General discussion and future perspectives
CHAPTER 9

Introduction
Colorectal cancer (CRC) is one of the most prevalent solid cancers in both men and women in developed countries. Annually, 1.2 million cases are recorded worldwide, and more than 600,000 patients die of this disease.1 While surgical resection of the primary colorectal carcinoma is the preferred treatment, it has been associated with higher risk for metastases development.2-4 Approximately 20-25% of all patients already have metastatic disease upon diagnosis of CRC.5 However, another ~10-25% of patients who do not have visible evidence of metastases at the time of diagnosis and in whom the tumor is removed surgically, will develop metastases within 5 years.6 This supports the presence of minimal residual disease that grows out into metastases after surgery. The liver is the major target organ for development of colorectal metastases, and accounts for ~70% of colorectal-related deaths.7

Previously, it was demonstrated that peritoneal surgery resulted in vascular damage in the liver that caused the exposure of sub-endothelial extracellular matrix (ECM) proteins.4 These are ligands for adhesion molecules like integrins that are expressed on the cells surface.8 Importantly, adhesion of tumor cells to the exposed ECM was observed. This supported that performing surgery in the abdominal cavity initiated systemic inflammatory responses, which enabled tumor cell adhesion in the liver. Therefore, I investigated the relation between surgery-induced inflammation and liver metastases development in more detail to gain new insights in this phenomenon (chapter 2-6). Furthermore, in chapters 7 and 8 it is shown that surgery-induced liver metastases outgrowth can be prevented with peri-operative monoclonal antibody therapy. The implications for future perspectives are furthermore discussed in this chapter.

Role of integrin molecules in metastases development
Development of metastases is usually a highly inefficient process. Firstly, tumor cells need to detach from the primary tumor by down regulating the expression of adhesion molecules, and enter lymphatic or blood vessels. Secondly, disseminated tumor cells have a limited life span when they are unable to adhere, and can be rapidly cleared by the immune system. However, previous studies supported that surgical trauma increased the risk of metastases development.9, 10 It was demonstrated that animals undergoing laparotomy (opening of the peritoneal cavity) developed more liver metastases compared to non-operated control animals.4 Therefore, it was previously proposed that during surgery tumor cells may be spilled from the primary tumor without the need of reduced expression of adhesion molecules on cells surface.4 Because of high expression of adhesion molecules on tumor cells and surgery-induced exposure of ECM proteins, to which these cells adhere preferentially, metastases formation is increased. It was demonstrated in animal models that blockade of integrins α2 or β1 prevented tumor cell adhesion in the liver or peritoneal cavity, respectively, whereas α5 was not involved.2, 4 Importantly, when integrin α2 on tumor cells was blocked, development of liver metastases was successfully prevented, supporting that
integron α2 was essential for tumor cell adherence. In chapter 2 we investigated the expression of integrins α2, α5 and β1 in primary colorectal tumors and correlated this with the survival of patients. In this retrospective study we found that high expression of integrin α2 positively correlated with lower overall survival. Moreover, patients with high integrin α2 expression in the primary tumor had higher risk of metastases development. Unfortunately, this did not reach statistical significance, which may be due to limited numbers of participating patients. In a previous study higher expression levels of integrin α2 in colorectal liver metastases was observed, compared to lung metastases.\textsuperscript{11} Therefore I speculate, the liver may contain a specific composition of ECM proteins, which favors the adhesion of tumor cells with high levels of α2 integrins expression. Adhesion of these cells may result in formation of liver metastases. Alternatively, it is also possible that because of high expression of integrin α2 tumor cells from colorectal cancers are able to adhere easily in the first organ they pass, which is the liver, and grow out into metastases. We did not find any correlation between integrins α5 or β1 expression and patients’ survival. Previously, it was shown that blockade of integrin β1 was not sufficient to prevent tumor cells adherence in the liver.\textsuperscript{4} Strikingly, tumor cells adhesion to the peritoneal cavity was inhibited when cells were incubated with antibody against integrin β1.\textsuperscript{2} Furthermore, it was demonstrated that high levels of integrin α5 gave rise to kidney metastases formation.\textsuperscript{12} All these data indicate that expression of specific integrins on tumor cells facilitate metastases formation in different target organs.

Because expression of integrins on tumor cells mediates metastases formation, blockade of integrins on tumor cells may represent an attractive therapeutic strategy. However, resection of the primary tumor unavoidably introduces a wound in the peritoneal cavity and the intestines. Wound healing is a process in which the integrins also play a central role.\textsuperscript{13,14} Thus, blockade of integrins on tumor cells with the intention to prevent tumor cells adhesion, may impair wound healing as well. Hampered wound healing may cause post-surgical anastomic leakage. Since patients with anastomic leakage were shown to have serious complications and poorer survival,\textsuperscript{15} I strongly caution against these therapies before extensive research has been performed to prove safety of this approach.

\textbf{Role of surgery-induced inflammation in development of liver metastases}

Previous studies demonstrated that surgery caused the release of inflammatory mediators, including reactive oxygen species (ROS).\textsuperscript{16-18} These compounds were suggested to be involved in tumor development by facilitating tumor cells adhesion.\textsuperscript{19} Incubation of endothelial monolayers with ROS for 12 hours was shown to result in up-regulation of adhesion molecule expression on endothelial cells such as endothelial-selectin (E-selectin), inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).\textsuperscript{19} These adhesion molecules were suggested to be involved in enhanced tumor cells adhesion to endothelial cells. In chapter 3 we now demonstrate that incubation of endothelial monolayers with comparable amounts of ROS resulted in damage of the monolayers. Subsequently,
intercellular gaps were formed that led to exposure of sub-endothelial ECM components to which tumor cells adhered preferably. Furthermore, we showed that surgery led to impairment of liver vasculature both in experimental animal models and in human liver samples. Treatment of animals with a ROS scavenger prevented vascular damage. Importantly, when rats were treated with a ROS scavenger we observed significantly less tumor cell adherence in the livers compared to rats that were treated with the vehicle only. When liver resident macrophages, Kupffer cells (KCs) were depleted, we also observed less tumor cells adhesion, suggesting that KCs are involved in ROS production. Unfortunately, treatment with ROS scavenger was not sufficient for prevention of metastases development. Previous studies demonstrated that KCs recognize and kill malignant cells in a ROS dependent manner.\textsuperscript{20, 21} Thus, scavenging of ROS during surgery likely acts as a double-edged sword. First, surgery stimulates the production of ROS by KCs, which leads to disruption of endothelial lining. This causes the exposure of sub-endothelial ECM proteins to which circulating tumor cells bind preferably. Second, interruption of ROS production and/or scavenging system may imbalance the tumor cell killing by KCs, which also requires ROS. This may finally lead to outgrowth of metastases from adhered tumor cells. Thus, overall scavenging of ROS may not have beneficial clinical effects in prevention of liver metastases formation.

**Role of bacterial products in metastases development**

Resection of the primary colorectal tumor furthermore resulted in translocation or spillage of bacterial products from the gut lumen to the peritoneal cavity.\textsuperscript{15, 22-26} Interestingly, patients with positive bacterial translocation or anastomotic leakage had poor clinical outcome.\textsuperscript{15, 24} Bacterial products are potent activators of strong immune responses, which may be involved in mediating damage to liver vasculature. This subsequently may lead to liver metastases formation. Because in our experimental laparotomy model the intestines were not resected, the additional effects of bacterial components on metastases formation were not investigated. Therefore, we developed a second experimental colectomy model in which a small part of the colon was removed surgically, that was followed by end-to-end anastomosis (chapter 4). Smear samples were taken from the peritoneal surface of the colon of rats undergoing colectomy at the begin and the end of the surgical procedure. Bacterial outgrowth was significantly increased in the samples that were taken from the anastomosis. Moreover, we observed decreased levels of tight junction protein expression in the livers of rats that received colectomy, compared with the livers of control rats or rats that underwent laparotomy. Subsequently, enhanced numbers of adhered tumor cells were detected in the livers of rats that underwent colectomy, compared to the livers of control rats or rats from the laparotomy group. Importantly, rats from the colectomy group developed significantly more liver metastases. Previous studies demonstrated that severity of surgical trauma correlated with development of liver metastases development.\textsuperscript{27} Thus, one explanation might be that rats undergoing colectomy developed more liver metastases because of higher extent of surgical trauma. However, in our colectomy model we also found significantly increased bacterial contamination
after surgery, which also is observed in patients. This may lead to systemic exposure
to bacterial products. Importantly, patients with bacterial translocation or anastomotic
leakage after surgery had significantly shorter disease-free survival and lower overall
survival. Moreover, local and systemic recurrence rates were higher in patients
with anastomotic leakage after surgery.\textsuperscript{15} This indicated that exposure to bacterial
components during surgery has a negative effect on long term clinical outcome.\textsuperscript{15, 24, 28}

Therefore, we propose that enhanced tumor development in our colectomy model
is mainly caused by bacterial products that are released or spilled during resection
in the colon.

Bacterial products are potent initiators of inflammatory immune responses through
interacting with Toll-like receptors (TLRs). For instance, the bacterial outer membrane
component LPS is the main ligand for TLR4. This receptor is expressed by wide variety
type of immune cells such as polymorphonuclear cells (PMNs) and KCs.\textsuperscript{29} Previously,
it was demonstrated that abdominal surgery led to attraction of high numbers of
PMNs to the peritoneal cavity.\textsuperscript{30} Interestingly, depletion of PMNs prevented local
recurrence of tumors. Furthermore, it was demonstrated that incubation of PMNs
with mesothelial monolayers enhanced the expression of adhesion molecules on
mesothelial cells. These molecules mediated increased tumor cells adherence.\textsuperscript{31}
Thus, these data suggested that PMNs might be involved in development of tumors.
Therefore, in chapter 5 we investigated the potential role of PMNs in initiation of
liver metastases development. In the livers of rats that had received laparotomy,
increased numbers of PMNs were observed, compared to the livers of control
rats. Moreover, the numbers of PMNs was further increased when rats underwent
colectomy. As we observed enhanced bacterial translocation in rats after colectomy
(see above), exposure to bacterial components likely led to sequestration of PMNs
in the liver. This is supported by previous studies that showed that LPS injection
resulted in accumulation of PMNs in the livers of mice.\textsuperscript{32, 33} Importantly, the numbers
of adhered tumor cells in the livers of rats either undergoing laparotomy of
colectomy were elevated as well, compared to control rats. This suggested a relation
between high numbers of PMNs and tumor cells adherence. As was demonstrated
previously,\textsuperscript{34} incubation of PMNs with LPS rapidly induced ROS release. In chapter 3
we observed that ROS had detrimental effects on endothelial monolayers. Therefore,
we investigated whether incubation of endothelial monolayers with PMNs and LPS
causes endothelial damage. When endothelial monolayers were incubated with
PMNs and LPS, formation of intercellular gaps were observed. This consequently
led to exposure of sub-endothelial ECM proteins, as we demonstrated in chapter 3.
Furthermore, injection of rats with LPS resulted in high numbers of both PMNs and
tumor cells in their livers. This suggested that LPS led to significant attraction of PMNs
to the liver that subsequently caused damage to the liver vasculature, resulting in
increased tumor cell adhesion. Importantly, both PMN and tumor cell accumulation
that was caused either by laparotomy or LPS administration (without surgery) was
prevented when rats were treated with a ROS scavenger. Thus, these data suggested
that activation of PMNs by LPS led to ROS production, which subsequently caused
damage to the liver vasculature and facilitated tumor cell adhesion.
Additionally, it was shown that LPS activates KCs.\textsuperscript{35} Furthermore, previously it was suggested that macrophages play a role in liver metastases development.\textsuperscript{21} Therefore, we evaluated the involvement of KCs in initiation of metastases development (chapter 6). When rats were injected with LPS (in the absence of surgery), tumor cell adherence in the livers was ameliorated significantly, compared to the livers of rats that were injected with saline. A previous study suggested that LPS increased the expression of adhesion molecules on tumor cells.\textsuperscript{36} Therefore, we tested whether LPS influenced tumor cells adhesion to either different ECM coatings or endothelial layers. Incubation of tumor cells with varying concentrations of LPS for different time points, did not affect the adherence of tumor cells on various ECM coatings (data not shown). Moreover, incubation of tumor cells or endothelial monolayers with LPS did not enhance tumor cell adherence to endothelial layers. These data indicated that tumor cell adhesion that was increased by LPS injection of rats was not caused by enhanced or altered adhesion to the endothelial lining.

KCs are however in close proximity of sinusoidal endothelial cells.\textsuperscript{37} Interaction of LPS with its receptor on KCs was furthermore shown to induce the release of inflammatory mediators including ROS.\textsuperscript{29, 35, 38} Subsequently, release of ROS by KCs may be harmful for the integrity of liver vasculature. Therefore, we tested the effect of LPS to co-cultures of endothelial cells and macrophages. We found that addition of LPS had detrimental effects on endothelial monolayers and caused intercellular gaps formation. Moreover, endothelial damage was prevented when ROS scavengers were added. This indicated that tumor cell adherence that was enhanced by LPS injection was due to damaged liver vascular lining. Importantly, depletion of KCs or using a ROS scavenger \textit{in vivo} significantly reverted tumor cell adherence that was enhanced by LPS injection. Thus, LPS-mediated tumor cells adherence is ROS dependent and KCs play an important role in this process.

In chapter 5 we observed that the highest numbers of tumor cells had adhered after 1.5 hours following laparotomy, while the numbers of PMNs was maximal after 6 hours. This suggested that tumor cells adherence might be PMNs independent. Because we found that KCs play a pivotal role in tumor cells adherence, we studied whether KCs are involved in accumulation of PMNs and tumor cells after laparotomy or LPS injection. Interestingly, the livers of rats in which KCs were depleted contained significantly less PMNs and tumor cells after laparotomy or LPS injection (Figure 1). Thus, KCs play a regulatory role in sequestration of PMNs and tumor cells in the liver. Therefore I hypothesize that activation of KCs results in release of ROS, which damages the liver vasculature. Subsequently, sub-endothelial ECM is exposed to which both PMNs and tumor cells adhere in high numbers (Figure 2). Additionally, as soon as PMNs are adhered and activated they may also release ROS and exacerbate liver vasculature damage. Our data are supported by a previous study, which demonstrated that PMNs sequestration in the liver during inflammation is diminished when KCs were depleted or non-functional.\textsuperscript{39} Furthermore, it was demonstrated that increased binding of PMNs in the liver vasculature after LPS treatment was due to altered interaction with ECM.\textsuperscript{33} The authors speculated that LPS-induced ROS production led to modification to ECM in the liver that facilitated the increased PMNs adherence.
Figure 1: KCs regulate the accumulation of PMNs and tumor cells in the liver. a: the numbers of PMNs and tumor cells in the livers of control and KCs depleted rats after laparotomy. b: PMNs and tumor cells in livers of control or KCs depleted rats, that were treated with saline or LPS. green=His48+ cells; blue=cell nuclei. *p<0.05, **p<0.01, ***p<0.001

Antibody therapy
Thus, surgery leads to undirected activation of KCs resulting in undesired damage of liver vasculature and increased tumor cell adhesion. However, directed activation of KCs that specifically target tumor cells was suggested to be advantageous in prevention of liver metastases development.\textsuperscript{40} Anti-tumor immune responses can be stimulated with the use of monoclonal antibodies (mAb). Previously it was demonstrated that mAbs may reduce tumor growth directly by inhibiting tumor cell proliferation, induction of programmed cell death (apoptosis) or sensitizing tumor cells for chemotherapy.\textsuperscript{41} Alternatively, mAbs may also control tumor progression indirectly by involving the immune system. One of the indirect mechanisms is complement dependent cytotoxicity (CDC).\textsuperscript{42} In this process, mAb binding to antigens activates molecules of the classic complement pathway, resulting in perforation of the cell membrane, leading to cell death. Additionally, mAb can form a bridge between tumor cells and Fc receptor-expressing immune cells, which can lead to lysis of tumor cells via a process referred to as antibody-dependent cellular cytotoxicity (ADCC).\textsuperscript{43} Traditionally, natural killer (NK) cells have been considered as main effector cells for ADCC.\textsuperscript{44} After formation of immune synapses between the mAb-opsonized target cells and NK cells, target cells are eliminated by directed release of cytotoxic compounds from NK cells.

Alternatively, a role for macrophages was proposed in tumor targeting antibody immunotherapy. A previous study demonstrated that liver metastases development was successfully prevented by mAb treatment, which was dependent on the presence of either FcγRI or FcγRIV.\textsuperscript{45} Since monocytes/macrophages are the only cells that express both receptors in mice, a role for these cells was strongly supported. Recognition of mAb-coated target cells by macrophages or monocytes leads to phagocytosis and subsequent degradation in a process that is referred as antibody-dependent phagocytosis (ADPh).\textsuperscript{46}
Figure 2: Schematic overview of the liver microvasculature before and after surgery. Surgery-released factor(s) such as LPS cause the initiation of systemic immune responses that result in activation of the liver resident macrophages, KCs. Activated KCs release ROS that damages the sinusoidal endothelial cells and leads to exposure of sub-endothelial extracellular matrix (ECM) proteins. Circulating PMNs and tumor cells preferably adhere to exposed ECM. Additionally, adhered PMNs can produce ROS as well, and may exacerbate the damage to the sinusoidal microvasculature, stimulating the adherence of tumor cells that grow out in liver metastases.
In a previous study, co-localization of tumor cells and KCs in the liver was observed. Interestingly, treatment with mAb *in vivo* increased co-localization with 14%. Nonetheless, even though mAb therapy only marginally increased co-localization of tumor cells and KCs, massive impact on survival was observed, as mAb treated animals did not develop liver metastases, in contrast to isotype-treated controls. Since depletion of KCs was shown to abolish the anti-tumoral effects of mAbs, an essential role for KCs in tumor prevention was proposed. However, the exact mechanism of how KCs are involved in mAb-mediated prevention of tumor development remained unknown. To understand these results and investigate the mechanisms of effective mAb therapy in more detail, we therefore performed intravital microscopy (in chapter 7). Kupffer cells were able to sample small parts of tumor cells when mice were treated with PBS or an isotype control, but phagocytosis of whole tumor cells was limited. Still, these results explained why co-localization between Kupffer cells and tumor cells was observed with immunohistochemistry experiments. By contrast, Kupffer cells phagocytosed complete tumor cells rapidly when mice were treated with tumor specific mAb. Thus, although no difference was observed in the numbers of tumor cells that were in contact with KCs, a significantly increased number of tumor cells were ingested by KCs of mice that had been treated with specific anti-tumor mAb. Furthermore, the fate of tumor cells 24 hours after injection was investigated. Although red fluorescent dye was still visible (indicative of tumor material), particles were significantly smaller in mAb-treated mice, supporting breakdown inside macrophages. 3D reconstruction confirmed that tumor cells in the livers of untreated mice poorly co-localized with Kupffer cells. Furthermore, large clusters of tumor cells were observed, which supports outgrowth. In contrast, most tumor material was encapsulated by Kupffer cells in mAb-treated animals, indicating effective degradation of tumor cells.

Recently, mice were injected with tumor cells and mAb against tumor cells in the peritoneal cavity. After peritoneal lavage, interactions between cells were studied microscopically. Interestingly, formation of immune synapses between tumor cells and immune cells after mAb treatment was demonstrated, suggesting the elimination of tumor cells via ADCC. In contrast, we observed the liver of mice constantly after injection of tumor cells with an intravital microscope. We found that treatment with mAb led to effective uptake of tumor cells by KCs through ADPh within minutes after injection. Moreover, depletion of KCs abrogated mAb-mediated anti-tumoral response. This strongly indicated that mAbs therapy leads to ADPh in the liver and KCs are the main effector cells in this process.

Importantly, previous studies demonstrated the presence of circulating tumor cells in peripheral blood of colorectal cancer patients. Moreover, the numbers of circulating tumor cells were increased during or after surgery, especially in portal blood, suggesting the dissemination of tumor cells by surgical manipulation. Increased numbers of circulating tumor cells furthermore correlated with poor patient prognosis. Thus peri-operative mAb treatment may eliminate disseminated tumor cells. Successful mAb therapy however depends on the
expression of an (specific) antigen by tumor cells. Epidermal growth factor receptor (EGFR) is up regulated in 60-80% of colorectal cancer cases and was therefore suggested as a potential target for mAb therapy. The anti-EGFR mAb Cetuximab is an antibody that is already approved for clinical use. It was demonstrated that binding of Cetuximab to EGFR prevented cell proliferation and therefore inhibited tumor progression. However, in patients with established colorectal tumors Cetuximab treatment resulted in disappointing response rates of 11% that were increased to 23% when mAb therapy was combined with chemotherapy. Previous studies suggested that therapeutic actions of Cetuximab was mainly dependent on the RAS/RAF signaling cascade. Interference with RAS/RAF signaling results in disruption of several processes such as cell cycle progress and apoptosis, which finally prevents tumor growth. Mutational changes in these proteins impair the response to anti-EGFR mAb therapy, which is likely the cause of disappointing clinical results. However, anti-EGFR mAb may be used successfully in patients with colorectal tumors who undergo surgical resection of the tumor, as binding of mAbs to EGFR on circulating tumor cells may result in phagocytosis by macrophages.

In chapter 8, we demonstrate that incubation of tumor cells with a mAb against EGFR (Zatulumumab) effectively enhanced tumor cell phagocytosis and killing by macrophages. This was only dependent on surface expression of EGFR on malignant cells. Importantly, cell lines with mutated proteins of RAS or RAF were also efficiently phagocytosed and killed. Subsequently, after phagocytosis, lysosomal fusion with the phagosome led to cancer cell degradation. Thus, anti-EGFR mAbs may be used successfully for prevention of surgery-induced liver metastases. Importantly, mutations of cell signaling proteins do not affect this process. As we showed in animal models, surgery-induced liver metastasis is successfully prevented by mAbs that target tumor specific antigens. Therefore, I propose that clinical studies must be implemented as soon as possible to investigate this in patients.

Conclusions
In this thesis I show that surgery-induced inflammation and/or bacterial products that are spilled during surgery cause the activation of KCs. Activated KCs release the inflammatory mediator ROS that damages the sinusoidal endothelial lining and disrupts liver microvasculature. Subsequently, this leads to exposure of sub-endothelial ECM proteins. Circulating tumor cells and PMNs adhere to exposed ECM via their adhesion molecules like integrin proteins (Figure 2). However, experimental therapy using an anti-oxidant to prevent metastases formation was not successful. This may be due to interference with ROS dependent tumor cell killing by macrophages. Importantly, injection of tumor specific mAb led to effective phagocytosis of tumor cells by KCs and therefore was sufficient for prevention of liver metastases formation.

Future perspectives and recommendations
Detailed knowledge of inflammatory responses that occur in the period during and immediately after surgery is pivotal for development of new therapeutic strategies to prevent metastases development. We demonstrated that abdominal surgery
and the bacterial component LPS – is spilled during surgery - stimulated tumor cell adhesion. Thus, improvement of surgical techniques to minimize surgical trauma, bacterial translocation and spillage of tumor cells may improve patients’ outcome. However, surgical resection of the primary colorectal tumor inevitably leads to tissue damage. Therefore, additional therapies are required to prevent surgery-induced inflammatory responses. For instance, prevention of the interaction between LPS and its receptor TLR4 on immune cells may avoid undesired activation of macrophages that facilitates tumor cell adherence. Effects of LPS scavengers or TLR4 antagonists on tumor cells adherence should be investigated in more detail. Furthermore, spillage of bacteria may also result in enhanced concentration of other bacterial components such as lipoproteins, lipopeptides or flagellin. Each of these bacterial components can trigger different TLRs such as TLR1, 2, 5 and 6 and initiate similar potent immune responses, compared to TLR4. To find out which bacterial product(s) or TLRs are involved in enhanced tumor cells adhesion, animals with specific knock outs of these receptors or TLR signaling pathway proteins may be used. Detailed knowledge about the role of bacterial products in tumor development may serve to design novel targeted therapies. Moreover, clinical studies can be performed investigating whether selective decontamination of the digestive tract, eliminating the bacterial presence in the intestines, may prevent recurrence of local or distant metastases.

Furthermore, we demonstrated that undirected activation of KCs during surgery facilitated tumor cell adhesion. However, we also showed that KCs are the most important effector immune cells in mAb therapy against circulating tumor cells. Thus, directed activation of KCs may improve their anti-tumoral actions and prevent tumor formation. Therefore, I hypothesize that pre- or peri-operatively administration of mAbs against tumor antigens, which induce efficient ADPh, will inhibit metastases development. Clinical studies should be executed to investigate the effects of mAbs on surgery-induced liver metastases development.
CHAPTER 9

Reference list

27. van Grevenstein,W.M. et al. Surgery-derived reactive oxygen species produced by polymorphonuclear