence of Kupffer cells. All Kupffer cells were depleted 3 days after injection of clodronate-liposomes (Fig. 5*A*). Return of Kupffer cells was observed after 6 days. The number of Kupffer cells gradually increased over time, and, at day 13, no significant difference in Kupffer cell number was observed compared with control mice. After depletion of Kupffer cells, the number of liver metastases increased ~10-fold when mice were treated with PBS, supporting the potent capacity of Kupffer cells to eliminate tumor cells even in the absence of mAb (Fig. 5*B*). Additionally, whereas the tumor did not always take in control mice,

all Kupffer cell-depleted mice developed liver metastases, because of lack of this innate cytotoxic capacity. TA99 mAb treatment was furthermore highly effective in control mice, but ineffective in ~45% of Kupffer cell depleted animals, supporting that Kupffer cells are (partly) involved in successful mAb therapy (Fig. 5B).

Discussion

Surgical removal of the primary tumor is the only treatment that can provide long-term, disease-free survival of patients with colorectal carcinoma (36). Unfortunately, even in patients who are eligible for intentionally curative surgery and do not have evidence of metastases at the time of resection, development of secondary disease remains a major problem. We previously showed that surgery, which is the appropriate and necessary initial treatment, paradoxically augments the development of liver metastases (1, 4, 5). Postoperative treatment aiming to target minimal residual disease might, therefore, improve clinical outcome. As such, we studied the potential of therapeutic mAb to prevent tumor outgrowth in the liver after a surgical procedure. We observed that TA99 mAb therapy completely prevented development of liver metastases in 90–95% of the mice and significantly reduced the number of metastases in the remaining 5–10%. Additionally, no metastases were observed in other organs, and mice remained completely tumor free for at least 20 wk, which indicates that preventive postoperative TA99 mAb therapy is curative and does not redirect the tumor burden to other organs. Because the syngeneic B16F10 melanoma model is not necessarily representative for development of liver metastases after removal of a primary colon carcinoma, we also performed experiments in rats. In this study, a syngeneic model was used in which anti-CC531s mAb were given after i.p. surgery and intraportal inoculation of CC531s colon carcinoma cells. Similar to the murine experiments with B16F10, rats did not develop liver metastases after mAb treatment (data not shown), supporting that postoperative mAb therapy may benefit patients with colorectal cancer. Moreover, since the spleen was not removed in the rat model, it is likely that the removal of the spleen in the mouse model did not negatively influence effective mAb therapy.

In nude mice, Ab treatment was previously demonstrated to reduce liver metastasis outgrowth as well (37, 38). However, this may not represent the most optimal model to further unravel the mechanisms of Ab therapy as these mice lack T cells, which is compensated by an increased number of NK cells. The underlying mechanisms of therapy with an Ab of the IgG2a subclass were, therefore, assessed in immunocompetent animals and in mice deficient for either complement factors or distinct leukocyte Fc γ R. TA99 mAb treatment was still effective in C1q^{-/-} mice. Since binding of C1q to IgG Abs is crucial for activation of the classical pathway of complement, a major role for complement in TA99 mAb therapy can be excluded, which is in accordance with earlier data showing that treatment of mice with cobra venom factor—hereby depleting complement—did not affect therapeutic efficacy of mAb TA99 in a B16F10 lung metastases model (39). However, in contrast to the lung metastases model, in which CR3 proved crucial for effective therapy (34), absence of CR3 did not diminish TA99 mAb therapeutic efficacy against the development of liver metastases. CR3 is required for efficient immunological synapse formation between neutrophils and their targets, since absence of CR3 prevented Ab-mediated tumor cell killing by neutrophils (40). Neutrophils are, therefore, presumably involved as effector cells in the prevention of lung metastases development after Ab therapy. In the liver, however, a role for neutrophils is less likely, as therapy was not dependent on the presence of CR3. This is strengthened by the observation that additional treatment with G-CSF—which in-

creases the number of neutrophils in the blood—augmented therapeutic efficacy in the lung metastases model (34) but did not enhance therapeutic activity in the liver metastases model (data not shown).

Therapeutic activity against liver metastases formation was completely abrogated in FcR γ -chain^{-/-} mice, indicating an essential role for activatory Fc γ R, which is in accordance with earlier reports that describe a critical role for Fc γ R in Ab therapy (21, 22, 41). However, the relative contributions of individual Fc γ R classes in TA99 mAb therapy are incompletely understood. In the lung metastases model, contradictory results have been reported, ascribing a central role for either Fc γ RI (42) or Fc γ RIV (43). As the mechanisms of Ab therapy and the responsible effector cell populations appear to differ between the liver and lung models, we investigated which Fc γ R was involved in TA99 mAb-mediated prevention of liver metastases. A role for Fc γ RIV seemed imperative, because TA99 treatment was still effective in Fc γ RI^{-/-}, Fc γ RIII^{-/-}, Fc γ RI/III^{-/-}, and Fc γ RI/II/III^{-/-} mice, hereby apparently excluding a role for all other Fc γ R. Unexpectedly, blocking Fc γ RIV did not influence therapeutic efficacy either. Only when Fc γ RI and Fc γ RIV function was simultaneously inhibited, development of liver metastases was no longer prevented by TA99 mAb treatment. Thus, absence of either Fc γ RI or Fc γ RIV is completely compensated by the remaining receptor, and curative Ab therapy is only diminished when both receptors are lacking. This observed redundancy is remarkable, since both receptors have different affinities. Fc γ RI is a high affinity receptor and has been considered to play a limited role in immune complex-mediated effector functions because of the competition with circulating monomeric IgG2a for its binding site (24). By contrast, Fc γ RIV binds IgG2a immune complexes. Tumor cells, which are opsonized with IgG2a will function as immune complex, explaining why Fc γ RIV is sufficient for therapeutic efficacy. However, in absence of Fc γ RIV, Fc γ RI is able to bind mAb-opsonized tumor cells, suggesting that Fc γ RI is either not completely saturated by monomeric IgG2a or that immune complexes (like mAb-opsonized tumor cells) compete for binding with monomeric IgG2a. This latter was previously shown using an IgG2a anti-RBC-induced autoimmune hemolytic anemia model (44). In this model, high IgG2a densities that bound to RBC could efficiently compete with circulating monomeric IgG2a for Fc γ RI binding on phagocytes, indicating that Fc γ RI participated in erythrophagocytosis. Fc γ RI may, therefore, play an important role in immune clearance of mAb-opsonized tumor cells as well, explaining why Fc γ RI can compensate for the lack of Fc γ RIV.

NK cells represent a prominent effector cell population for the natural defense against tumor development (45). However, as they neither express Fc γ RI nor Fc γ RIV, and Fc γ RIII—which is expressed by NK cells—proved not to be involved in TA99-mediated therapy, NK cells can be excluded as effector cells in postoperative mAb therapy of liver metastases. Furthermore, although murine neutrophils express Fc γ RIV, expression of Fc γ RI is either absent or present at very low levels (46), which renders their involvement also less likely. This is supported by our data in CR3^{-/-} mice, as well as the fact that G-CSF treatment did not enhance Ab therapy. We, therefore, hypothesized that macrophages, which express Fc γ RI as well as Fc γ RIV, represent the main effector cell population for TA99-induced anti-tumor effects in the liver. Kupffer cells, which are resident liver macrophages, are not only essential for clearance of bacteria that invade the portal circulation during inflammation (47) but were demonstrated to trap and phagocytose tumor cells entering the liver, as well (48, 49). Freshly isolated Kupffer cells were additionally shown to mediate effective tumor cell killing (50), and significant outgrowth of tumor cells in the

liver has been observed upon depletion of macrophages in rats (25) and mice, which strongly supports an important innate role for Kupffer cells in the defense against development of liver metastases. Moreover, TA99 mAb therapeutic efficacy was abrogated in ~45% of Kupffer cell-depleted mice, suggesting that Kupffer cells contribute to ADCC. The fact that Kupffer cells reappear in the liver after 6 days at which time mAb is still present in the circulation may explain why mAb treatment was still effective in 55% of mice. It was previously shown in vitro that Ab can recruit macrophages as effector cells (51). Furthermore, Ab-induced depletion of B cells was mediated by macrophages, supporting that Kupffer cells may mediate enhanced tumor cell killing after mAb therapy (52). We, therefore, propose that development of liver metastases after mAb treatment is mainly inhibited because Kupffer cells arrest and kill circulating tumor cells (53). Since Kupffer cells are not very effective APCs, this hypothesis may also explain our observations that TA99 mAb treatment did not induce memory responses (data not shown, $n = 3$). Moreover, Kupffer cells are abundantly present in the liver, suggesting high E:T ratios, which might explain the more efficient antitumor responses in the liver compared with lungs. Although the average number of lung metastases per mouse was reduced with ~70–95%, complete prevention was not observed, in contrast to the liver model in which 90–95% of the mice did not develop any metastasis after immediate postoperative treatment (21, 34, 39).

In conclusion, in our model, TA99 (IgG2a) Ab therapy, aimed to inhibit surgery-induced development of liver metastases, was not dependent on activation of the classical complement pathway but required the presence of myeloid activatory Fc γ R. Interestingly, Fc γ RI and Fc γ RIV can completely compensate each other's function, and curative Ab therapy in the liver is only abrogated when both receptors are absent. Furthermore, mAb therapy was able to prevent outgrowth of tumors in 90–95% of mice, presumably partly through arrest of circulating tumor cells by Kupffer cells. Thus, provided that mAb are given immediately after surgery, adjuvant Ab therapy after resection of primary colorectal cancer can help to limit the development of liver metastases, which may significantly improve patient outcome.

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Disclosures

The authors have no financial conflict of interest.

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