Discussion
Tumour immunology is a broad field comprising all interactions of the immune system with cancer. It is clear that there is a prominent interplay between immune and tumour cells, which influences the development of the tumour. Moreover, different immune cells can nowadays be targeted to attack the tumour with as ultimate goal the clearance of tumour cells throughout the whole body. In this thesis I investigated the potential of enlisting macrophages in tumour therapy. On the one hand I determined if efficacy of antibody-dependent phagocytosis can be improved through engineering of monoclonal antibodies (mAbs), and whether anti-EGFR mAbs are suitable to eliminate circulating tumour cells in patients with colorectal cancer. On the other hand, I have studied which proteins - secreted by either mamma or colon carcinoma cells – influence macrophage polarization in order to identify candidate molecules that can be used to enhance cytotoxicity of macrophages.

**Antibodies and Receptors**

There are several isotypes of antibodies present in mammals. In human and mouse immunoglobulin G (IgG), IgA, IgD, IgE and IgM are present. IgGs can furthermore be divided into four subclasses in human (IgG1, IgG2, IgG3 and IgG4) and in three in mice (IgG1, IgG2a and IgG2b). There is a great homology between both species, and mice are regularly used as a model system to study human antibody and Fc receptor biology, although some interspecies differences need to be taken into account when interpreting results. Murine IgG2a (mIgG2a) is the homologue of human IgG1 (hIgG1), and both antibodies activate the immune system effectively. Antibodies can activate various aspects of the immune system including the complement cascade and Ig specific receptors such as the IgG Fc receptor (Fcγ receptor). Mammals have multiple Fcγ receptors that interact with the IgG subclasses with a varying affinity.

**Table 1. Interactions of Fcγ receptors with different IgG isotypes (reviewed in\(^2\))**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Function</th>
<th>Affinity</th>
<th>Interacting IgG (in sequence of affinity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>Activating</td>
<td>High</td>
<td>IgG1=IgG3&gt; IgG4</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Activating</td>
<td>Low</td>
<td>IgG1&gt;IgG3&gt; IgG2&gt; IgG4</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Activating</td>
<td>Low</td>
<td>IgG1&gt;IgG3&gt; IgG4&gt; IgG2</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td>Inhibitory</td>
<td>Low</td>
<td>IgG3&gt;IgG1=IgG4&gt; IgG2</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Activating</td>
<td>Intermediate</td>
<td>IgG3&gt;&gt;IgG1&gt;&gt;IgG4&gt;&gt;IgG2</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Activating</td>
<td>Intermediate</td>
<td>IgG3&gt;&gt;IgG1&gt;&gt;IgG4&gt;IgG2</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td></td>
<td>Low</td>
<td>IgG3&gt;IgG1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>Activating</td>
<td>High</td>
<td>IgG2a&gt;&gt;IgG2b, IgG3</td>
</tr>
<tr>
<td>FcγRII</td>
<td>Inhibitory</td>
<td>Low</td>
<td>IgG2a=IgG2b=IgG1</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>Activating</td>
<td>Low</td>
<td>IgG2a=IgG2b=IgG1</td>
</tr>
<tr>
<td>FcγRIV</td>
<td>Activating</td>
<td>High</td>
<td>IgG2a=IgG2b</td>
</tr>
</tbody>
</table>

The human FcγRI and FcγRIIa are the receptors with the highest affinity for IgGs (Table 1, and chapter 1\(^2\)). Additionally, a common polymorphism in the FcγRIIa and FcγRIIIa has been associated with increased effect of therapeutic anti-cancer antibodies currently used in the clinic. The mouse equivalents of human FcγRI and FcγRIII are murine FcγRI and FcγRIV, being evolutionary orthologous genes. In various mouse metastasis models these receptors showed to be essential. For example, whereas in the liver both the FcγRI and IV have redundant functions, in a lung metastasis model two papers independently described either FcγRI or the
FcγRIV as the primary effector receptor\textsuperscript{9,10}. Fcγ receptors are expressed by various effector cells that include myeloid cells such as macrophages and neutrophils but also natural killer (NK) cells (Table 2\textsuperscript{3,11–13}). Each of these cell types express a different set of Fcγ receptors in both human and mice. Due to the homology of murine IgG2a and human IgG1, either antibody can be used as therapeutic antibody in mouse models of diseases, because of cross reactivity between human and mouse Fcγ receptors. mIgG2a is more effective than hIgG1, but mouse macrophages can efficiently induce tumour killing in the presence of either chimeric, humanized or fully human IgG1 and IgG2 via murine Fcγ receptors\textsuperscript{14}. Human IgG3 has the best interaction with FcγRI and FcγRIII. Furthermore, hIgG3 can very effectively activate the complement pathway, and induces effective immune effector functions, although this may depend on the target and potentially be affected by the allotype\textsuperscript{1,15–18}. However, human IgG3 has a short half-life. As such, most therapeutic antibodies are of the IgG1 subclass, which has longer half-life and also potently induces effector functions and complement activation. The vast majority of current therapeutic antibodies are chimeric or humanized antibodies with variable regions originating from a mouse antibody combined with human IgG1 constant domains. Both human and mouse antibodies have a glycan in the Fc domain that is involved in the interaction with the high and intermediate affinity receptors (human FcγRI and IIIa and mouse FcγRI and FcγRIV)\textsuperscript{19–21}. Without these glycans this interaction is significantly reduced. Variation in the glycan composition can result in changes in immune responses.

<table>
<thead>
<tr>
<th>Human</th>
<th>FcγRI</th>
<th>FcγRIIa</th>
<th>FcγRIIB</th>
<th>FcγRIIIa</th>
<th>FcγRIIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCs</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Uninflamatory monocytes</td>
<td>+</td>
<td>+</td>
<td>+\textsuperscript{§}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resident monocytes</td>
<td>+</td>
<td>+</td>
<td>+\textsuperscript{§}</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+\textsuperscript{*}</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NK cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse</th>
<th>FcγRI</th>
<th>FcγRII</th>
<th>FcγRIII</th>
<th>FcγRIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Inflamatory monocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Resident monocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>-</td>
<td>+</td>
<td>+\textsuperscript{§}</td>
<td></td>
</tr>
<tr>
<td>NK cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{*}upon activation \textsuperscript{§}organ specific

**Improving monoclonal antibody therapy.**

Because of the overall effector capacity and the long half-life, the majority of the current therapeutic antibodies are based on human IgG1. The effector mechanisms of these therapeutic antibodies is described in chapter 1\textsuperscript{2}. One of the first therapeutic mAb was rituximab (mabThera), which was approved in 1997 by the FDA. Rituximab is an anti-CD20 mAb that was developed for the treatment of malignant B cells (e.g. non-Hodgkin’s lymphoma)\textsuperscript{22,23}. In the following period many other therapeutic
antibodies were developed including cetuximab\textsuperscript{24} (Erbitux, an epidermal growth factor receptor (EGFR) blocking mAb, and trastuzumab\textsuperscript{25} (Herceptin, anti-Her2/neu blocking mAb), which are used to treat colorectal and head/neck cancer, and breast carcinoma respectively. In addition to direct effects on tumour cells (e.g. blocking of growth factors, induction of apoptosis), these antibodies can also trigger immune responses, either by complement activation or by binding to Fcy receptors that are expressed by monocytes, macrophages, NK cells and neutrophils\textsuperscript{4}. As a result of binding to Fcy receptors immune cells get activated to induce potent anti-tumour immune responses targeting the tumour. Although these therapies are promising for the treatment of cancer, they still need to be improved. Therefore, multiple approaches aim to improve the activation of the immune system by combining with adjuvants such as toll like receptor (TLR) agonists or by modification of the antibody\textsuperscript{3, 26, 27}. In this thesis I investigated two modifications of therapeutic mAbs.

The first modification targeted the interaction between the Fc domain of IgG and the Fcy receptors. Both human and mouse antibodies have a glycan in the Fc domain that is involved in the interaction between the antibody and the high and intermediate affinity receptors (human FcyRI and IIIA and mouse FcyRI and FcyRIV)\textsuperscript{19–21}. The core of this glycan often contains a fucose. We produced mAbs without core-fucose in the human IgG1 Fc domain of humanized anti-gp75 (TA99) mAbs (chapter 2). As a result improved binding to the human FcγRIIIa was observed, resulting in enhanced killing of tumour cells by effector cells (chapter 2)\textsuperscript{28–34}. Moreover, we demonstrated that mouse models are suitable to test the in vivo efficacy of glycan modified antibodies in order to predict their effect in patients. Treatment with afucosylated humanized IgG1 TA99 led to significant better protection for tumour outgrowth in a peritoneal metastasis model compared to treatment with wildtype hIgG1 TA99. We demonstrated that in vitro both human macrophages and NK cells efficiently killed B16F10-gp75 tumour cells in the presence of afucosylated IgG1 TA99. However, it is unlikely that NK cells played a significant role in targeting the tumour in the mouse. One of the reasons is that mouse NK cells lack FcγRI and FcyRIV. Additionally, there were only low numbers of NK cells present in the peritoneal cavity. The therapeutic efficacy of both wildtype and afucosylated TA99 mAbs was completely abrogated in mice lacking FcyRIV, demonstrating that effector cells have to express this receptor. The most abundant cells in the peritoneal cavity expressing FcyRIV are macrophages and monocytes. Additionally, after injection of fluids in the peritoneal cavity, we observed a high influx of neutrophils. Upon activation neutrophils can induce FcyRIV expression. Therefore, they could also be involved in tumour killing, although neither we nor others were able to demonstrate potent tumour killing by human or mouse neutrophils in the presence of IgG\textsuperscript{35, 36}. In fact, it was previously demonstrated that IgA is a more potent antibody to activate neutrophils and initiate tumour cell killing in vitro\textsuperscript{37, 38}. Nonetheless, treatment with anti-EGFR IgA mAbs resulted in elimination of B16F10-EGFR tumour cells by macrophages, but not neutrophils in a murine intraperitoneal metastasis model on\textsuperscript{39}. Moreover, we and others previously demonstrated that macrophages are essential for successful mAb treatment of liver metastases\textsuperscript{8, 40}. Taken together, this supports that macrophages and not neutrophils were the primary effectors cells in our model. As second modification we changed the human IgG1 Fc tail into an IgG3 backbone. Mouse macrophages were less efficient in mediating antibody dependent phagocytosis (ADCP) of tumour cells in the presence of hIgG3 compared to hIgG1. Additionally, treatment with hIgG3 TA99 was not better in preventing tumour outgrowth in mice compared to hIgG1 TA99 (chapter 3)\textsuperscript{Braster et al. submitted}. A major drawback of IgG3 is the
lack of interaction with the neonatal Fc receptor (FcRn). FcRn is expressed by many cells in the body, particularly on immune and endothelial cells\textsuperscript{17,41}. After internalisation of antibodies by pinocytosis, FcRn binds IgG in endosomes at low pH, and recycles the antibody back to the surface, releasing IgG at neutral pH in the bloodstream. All mouse and human IgG subclasses bind FcRn efficiently, except hIgG3. Therefore the half-life of IgG3 is shorter, as it is more easily degraded, making it less suitable as therapeutic mAb\textsuperscript{42}. The essential amino acid involved in binding to FcRn is a Histidine at position 435 in the Fc domain. By mutating the arginine at position 435 in the hIgG3 Fc domain into a histidine the binding to FcRn should be similar to hIgG1, resulting in enhanced half-life \textit{in vivo}\textsuperscript{17}. It has been reported by other groups that swapping parts of anti-CD20 hIgG1 into a hIgG3 backbone, or using whole hIgG3, results in enhanced complement dependent cytotoxicity (CDC) and antibody dependent cell cytotoxicity (ADCC) of lymphomas\textsuperscript{16,43,44}. However, we did not observe any improved therapeutic effect of IgG3 with prolonged half-life compared to treatment with IgG1 antibodies in the peritoneal metastasis model. These findings suggest that using an IgG3, even with enhanced half-life will not improve current therapeutic mAbs.

**Changing anti-EGFR mAb therapy**

EGFR-blocking antibodies are often used to treat patients who have either primary head or neck cancer or metastatic colorectal cancer without mutations in KRAS or BRAF. Optionally these treatments are combined with chemotherapies\textsuperscript{45}. The aim of this therapy is to constantly opsonize all EGFR on the tumour cells, hereby preventing binding of EGF and inhibiting the signalling pathway, which will result in cell cycle arrest and apoptosis\textsuperscript{46}. When these patients do not have mutations in the EGFR signalling pathway they are treated with repeated high doses of the IgG1 mAb cetuximab (Erbitux) or panitumumab (Vectibix), which is a human IgG2. The first infusion of cetuximab is 400mg/m\textsuperscript{2} in two hours, or 6mg/kg for panitumumab in one hour. There is no difference in effect observed for patients treated with chemotherapy and either cetuximab or panitumumab\textsuperscript{47}. Zalutumumab, which is a fully human IgG1, was developed as alternative for cetuximab and panitumumab.

The primary tumour of colorectal cancer patients is preferentially surgically removed and provides the best chance for cure. Unfortunately, 20-45% of the patients will develop liver metastases even when at time of diagnosis no metastatic tumours are detected\textsuperscript{48}. It was shown that surgical opening of the peritoneal cavity inflicts trauma that leads to enhanced tumour outgrowth in both the liver and peritoneum of rats\textsuperscript{49,50}. Furthermore, during surgery tumour cells can release from the primary colorectal tumour and end up in the portal circulation that is filtered by the liver\textsuperscript{51–53}. Increased CTC numbers in the portal circulation is associated with decreased overall survival of colorectal cancer patients\textsuperscript{54}. We previously demonstrated in rats that surgical trauma results in a systemic inflammatory response that leads to damage of the endothelium\textsuperscript{49}. As a consequence, there is enhanced adhesion of CTCs to the micro-vasculature of the liver.

Bacterial products, which can be released into the circulation because of resection of the colon, also play a role in enhanced metastasis and worse prognosis. This was shown in patients with an anastomic leakage, who have poorer oncological outcome compared to patients without infectious complications\textsuperscript{55–57}. The presence of lipopolysaccharide (LPS), which is secreted by Gram negative bacteria, in the bloodstream is associated with enhanced adhesion of colorectal tumour cells to liver sinusoids\textsuperscript{58–60}. It was demonstrated that liver macrophages (Kupffer cells) have a dual role. On the one hand they get activated by bacterial products and release reactive
oxygen species that damage the endothelial layer to which CTCs can adhere. On the other hand, they are involved in eliminating CTCs, as massive outgrowth was observed in livers of rats of which Kupffer cells had been depleted. Thus, surgery creates a niche in the liver allowing adherence and outgrowth of CTCs. Nonetheless, surgery is also a window of opportunity as only small tumour fragments, either single cells or small clumps, remain after the surgical removal of the primary colorectal tumour. Therefore, this would be the ideal period to apply mAb therapy, as removal of minimal residual disease by enlisting immune system would prevent metastasis formation and may increase disease free survival. We previously demonstrated that administration of tumour targeting mAbs during surgery induced antibody-dependent phagocytosis by Kupffer cells, which resulted in elimination of CTCs and prevented metastases development in animal models. We therefore now investigated whether pre-operative mAb therapy can be used to treat patients with colorectal carcinoma undergoing surgery.

By treating colorectal cancer patients pre-operative with antibodies targeting the tumour we aim to opsonize blood CTCs. Approximately 80% of colorectal carcinomas express EGFR on their membrane. In chapter 4 we investigated the potential of anti-EGFR antibodies to be used in a pre-operative setting. One of the most important obstacles for pre-operative adjuvant therapy is the possibility that wound healing will be affected. Especially when antibodies are used that target a growth factor receptor is it essential to show that there is no effect on wound healing itself. Besides EGFR overexpressing tumour cells also healthy epithelial cells may be targeted. It has been shown that the high dose - used to block all EGFR on the tumour cell membrane - may induce several side effects such as fever, skin reactions like a rash and necrosis, headaches, diarrhoea and nausea/vomiting. These side effects are also unwanted during surgery. However, the majority of these side effects are due to repeated infusions of anti-EGFR mAbs and therefore is unlikely to occur after only one infusion prior to surgery.

In a phase 2 trial the effect of a combination therapy of chemo- and radiotherapy together with cetuximab prior to resection of a primary rectal tumour was tested in order to reduce the tumour growth before surgery. In this study patients were treated in the 9 weeks prior to surgery with a high dose chemotherapy (capecitabine), radiotherapy starting in week 4, 25 times 1.8 Gy, and infusion of 400mg/m² cetuximab in week 3 followed by a weekly dose of 250mg/m² for an additional 5 weeks. Four to six weeks after this treatment the tumour was surgically resected and post-operative chemotherapy was given. In 6 of patients (16%) a delayed post-operative wound healing was observed. This treatment regime results in a stable high serum concentration of approximately 150μg/ml from week three after the first infusion until therapy is stopped. With a half-life of approximately 100 hours it is predicted that ca. 9μg/ml was present in the circulation at the time of surgery. However, is it not clear whether delayed wound healing was due to cetuximab therapy, or the combination with chemo-and radiotherapy.

It has been demonstrated that antagonists or high doses of mAbs targeting the EGFR pathway can inhibit tumour proliferation and migration. Proliferation was inhibited by anti-EGFR mAbs targeting tumour cells with a wildtype EGFR pathway at concentrations higher than 1μg/ml. To safely use anti-EGFR antibodies such as cetuximab during the surgical period, we wanted to investigate whether wound healing will be unaffected, while CTCs will still be eliminated when a lower dose of therapeutic mAbs is used. In our experiments no effect on migration was observed in the presence of anti-EGFR mAbs. We
demonstrated in chapter 2 that immune cells such as human macrophages and NK cells are potent killers of tumour cells in the presence of tumour targeting mAbs. Similarly, the therapeutic antibodies cetuximab, zalutumumab and panitumumab induced effective ADCP by human macrophages at concentrations of 0,1μg/ml. Concentrations as high as 30μg/ml did not show significant improved ADCP compared to 0,1μg/ml. Macrophages killed tumour cells very efficiently by phagocytosis followed by lysosomal degradation as we demonstrated by time lapse imaging. Within the first hour the fast majority of tumour cells had been phagocytosed in the presence of zalutumumab. We demonstrated in an in vivo imaging model that Kupffer cells in the liver clear CTCs via ADCP, as well. Additionally, human NK cells were potent killers of tumour cells at low concentrations of cetuximab and zalutumumab. The fully human IgG2 anti-EGFR mAb panitumumab was not able to induce any tumour cell kill by NK cells. This is likely because they only express FcγRIIIa that does not bind IgG2 effectively. Hence, it may be more favourable to treat patients with IgG1 based mAbs such as cetuximab and zalutumumab for more effective clearing of CTCs.

Taken together, our data support that treating patients with an amount of anti-EGFR antibodies, preferably hIgG1 based, prior to surgery, which will result in a concentration of around 1μg/ml in blood, would efficiently opsonize and clear circulating tumour cells by myeloid cells in the liver, in particular Kupffer cells, and NK cells in the circulation. Moreover, it is unlikely that healing of the anastomosis will be affected at these low concentrations. By modifying mAb in such a way that immune cell activation is improved, for example by removing the core-fucose as described in chapter 2, effectivity might be improved and the dose reduced even further.

**Macrophages in the tumour microenvironment**

Although antibody therapy will efficiently clearing CTCs, mAbs are less effective for targeting solid tumours. The inability of mAbs to induce proper immune responses is probably caused by an immune suppressive tumour microenvironment. Therefore, we investigated if the microenvironment can be modified into a hostile anti-tumour milieu, in which tumour cells can be killed by immune cells. As previously described, various immune cells play an important role in the tumour development by regulating the immune status of the tumour microenvironment. By secretion of cytokines, growth factors and angiogenic factors these cells are able to create an environment that can enhance or suppresses tumour growth, depending on the circumstances. Myeloid cells, especially myeloid derived suppressor cells (MDSCs) and macrophages, are considered prominent suppressive cells in the tumour microenvironment. In the majority of types cancer types, tumour associated macrophages (TAMs) are described to promote tumour growth by stimulating angiogenesis, metastasis and immunosuppression. This was also demonstrated in various preclinical models. TAMs have been demonstrated to play a prominent role in stimulating growth of mamma- and various other carcinomas. Furthermore, high infiltration of TAMs is associated with a bad prognosis for patients. However, presence of TAMs does not always correlate with a worse prognosis. For instance, in colorectal cancer high macrophage infiltration was associated with improved prognosis. When macrophages were depleted in rats prior to inducing metastasis the total colon carcinoma load was significantly increased. As a consequence, overall survival was decreased, indicating that macrophages have a crucial role in inhibiting tumour growth in colon carcinoma. Remarkably, the absence of macrophages also resulted in reduced micro-vessel density and enhanced tumour differentiation, which points at a
pro-tumouricidal role for macrophages as well. This data demonstrates the different and complex functions that macrophages have during colon carcinoma development.

**Macrophage phenotypes**

The completely opposite effector functions of macrophages in various types of tumours can be explained by macrophage plasticity. At first macrophages were divided into two classes, namely classically activated or M1 macrophages versus alternatively activated or M2 macrophages. M1 macrophages were associated with T helper 1 lymphocytes and with inflammation induced by bacterial products and inflammatory factors such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α). This results in secretion of pro-inflammatory cytokines such as IL-12, IL-23 and TNF-α. Alternatively activated M2 macrophages are associated with T helper 2 lymphocytes and activation results in secretion of more anti-inflammatory mediators such as IL-10, C-C motif chemokine ligand 17 (CCL17) and CCL22. However, it became rapidly clear that the M1/M2 classification is oversimplified due to the plethora of macrophages functions in organisms. This resulted in the subdivision into additional subpopulations, with M1 and M2 as extremes on a scale. Macrophage phenotypes have also been depicted as colour wheel of macrophage activation. More recently a set of standard characteristics has been suggested, which proposes to describe macrophage activation by the source of the macrophages, the way they are activated and a selection of markers including cytokines, chemokines, and various receptors. Due to their plasticity macrophages are able to change in function. Therefore, in my opinion secretion of cytokines, chemokines and the direct effect of macrophages on the microenvironment is the most important characteristic of macrophage phenotypes. We previously demonstrated that resident macrophages in the colon carcinoma microenvironment can kill tumour cells. Progression of the tumour however can result in secretion of chemokines such as CCL2 that consequently recruits new monocytes/macrophages. By inhibiting monocyte recruitment with flavonoids, tumour outgrowth in these rats was significantly increased, supporting that also newly recruited macrophages gained a cytotoxic anti-tumour phenotype. However, when monocytes infiltrate in an immunosuppressive tumour microenvironment they will differentiate into a tumour promoting phenotype. For example, this was demonstrated in a mouse model for mammary carcinoma. The infiltration of macrophages as a response to CCL2 secretion resulted in enhanced tumour progression. Thus, the tumour microenvironment dictates the development of macrophages. The role of the tumour microenvironment on macrophages differentiation and the possibility to manipulate this process was further investigated in chapter 6. We hypothesised that by repolarizing TAMs in the tumour microenvironment a tumour suppressive, inflammatory milieu can be generated in solid tumours, activating effector cells such as NK cells and cytotoxic T cells to attack the tumour cells. Moreover, combining repolarisation of TAMs with therapeutic tumour targeting mAbs can have an improved effect compared to the current strategies.

**Macrophage re-polarisation**

In chapter 6 we demonstrated that colorectal carcinoma cell lines secrete products that drive monocytes to develop pro-inflammatory characteristics. On the other hand, mammary carcinoma cells skewed monocytes towards an immune-suppressive and wound healing phenotype. The latter was demonstrated previously with ovarian carcinoma cells. These data demonstrate that tumours themselves produce and secrete factors that influence macrophage phenotypes. Using proteomics to analyse
the secretomes of several colorectal- and mamma carcinoma cell lines we identified several proteins that are exclusively expressed by colorectal carcinoma cells. Among the candidates we hypothesised to be involved in macrophage priming, versican was the most promising since it was expressed and secreted by all tested colorectal carcinoma cell lines and none of the mamma carcinoma cell lines (chapter 6)\textsuperscript{96}. Versican is an extracellular matrix proteoglycan that has been described for its roles in various processes such as adhesion, proliferation as well as angiogenesis and migration\textsuperscript{97}. There is conflicting data about the effect of versican production by malignant tumours. Patients with high expression of versican in ovarian cancer have improved prognosis, whereas worse prognosis is correlated with high versican expression in endometrial and cervical cancers\textsuperscript{98,99}. We demonstrated that versican is involved in skewing monocyte differentiation towards an inflammatory phenotype. When we blocked versican with antibodies or suppressed expression by virally introducing short hairpinRNA (shRNA) in colorectal cell lines, monocytes that were stimulated with this supernatant expressed less inflammatory cytokines. Versican expression by malignant epithelial cells in the periphery of the tumour was correlated with improved disease free survival of patients with stage II or III type colon cancer\textsuperscript{100}. These data indicate that versican is one of the tumour-secreted factors influencing the tumour microenvironment by interaction with monocyte/macrophages. However, versican has glycosaminoglycan chains that are able to interact with many proteins and lipids. It is therefore likely that a combination of factors bound to versican may induce differentiation of monocytes/macrophages towards a specific phenotype\textsuperscript{101–103}. Given the fact that versican expression is correlated with poor prognosis in other carcinomas, with hindsight it may be a completed molecule to target in tumour therapy.

Two other identified interesting candidates were Fam3C and Fat1. Both proteins are often described in cancer\textsuperscript{104,105} and were exclusively found in the proteomic analysis of the secretomes of colorectal carcinoma and not mamma carcinoma (data not shown). However, mRNA analysis of colorectal and mamma carcinoma cell lines demonstrated that mamma carcinoma also expressed these proteins, albeit generally less than the colorectal cell lines (Figure 1A and C, methods as described in chapter 6). By introducing short hairpin RNA via lentiviral transduction we expected to see a similar effect as was observed with versican. We were able to reduce the mRNA expression in cell lines (Figure 1B and D, methods as described in chapter 6). Unfortunately, when human monocytes were stimulated with supernatant obtained from knock down versus sham (scrambled siRNA) cell lines no differences were observed in secretion of cytokines such as IL-6 and IL-8 by monocytes (Figure 1 E and F, methods as described in chapter 6). It is therefore unlikely that either Fam3C or Fat1 are involved in monocyte polarization.

**Future perspectives and recommendations**

I focussed in this thesis on both recruiting macrophages by (modified) therapeutic antibodies and on re-polarizing tumour associated macrophages. Either may improve current therapeutic strategies separately, but especially combined activation of cytotoxic macrophages by anti-tumour therapeutic antibodies may be an attractive future approach. Several recommendations can be made.
Timing and modifying therapeutic antibodies

A major advantage of therapeutic mAbs is their specificity to target proteins highly expressed on tumour cells. Currently anti-EGFR antibodies cetuximab and panitumumab are administered, often in combination with chemotherapy, to patients that already developed advanced metastases. Additionally, therapy is only indicated when the metastases express wildtype KRAS and BRAS, kinases downstream of EGFR. Although patients benefit from this treatment it is likely that only the direct effects of the antibody by blocking EGFR are involved. The immune system is likely not efficient in inducing tumour cell death. Therefore, we propose to change the moment of administration of anti-EGFR antibodies (chapter 4). By administering these antibodies prior to surgery circulating tumour cells and tumour cells that are detached from the primary tumour during surgery will be opsonized and eliminated by immune cells. As a result, the chance of metastasis formation should be reduced significantly and hopefully even completely prevented in patients. Moreover, because this strategy depends on immune effector cells and not on the blocking properties of therapeutic mAbs, mutations in the EGFR pathway are irrelevant. As a result, also patients with EGFR expressing tumours that harbour mutations can be treated, increasing the number of patients that might benefit from this treatment. Moreover, if it has been demonstrated to be beneficial for colorectal cancer this strategy could be applied for any resectable solid tumours. The therapeutic effectiveness of antibodies can be even further improved by modification of the mAbs. Several of these modifications are being investigated to improve the immune activating abilities. Recent studies primarily focused on the role of the glycans on the asparagine at position 297 in the Fc domain. These glycans are essential for effective interaction with Fcγ receptors. For example, removing the core-fucose as described in chapter 2 will enhance the tumour killing capacity of NK cells and macrophages significantly. On the contrary, sialic acids have been demonstrated to be essential in immune suppressive effects of intravenous IgG (IVIG). By removing these sialic acids no immune suppressive effects of IVIG were observed in mice. Therefore, it is interesting to test the combination of...
removing both the fucose and the sialic acids on therapeutic antibodies. Many other modifications are currently also investigated. For example, bi-specific antibodies (BsAb) are designed to bind two different targets. For instance, BsAb targeting a tumour antigen and CD3 may induce T cell mediated killing. Additionally, BsAb targeting two different receptors at the tumour cell to enhance the specificity have been described\textsuperscript{109,110}. Alternatively, nanobodies, which consist of only a functional heavy chain immunoglobulin that exists in Camelidae, are significantly smaller than regular immunoglobulins and have therefore improved penetration into the tumour for, for example, more efficient delivery of toxins into the tumour\textsuperscript{111}.

**Combination therapies**

Combination of chemotherapy, radiotherapy, and/or immunotherapy is the most effective way to treat cancer patients. In addition, combination of different immunotherapeutic strategies may improve outcome for patients. For example, tumour targeting antibodies can be used to trigger Fcγ receptor expressing innate immune cells together with activation of the adaptive immune system. The latter can be roughly divided into two major strategies, 1) vaccination and 2) T cell activation. The goal of vaccination is to activate the immune system in a ‘natural’ way. Therefore, antigen presenting cells, predominantly dendritic cells, are targeted to induce cytotoxic T cell responses\textsuperscript{112–114}. For the second strategy tumour specific T cells were isolated from the tumour, expanded \textit{ex vivo} and re-infused into the patient\textsuperscript{114–117}. However, cytotoxic T cells can be negatively regulated via receptors such as CTLA-4 and PD-1. Tumour cells or immune suppressive cells in the tumour microenvironment can express ligands such as PD-1L, preventing T cells to attack the tumour. Therefore, antagonists or checkpoint inhibitors have been developed to prevent inactivation of T cells. A positive side effect of blocking CTLA-4 on cytotoxic T cells is that regulatory T cells, which are also potent immune suppressive cells in the tumour microenvironment, lack a stimulus to maintain their inhibitory function, resulting in a more inflammatory milieu in the tumour\textsuperscript{118}. A combination therapy of checkpoint inhibitors and anti-tumour mAbs may recruit the whole spectrum of cytotoxic effector cells for efficient elimination of the tumour.

Alternatively, during mAb therapy the Fcγ receptor expressing cells could be more efficiently activated in solid tumours. TAMs are a good candidate to target simultaneously with mAb therapy to manipulate the tumour microenvironment. In this way tumour growth may be suppressed and tumour killing properties of macrophages and other effector cells such as NK cells and T lymphocytes are enhanced. In colorectal cancer high macrophage influx is associated with good prognosis, whereas in the majority of the other types of cancer this is correlated with a worse prognosis. As a result, it is well accepted that macrophages are a good target in the tumour microenvironment. Different strategies in which macrophages are manipulated to act against the tumour are summarized in chapter 5\textsuperscript{83}.

We demonstrated that versican polarizes monocytes towards inflammatory macrophages, however we do not know the mechanism (chapter 6)\textsuperscript{96}. TLR-2 has been proposed as receptor for versican\textsuperscript{119}. The use of bacterial components to target TLRs is already an old strategy to activate the immune system and more particular macrophages\textsuperscript{91,92}. Bacillus Calmette-Guérin (BCG; Mycobacterium bovis) is widely used for treatment of patients with bladder carcinoma and is currently investigated for the treatment of colorectal carcinoma\textsuperscript{120,121}. Additionally, targeting TLR2, 3 and 4 with PAM3CSK4, Poly I:C and LPS or the synthetic counterpart liposome-encapsulated
muramyl-tripeptide phosphatidyl-ethanolamine (MTP-PE) has been shown to have a positive effect on macrophage-induced killing of tumour cells. Macrophage activation in some cases also (indirectly) results in cytotoxicity of NK cells and cytotoxic T cells\textsuperscript{122-124}. Therefore, the use of TLR agonists in combination with antibodies may represent a beneficial therapeutic strategy.

Alternatively, several clinical trials aim to use or block factors that influence macrophage polarization. Blocking the colony stimulating factor 1 (CSF-1, M-CSF) receptor resulted in regression of established tumours and enhanced survival of mice in a human xenograft glioma model\textsuperscript{125}. The repolarisation of macrophages into an inflammatory phenotype by the secretion of granulocyte macrophage colony stimulating factor (GM-CSF), possibly in the presence of IFN-\gamma secreted by glioma cells, was suggested to be the effect of M-CSF receptor blocking\textsuperscript{125}. This was supported by a study involving tissue from colorectal cancer patients in which a correlation between GM-CSF and improved prognosis was observed\textsuperscript{126}. However, a direct correlation with macrophages was not demonstrated\textsuperscript{127,128}. A less effective but still extensively studied protein is IFN-\gamma. Injecting tumour bearing rats with IFN-\gamma resulted in a small decrease in tumour burden compared to control animals\textsuperscript{129}. Currently two clinical trials with IFN-\gamma are ongoing but have not been published yet (NCT01957709 and NCT02197169, clinicaltrials.gov).

**Concluding remarks**

The data presented in this thesis demonstrates that both monoclonal antibodies and targeting of macrophages may have a great potential to improve current anti-cancer therapies (Figure 2). Colorectal cancer patients might greatly benefit from pre-operative treatment with anti-EGFR antibodies to decrease the chance on metastasis. Removing the core-fucose of mAbs enhances activation of macrophages in the liver and in tumours as well as NK cells, making current therapeutic antibodies more effective. Tumour associated macrophages can be manipulated to create an anti-tumour microenvironment.

In my opinion both the innate and adaptive immune system should be effectively activated during cancer treatment. To remove (resectable) primary tumours a combination of surgery, radio- and chemotherapy is the most suitable strategy. However, to prevent relapse and metastasis a good strategy will be the combination of antibody therapy and vaccination to one hand eliminate non detectable metastasis and circulating tumour cells and on the other hand to induce long term adaptive immune responses, respectively.
FIGURE 2. Summarizing figure
(A) Liver macrophages (Kupffer cells), are efficient in capturing and killing circulating tumour cells through either human IgG1 (green) via FcγRIII (red) or IgG2 (orange) via FcγRI or FcγRIIa (blue and yellow resp.) (B) The Fcγ receptor expressing cells in the bloodstream may contribute to killing of tumour cells. NK cells were able to kill tumour cells in the presence of IgG1, but not IgG2 (C) Myeloid cells, predominantly macrophages, MDSCs and to a lesser extend neutrophils as well as regulatory- and helper-T cells manipulate the tumour microenvironment.


29. Shields, R. L. *et al.* Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human


48. Mirnezami, A. et al. Increased local recurrence and reduced survival from colorectal cancer following


