INTRODUCTION PART I

MACROPHAGES AND TUMOURS:
ROLE IN DEVELOPMENT, PROGNOSIS AND
THERAPEUTIC POSSIBILITIES
& OUTLINE OF THE THESIS

CHAPTER 1
Outline of the thesis

Macrophages play an important role in the development of colorectal tumours and furthermore contribute to surgery induced colorectal metastasis initiation. However they also can be a therapeutic target in abrogating colorectal metastasis development. In this thesis I try to elucidate the complicated role macrophages play in colorectal cancer development. The thesis is divided into two parts. In part one I will discuss the role of macrophages in tumour development, whereas in part II I focus on the role of macrophages in initiating and inhibiting surgery induced colorectal metastasis formation. In chapter 2 I demonstrate that both mature macrophages and newly infiltrated monocytes can reduce development of peritoneal colorectal metastases. Peritoneal macrophages are shown to be cytotoxic against colon carcinoma cells and infiltration in colon carcinoma tumours therefore is beneficial for survival. These data are conclusive with patient studies, which show macrophage infiltration into colorectal tumours to be beneficial for patient survival. As however clinical data show opposite effects on patient survival after macrophage infiltration into breast carcinoma, we study in chapter 3 how carcinoma cells themselves can influence macrophage phenotype. We show that breast carcinoma cells can enhance pro-tumour properties of macrophages, whereas colon carcinoma cells polarize monocyte towards a more cytotoxic anti-tumour phenotype. In chapter 4 I review the role of macrophages in initiating and inhibiting surgery induced colorectal metastasis development. It has become clear that surgery itself, although mandatory to secure patient survival, contributes to metastases development. Surgery induces systemic effects leading to damage of endothelial linings and exposure of extracellular matrix. Together with enhanced tumour cell shedding from the primary tumour after surgery, this exposure of extracellular matrix leads to more tumour cell adhesion and subsequently to heightened metastasis outgrowth. In chapters 5 we show that macrophages become activated after surgery and subsequently cause endothelial cell damage. Macrophages therefore play an important role in initiating enhanced tumour cell adhesion after surgery. However, in chapters 6 to 9 we demonstrate that macrophages can also be used to diminish metastases outgrowth by treating animals with different mAbs targeting tumour cells. In chapter 6 we show that both resident macrophages of the liver (Kupffer Cells) as well as infiltrating monocytes are able to prevent surgically induced colorectal liver metastases by monoclonal antibody therapy. By using human mAb targeting the human epithelial growth factor receptor on colon carcinoma cells we demonstrate in chapter 7 that we can extrapolate our data towards the human situation in the future. In chapter 8 we use intravital microscopy to show that also in vivo mAb therapy leads to antibody dependent phagocytosis of tumour cells by macrophages. In chapter 9 we try to enhance the efficiency of mAb therapy to reduce metastases development by changing the isotype of monoclonal antibodies. We use different human IgG1 and IgG3 mAb, targeting an identical tumour antigen to elucidate their effects on metastases development. Finally, chapter 10 addresses the clinical implication of the experimental data from this thesis, and therapeutic strategies are recommended.
Introduction

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 and these numbers are projected to continue rising, with an estimated 13.1 million deaths in 2030 (World Health Organisation, Globocan 2008) 1. Cancer arises from one single cell. The transformation from a normal cell into a tumour cell is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumours. Cancer is mostly caused by alteration in oncogenes and tumour suppressor genes 2. Hereditary genetic defects in these genes attribute only to 5-10% of all cancer cases, whereas the remaining 90-95% have their origin in the environment and lifestyle 3. Different physical, chemical or biological carcinogens, like alcohol, sun exposure, environmental pollutants, infections, obesity and diet (fried food, red meat) are well known to cause mutations in oncogenes and tumour-suppressor genes. A single genetic change however is rarely sufficient for the development of a malignant tumour, and cancer development is a multistep process of sequential alterations in, often many, genes in cancer cells 2. Oncogenes encode proteins that control cell proliferation or apoptosis and mutations in these genes lead to uncontrolled cell proliferation. As the tumour grows it needs more and more oxygen and nutrients and it therefore will interact with the surrounding tissues. Different cell types like fibroblasts and endothelial cells will infiltrate the growing tumour mass, leading to angiogenesis and vascularisation of the tumour 4. Besides fibroblasts and endothelial cells will different immune cells from the host microenvironment infiltrate the growing tumour, where they can extensively influence tumour progression and development 5. Macrophages are an important component of the innate immune system and they form a major component of tumours where they can directly influence tumour development and growth. After infiltration into tumours, macrophages are polarized by factors in the tumour microenvironment and can have both anti- and pro-tumour properties. Depending on the activation state of macrophages in the tumour, macrophages can positively or negatively influence patient survival. Exactly how macrophages in tumours are polarized to become pro- or anti-tumourigenic is still unknown, however it has become clear that macrophages form a complicated synergistic relationship with malignant cells in tumours. Tumour cells affect macrophage phenotype directly and influencing macrophage polarization may therefore have therapeutic effects leading to enhanced patient survival. Exactly how macrophages contribute in cancer development and how the function of macrophages in tumours can be altered is still a controversial subject, which needs further research.
Chapter 1

Immune cell infiltration

The presence of large immune cell infiltrates in solid tumours was already described over a century ago and it has become clear that the innate immune system can play a role in both initiation and development of cancer. In normal circumstances, inflammation is induced by innate immune cells after infection or injury, in order to remove potential harmful micro-organisms, after which homeostasis is restored. When the innate immune system is however unable to clear the infection or there is unceasing tissue injury, inflammatory responses are sustained, resulting in a chronic inflammation, which predisposes to the development of cancer. For instance, excessive reactive oxygen species (ROS) production by either macrophages or neutrophils can lead to DNA damage, which can trigger the onset of cancer formation. However, to become a clinical manifest tumour, initial cancer cells have to interact with their environment, which leads to attraction of stromal supporter cells like endothelial cells and fibroblasts. Furthermore, an influx of immune cells in tumours is often observed as well. Together these cells create a micro-milieu, in which cytokines, chemokines, growth factors, and angiogenic factors are produced that allow further tumour development.

Macrophages represent one of the most prominent leukocyte populations present within tumours, and accumulating evidence supports a key role for these cells in tumour development. They derive from circulating monocytes that are recruited towards the tumour by chemoattractants, which can be locally produced in the tumour itself. After extravasation monocytes differentiate into mature macrophages. Depending on the local micro-milieu, macrophages can obtain different functional phenotypes, ranging from tumour cell killing to directly promoting tumour growth, supporting angiogenesis and inducing metastasis. In this chapter I will address the diverse roles of macrophages in tumour development, demonstrating the delicate balance between anti- and pro-tumorigenic functions of tumour infiltrating macrophages. Furthermore we will refer in detail on how interfering with either anti- and pro-tumorigenic functions of tumour infiltrating macrophages can lead to new therapeutic stratagies, reducing tumour growth and enhancing patient survival.
The importance of macrophages in the progression of cancer is supported by many retrospective studies that link tumour stage and patient prognosis to the number of macrophages in malignant tissues. For instance, extensive immune cell infiltration - which consisted primarily of macrophages -, in mamma carcinoma tumours was associated with augmented malignancy and enlarged tumour size. Moreover, increased staining of CD68-positive macrophages in tumours from patients with invasive breast carcinomas correlated with worse prognosis. Overall is the extent of macrophage infiltration into the tumour an independent prognostic factor for disease free-, relapse free- and overall survival. Furthermore, the correlation between increased macrophage infiltration into the tumour and poor survival is currently not only evident for invasive breast carcinomas, but also for a variety of other human malignancies, including bladder carcinoma, cervix or endometrial tumours, melanoma, oesophagus carcinoma, renal cell carcinomas, cholangiocarcinoma and non-Hodgkin lymphoma (see also table 1 for more details). In these malignancies it was demonstrated that patients with tumours containing high levels of infiltrated macrophages had either worsened clinical stage of tumours, higher neovascularisation or vascular invasion, increased disease progression, more distant or lymph node metastases, or decreased 5 year (disease free) survival.
Table 1: Prognosis and malignant tissue associations. The table shows the relationship between tumor infiltrating macrophages (CD68+) and various markers and tissues, as well as the corresponding conclusion for each association.

<table>
<thead>
<tr>
<th>Prognosis</th>
<th>Malignancy</th>
<th>Markers &amp; tissues</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>Poor</td>
<td>Breast</td>
<td>CD68 &amp; MCP-1</td>
<td>Y CD68+ ma corresponded with Y MCP-1 expression and poor patients prognosis (&lt; 5 year survival)</td>
</tr>
<tr>
<td>Poor</td>
<td>Breast</td>
<td>CD68, vWF and CD31</td>
<td>Y ma number δ worsened relapse-free and overall survival and enhanced vascular grade. However high dense ma areas in tumour δ low angiogenesis</td>
</tr>
<tr>
<td>Poor</td>
<td>Breast</td>
<td>CD68, Ki67 &amp; CD34</td>
<td>Y tumour grade and tumour cell proliferation associated with Y infiltration of CD68+ ma. Y CD34+ microvessel density positively correlated with ma presence, linked to poorer cancer-specific survival</td>
</tr>
<tr>
<td>Poor</td>
<td>Breast</td>
<td>CD68, vWF, HE</td>
<td>- Diffuse inflammation, consisting primarily of ma in tumour, associates with Y tumour grade, Y size and Y tumour necrosis. - Correlation between diffuse ma inflammation and vascularity - Ma hotspots not associated with vascular hotspots.</td>
</tr>
<tr>
<td>Poor</td>
<td>Bladder</td>
<td>CD68 &amp; CD34</td>
<td>Mean ma number and microvessel count in invasive bladder cancer Y compared to superficial bladder cancer: ma numbers Y correlated with microvessel density Y, tumour grade Y and presence of distant metastases Y</td>
</tr>
<tr>
<td>Poor</td>
<td>Cervix</td>
<td>CD68, IL8 &amp; VWF</td>
<td>Ma number in tumour correlates with IL8 levels in tumours. Infiltrated ma correlate with microvessel count. Infiltrated ma are source of IL8 leading to increased vasculature and increased clinical stage of tumours</td>
</tr>
<tr>
<td>Poor</td>
<td>Melanoma</td>
<td>CD68 &amp; VWF</td>
<td>Vascular density and ma infiltration closely associates in advanced melanomas. Depth of tumour invasion correlates with ma infiltration. However neovascularisation of tumour precedes ma infiltration in early stage melanomas</td>
</tr>
<tr>
<td>Poor</td>
<td>Uveal melanoma</td>
<td>CD68 &amp; PAS</td>
<td>Number of ma associated with tumour diameter and microvessel density. Association between increased melanoma-specific mortality and increasing number of CD68+ ma</td>
</tr>
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13. Bladder CD68 & CD34: 63 bladder cancer specimens (40 superficial and 23 invasive bladder cancers)
14. Cervix CD68, IL8 & VWF: 80 cervical cancer tissues
15. Melanoma CD68 & VWF: 37 primary cutaneous malignant melanomas
16. Uveal melanoma (choroidal or ciliary body) CD68 & PAS: 139 uveal melanomas semi-quantitatively scored
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<tr>
<th>Poor</th>
<th>Oesophagus</th>
<th>CD68, MCP-1, CCR2, Ki-67 &amp; Factor VIII 56 esophageal squamous cell carcinomas</th>
<th>MCP-1 expression by cancer cells associated with increased monocyte count, microvessel density (MVD) and Ki-67 labeling. Monocytic count correlated with MVD. Monocyte count higher in tumours of patients with venous invasion and distant metastases. 5 year survival rate in patients with high monocyte count or MCP-1 expression worse then patients with low monocyte count or without MCP-1 expression</th>
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<tr>
<td>Poor</td>
<td>Renal Carcinoma</td>
<td>CD68, Factor VIII, S100, HLA-DR &amp; Ki-67 96 renal cell carcinoma tumours</td>
<td>In multivariate analysis: High mø count, high MVD and high proliferation index were significant factors for poor prognosis. Positive correlation between mø and MVD. Low MVD group with high mø count demonstrated poor prognosis in univariate analysis. High DC count (S100) tendency to correlate with favourable prognosis.</td>
</tr>
<tr>
<td>Poor</td>
<td>Clear cell renal cell carcinoma (ccRCC)</td>
<td>CD68, CD163, CD204 Tissue microarray of 66 ccRCC cases</td>
<td>Nuclear grade of ccRCC tumours associated with number of CD163+ and CD204+ mø and TNM classification associated with number of CD163+ mø. Infiltration of CD163+ mø correlated with reduced OS. No correlation between OS and number of CD204+ mø</td>
</tr>
<tr>
<td>Poor</td>
<td>Cholangiocarcinoma (CCA)</td>
<td>MAC387 &amp; MMP9 in cells along leading edge of tumour 50 CCA tissues</td>
<td>High density of MAC387+ mø associated with poor prognosis parameters (non-papillary and mass forming type CCA). OS significantly reduced in patients with high density of MAC387. Density of MAC387+/MMP9+ mø is independent factor in poor patient outcome</td>
</tr>
<tr>
<td>Poor</td>
<td>Intrahepatic cholangiocarcinoma (ICC)</td>
<td>CD68, CD163 &amp; FOXP3 39 ICC patients</td>
<td>Positive correlation between CD68 and CD163 mø and vessel number and number of regulatory T-cells. Density of both CD68+ and CD163+ mø did not correlate with overall survival, however, ICC with high CD163+ mø showed poor disease free survival</td>
</tr>
<tr>
<td>Poor</td>
<td>Non-Hodgkin's lymphoma</td>
<td>CD68, Factor VIII, and T/B-cell markers 71 B-cell non-Hodgkin's lymphomas</td>
<td>High grade lymphomas demonstrate higher vessel density and increased mø infiltration.</td>
</tr>
<tr>
<td>Poor</td>
<td>Lung adenocarcinoma</td>
<td>CD68 and D2-40 65 Lung adenocarcinomas</td>
<td>Tumour mø count associated with tumour staging (p-TNM) and lymph node metastasis. Inverse relation between peritumoural LMVD and OS. Tumour mø number correlate with LMVD.</td>
</tr>
<tr>
<td>Poor</td>
<td>Gastric carcinoma</td>
<td>25F9 (tissue mø). Ratio tumour cells/mø determined 66 Gastric carcinomas</td>
<td>Patients with better prognosis had less mø in tumour infiltrate and high tumour/mø ratio compared to patients with poor prognosis</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Malignancy</td>
<td>Markers &amp; tissues</td>
<td>Conclusion</td>
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</table>
| **Good** | Non small cell lung cancer (NSCLC) | CD68 and tryptase  
Tumours of 175 patients with NSCLC | Increasing tumour islet mø density is favourable independent predictor of survival. Islet/stromal mø ratio is strong favourable independent prognostic factor | 33 |
| **Good** | Non small cell lung cancer | CD68/HLA-DR, CD68/C163  
100 patients (50 short survival (1y) and 50 long survival (5y)) retrospective | - No difference CD68/C163 in tumour islets and/or stroma between long or short surviving patients, no correlation with OS  
- CD68/HLA-DR in tumour islets or stroma of long survival patients  
- CD68/HLA-DR correlates with good OS and is independent predictor of patient’s survival time | 34 |
| **Good** | Non small cell lung cancer (NSCLC) | CD68 combined with  C163, HLA-DR, INOS, VEGF or TNFα  
40 patients with NSCLC | Infiltration of tumour islets with mø expressing INOS, HLA-DR & TNFα is increased in patients with extended survival. Mø infiltrated in patients with poor survival showed high expression of CD163 & VEGF | 35 |
| **Good** | Colon cancer | CD68, CD8  
446 colorectal cancer specimens | Patient survival increased with mean tumour front CD68+ mø infiltration. High mø hotspot score showed survival advantage also in curatively resected colon cancer cases. High infiltration of CD8+ CTL tended to follow high infiltration of CD68. | 36 |
| **Good** | Colon cancer | CD68 in invasive tumour front  
160 patients with TNM stage III/IV colon carcinoma | High mø infiltration correlates with low TNM tumour grade, presence of hepatic metastasis and pathological classification. CD68+ mø infiltration highly correlated to OS but not to liver metastasis free survival. | 37 |
| **Good** | Colorectal cancer (CRC) | CD68, CD3, CD34 & vWF  
70 moderately differentiated stage II/III CRC | No association between mø infiltration and MVD. Patients with high number of mø in invasive tumour front, and low vWF+ MVD are prognostically favourable. | 38 |
| **Good** | Colon carcinoma | CD68, VEGF & EGFR  
131 stage II/III colon carcinomas | Presence of mø expressing VEGF predictive of improved survival. VEGF or CD68 expression alone no association. EGFR expression correlated with trend towards worsened survival | 39 |
| **Good** | Colorectal cancer | CD8 & CD68 in invasive margin of tumour  
97 adenocarcinomas of colon/rectum | Patients with high level mø infiltration showed lower depth of tumour invasion, less vascular invasion into tumour and higher lymph node metastasis negative cases. OS was higher in patients with both high CD68+ mø and CD8+ infiltration. | 40 |
| **Good** | Colorectal cancer | CD68/C163, S100/CD11c/CD1a  
40 CRC samples | Better survival in patients with high stromal and epithelial S100+ DC infiltration. No significant association between CD68+ mø presence and survival. Trend for better survival in patients with increased C163+ mø | 41 |
| **Good** | Gastric cancer | CD68, CD8 & TUNEL  
Tissue specimens of 84 advanced gastric carcinoma (41 pT1, 43 pT2 stage) | mø infiltration into cancer nests associates with increased tumour cell apoptosis index, increased nest CD8+ TIL and increased 5-year disease free survival rate. | 42 |
| **Good** | Gastric cancer (diffuse & intestinal type) | MMP1/9, uPAR(R) & CD68  
Tumours of 104 patients with gastric cancer (26 diffuse, 78 intestinal type), | Diffuse type tumours show decreased mø number and lower MMP9/1 & uPAR expression. Mø number higher in intestinal type tumours without liver metastasis. | 43 |
| **Good** | Prostate cancer | CD68  
81 prostatectomy specimens of prostate cancer patients | High total mø area in tumours is independent factor for disease free survival after surgery. Reduced total mø density associated with presence of positive lympe nodes. | 44 |
| **Good** | Osteosarcoma (High-Grade) | CD14, C163, HLA-DR & CD31  
+ micro-arrays  
Cohort 1: 53 diagnostic biopsies (34 with metastasis, 19 without) for gene profiling  
Cohort 2 & 3: 88 (TMA) or 20 patients: pretreatment, postchemotherapy and metastatic samples | - High expression of macrophage genes in biopsies of patients without metastases (cohort 1)  
- Metastases free survival correlates with CD14 in biopsies (cohort 2)  
- CD14ñ correlates with better OS in biopsies (cohort 2 & 3)  
- No correlation CD14/C163 or CD14/HLA-DRα with OS  
- CD14ñ correlates with MVDñ (CD31)  
- No correlation MVD and OS | 45 |
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<th>Conclusion</th>
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<tr>
<td>Poor/No-correlation</td>
<td>Breast</td>
<td>CD68, CD4 &amp; CD8</td>
<td>CD68 cell density alone no significant correlation with overall survival (OS). CD4+ high or CD8+ low correlates with reduced OS. CD68+ cell infiltration inversely correlates with CD8+ density. CD68+high/CD4+high/CD8+low signature in tumour correlates with reduced OS and relapse-free survival.</td>
<td>49</td>
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<tr>
<td>Poor/beneficial</td>
<td>Endometrial</td>
<td>CD68, Histological location of infiltrated mő in tumour was assessed. 70 endometrial cancer tissues</td>
<td>Increased mő numbers in necrotic tumour centre associates with increased clinical stage and decreased relapse free survival. Increased mő presence in tumour stroma correlates with presence of lymph node metastases. Aggregation of mő within cancer cell nests or in close contact with cancer cells has a beneficial effect on relapse free survival</td>
<td>16</td>
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<td>Good/poor correlation</td>
<td>Non-Small cell lung cancer</td>
<td>CD68, CD31, TP, CD68 or Mő distinguished by morphological criteria 141 early stage I-II cases of NSCLC, treatment with surgery only</td>
<td>CD68+ in tumour islet correlates with good survival - CD68+ in tumour stroma correlates with poor survival</td>
<td>47</td>
</tr>
<tr>
<td>Good/no correlation</td>
<td>Nonsmall-cell lung cancer (NSCLC)</td>
<td>CD68, CD31, TP, CD68 or Mő distinguished by morphological criteria 141 early stage I-II cases of NSCLC, treatment with surgery only</td>
<td>High TP tumour cell reactivity but not mő infiltration showed correlation with vascularisation degree. High mő infiltration related to low lymph node metastases incidence, but no correlation to prognosis was seen</td>
<td>50</td>
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<tr>
<td>No-correlation</td>
<td>Ovarian cancer</td>
<td>CD68 &amp; CD31 19 benign and 37 borderline or malignant specimens</td>
<td>No differences in mő numbers between benign, borderline or malignant tumours. No differences in mő number between areas of high and average microvessel density. Overall total MVD did correlate with mő number</td>
<td>48</td>
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<tr>
<td>No-correlation</td>
<td>Cervical carcinoma</td>
<td>CD68 &amp; CD31/UEA-1 75 squamous cell carcinomas of the uterine cervix</td>
<td>Mő density correlated with microvessel count. Microvessel count correlated with tumour stage, but not with survival. No correlation found between mő number and any clinical parameters.</td>
<td>51</td>
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<tr>
<td>No-correlation</td>
<td>Melanoma</td>
<td>CD68, HLA-DR, S100, CD4/8 Cutaneous melanoma samples from 47 patients with stage I and II melanoma</td>
<td>Mő density did not correlate with survival. High density of CD8 or HLA-DR cells was independent factor favouring survival.</td>
<td>52</td>
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<tr>
<td>No-correlation</td>
<td>Lung cancer</td>
<td>CD68, CD3, S100, B- and NK-cells 710 cases of primary lung cancer</td>
<td>No correlation between overall immune cell infiltration and survival. No correlation between infiltration of mő and survival. High CD3 and S100 (DCs) density related to improved survival and high NK cell number showed a trend towards enhanced survival. High intratumoral CD68+ mő infiltration strongly associated with infiltration of both CD3 and NK-cells</td>
<td>53</td>
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<tr>
<td>No-correlation</td>
<td>Astrocytoma</td>
<td>mő &amp; CD8 Frozen samples from 92 malignant astrocytomas</td>
<td>No correlation between mő number and survival. Parenchymal CD8 infiltration showed a trend for improved survival</td>
<td>54</td>
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<tr>
<td>No-correlation</td>
<td>Diffuse large B-cell lymphoma (DLBCL)</td>
<td>CD68, Ki67 DLBCL tissues of 176 patients</td>
<td>No correlation between mő and Ki67. Fl mő in patients &gt; 60 years, No correlation fl mő and progression free survival orOverall Survival</td>
<td>55</td>
</tr>
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CD68: monocyte/macrophage marker; CD163: scavenger receptor mő; CD204: mő scavenger receptor 1; vWF: Von Willebrand Factor, glycoprotein on endothelial cells; CD31: endothelial cell marker; CD34: microvessel marker; Ki67: proliferation marker; HE: Hematoxylin Eosin; IL8: interleukin-8, marker for angiogenesis, PAS: Periodic acid Schiff stain, histological staining for e.g. proteoglycans in microvessels; MAC387: marker for reactive/infiltrating mő; Factor VIII: marker for microvessels; S100: DC marker; HLA-DR: DC marker; D2-40: marker for lymphatic endothelial cells; LMVD: lymphatic microvessel density; MVD: microvessel density; FOXP3: marker for regulatory T-cells, CD3: T-cell marker; CD4: T-helper cell marker; CD8: cytotoxic T-cell marker; TP: Thymidine phosphorylase, marker for angiogenesis; CCL2, macrophage chemotactic protein-1; PD-1: Programmed death-1 receptor, negative immune response regulator; Tryptase: mast cell marker; EGFR: Epithelial growth factor receptor; CD11c/CD1a: DC markers; TUNEL: apoptosis staining; MMP1/9/11: matrix metalloproteinases; uPA(R): urokinase plasminogen activator (receptor); UEA-1: Ulex europaeus-aggulutin-1: binds L-fucose containing residues on endothelial cells,
Tumour infiltrating macrophages and patient prognosis

Thus, the current consensus indicates that the extent of macrophage infiltration into the tumour is a prognostic factor for poor prognosis. However, the role of macrophages in disease progression is not as unambiguous as proposed, which is illustrated by several conflicting studies in which this correlation was not found (see Table 2 for details). Interestingly, most studies on tumours from patients with colon carcinoma showed that increased macrophage infiltration is associated with decreased disease recurrence, diminished metastasis and increased patient survival \[36-40, 55\]. Therefore, for patients with colon carcinoma, macrophage infiltration is an independent prognostic marker for survival \[36\].

The dichotomy in the correlation between tumour infiltrated macrophages and patient prognosis indicates that macrophages can exert different functions depending on the tissue, location of the body or micro-milieu in which they reside. A study with endometrial carcinomas supports this notion, as high numbers of tumour infiltrating macrophages in the stromal compartment or hypoxic areas of the tumour correlated with lymph node metastases and reduced relapse free survival, whereas macrophage presence in close proximity to tumour cell nests was associated with improved survival \[16\]. Caution therefore needs to be taken when interpreting macrophage stainings of tumours as location of macrophage in tumours seems important in how it influences patient survival. The assertion that macrophages can achieve such contrasting effects on patient survival indicates that these cells themselves can influence tumour development. The fact that presence of macrophages can either lead to enhanced or worsened progression of the malignancy in patients, indicates that macrophages can exert different functions in the tumour depending on the tumour origin. This led to the theory that different types of macrophages exist, which each have their own characteristics ranging from tumour cell killing to directly enhancing tumour cell growth.
Macrophage diversity

Macrophages are a major component of the innate immune system and part of the mononuclear phagocytic lineage, which arises in the bone marrow where they develop from pluripotent stem cells (PPSCs). Under the influence of cytokines like colony-stimulating factor 1 (CSF-1), PPSCs develop into monocytes, which enter the blood circulation. After approximately 18 hours in the circulation, monocytes migrate from the circulation and settle in their target tissue where they are instructed by the local microenvironment to differentiate into mature tissue macrophages. Each tissue contains its own locoregional-specific tissue macrophages like the Kupffer Cells in the liver, the alveolar macrophages of the lung or macrophage-like type-A cells of the synovial lining. After a noxious insult like an infection or local injury tissues are first quickly infiltrated by neutrophils followed by activation and transmigration of circulating monocytes into tissues, where they form part of the mononuclear cell infiltrate, which is characteristic for inflammation. Here they differentiate into activated macrophages exerting different roles in clearing inflammation and helping with tissue repair. Macrophages are a highly heterogeneous cell population with diverse functional and phenotypical characteristics. During the classical route of macrophage activation, monocytes are recruited to sites of infection where they encounter microbial products, such as the bacterial wall protein lipopolysaccharide (LPS). Due to the presence of bacterial products, in combination with inflammatory mediators like interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or interleukin-1β, monocytes differentiate into so called ‘classically activated’ macrophages. As IFN-γ is released by activated type-1 helper (Th1) T-cells, ‘classically activated’ macrophages are also referred to as M1 macrophages. M1 macrophage activation is characterized by high ROS production, including nitric oxide (NO), which together with superoxide forms the strong oxidizing agent peroxynitrite, which facilitates the killing of micro-organisms and other pathogens. Furthermore, M1 macrophages efficiently present antigens and stimulate Th1 adaptive immune responses. As activated M1 macrophages produce and secrete high amounts of pro-inflammatory cytokines like IL-12, TNF-α, IL-6 and IL-23 they are considered potent effector cells that induce an inflammatory environment, which facilitates the killing of both micro-organisms and tumour cells.

By contrast, stimulation with IL-4, either with or without additional stimuli like IL-10, IL-13, glucocorticoids or vitamin D3 leads to polarization towards an ‘alternatively activated’ phenotype. These macrophages are also referred to as M2, because Th2 cells are a major source of IL-4. Markers for M2 macrophages include increased mannose receptor and dectin-1 expression, as well as increased production of IL10 and the chemokines CCL17 and CCL22. Additionally, they have decreased production of pro-inflammatory cytokines, and poor antigen-presenting capability. Furthermore, these cells have lowered NO production due to increased arginase metabolism, subsequently leading to diminished cytotoxic activity towards tumour cells. M2 macrophages further promote activation of Th2 responses and through production of immune-suppressive mediators, including IL-10, prostaglandin E2 (PGE2) and transforming growth factor β (TGF-β), pro-inflammatory Th1 responses are dampened. By contrast, M2 macrophages produce growth factors such as basic fibroblast growth factor (bFGF) and pro-angiogenic factors like vascular endothelial growth factor (VEGF). As such, they play a role in tissue remodelling, tissue repair and wound healing, but are also implicated in fibrosis.
Importantly, it has lately become clear that the qualification of macrophage differentiation into either M1 or M2 macrophages is oversimplified, as subtleties of macrophage activation have been described that result in slightly different functional characteristics. As such, classification of macrophages should be regarded as a conceptual view of a wide range of diverse macrophage differentiation with M1 and M2 phenotypes as extremes in a continuum of diverse activational states.

Figure 1 Macrophage classification
Under the influence of IL4, IL10 and IL13 do monocytes differentiate into alternatively or M2 macrophages, whereas stimulation with LPS and IFNγ leads to classically activated or M1 macrophages. A strict qualification of macrophage differentiation into either M1 or M2 macrophages however is oversimplified. Many different subtleties of macrophage activation have been described resulting in macrophages with slightly different functional characteristics. The classic M1 and M2 macrophage phenotype must therefore be seen as the extremes in a continuum of diverse activational states, with many intermediary activational states in between.
Part I

Direct effects on tumour growth

Because M2 macrophages are important in processes of wound healing, tissue remodelling and down regulation of immune responses, they have also been described to directly influence tumour growth, progression and metastasis. In biopsies from human breast, endometrial and pulmonary tumours a clear association was observed between production of for instance epidermal growth factor (EGF)\(^{70}\) by tumour infiltrated macrophages and poor patient prognosis\(^{14, 71-73}\). In macrophages of breast carcinoma were shown to directly interact with isolated breast carcinoma cells \textit{ex vivo}, suggesting a paracrine growth regulation of the primary tumour cells\(^{74}\). Similarly, it was demonstrated that mouse macrophages, which are cultured in close contact with mouse lymphoma cells, directly regulated the growth of lymphoma cells\(^{75}\).

Because M2 macrophages have increased expression of arginase I\(^{62}\), L-arginine is converted into L-ornithine, which is important in biosynthesis of polyamides that are key factors required for (tumour) cell proliferation\(^{76}\). L-arginine is a substrate for nitric oxide synthase (iNOS), which converts L-arginine into L-citrulline leading to production of nitric oxide (NO). Depletion of L-arginine in tumours by over-expression of arginase I resulted in decreased iNOS activity and NO production and subsequent reduction of anti-tumoral activity of tumour infiltrating macrophages\(^{65}\).

It was furthermore demonstrated that depletion of tumour promoting macrophages diminished growth of tumours in an \textit{in vivo} model for buccal pouch carcinoma in hamsters\(^{77}\). Lewis lung carcinoma growth was impaired in CSF-1\(^{-/-}\) mice, which lack functional macrophages\(^{78}\). Treatment of CSF-1\(^{-/-}\) mice with recombinant human CSF-1 led to re-infiltration of tumours with macrophages and subsequent induction of tumour growth, supporting the tumour-promoting role of macrophages.

Angiogenesis

Without developing a vasculature through angiogenesis tumours cannot grow beyond a size of approximately 1-2 mm\(^3\), due to lack of nutrients and oxygen\(^{79}\). Tumour infiltrating macrophages participate in the process of angiogenesis by producing various pro-angiogenenic factors including VEGF\(^{80, 81}\), platelet derived growth factor\(^{82}\), bFGF\(^{67}\), IL-8\(^{15}\), thymidine phosphorylase (TP)\(^{83}\) and TGF\(\beta\)^\(^{84}\). Clinical data showed a high correlation between the number of tumour infiltrating macrophages, increased tumour vascularisation in the tumour and decreased survival in breast carcinoma\(^{11}\), melanoma\(^{18}\), glioma\(^{85}\), esophagus carcinoma\(^{19}\), bladder carcinoma\(^{13}\) and prostate carcinoma\(^{30}\) patients.

A direct effect of macrophages on tumour angiogenesis was furthermore demonstrated in mouse models. Mammary epithelial cell restricted expression of the polyoma middle T oncoprotein in the MMTV-PyMT mouse model results in spontaneous induction of breast carcinomas. Crossing of these mice with op/op mice that lack a functional copy of the CSF-1 gene, resulted in a 50% reduction in vascular density in mammary tumours\(^{86}\). Moreover, deletion of macrophages neither led to increased tumour growth nor to augmented tumour incidence, but macrophage-deficient mice had a delayed onset of malignant progression of tumours as well as an decrease in pulmonary metastasis\(^{87}\). Over-expression of either CSF-1 or VEGF in mammary epithelial cells of MMTV-PyMT mice resulted in increased macrophage infiltration into premalignant lesions and promoted an early onset of the angiogenic switch.
Role of macrophages in tumour growth and progression

Additionally, enhanced tumour vasculature, augmented tumour invasion and promotion of tumour progression to malignancy were observed. Enhanced tumour vasculature, augmented tumour invasion and promotion of tumour progression to malignancy were observed. Tumour infiltrating macrophages overexpress legumain, a member of the asparaginyl endopeptidase family. Removal of tumour infiltrating macrophages in syngeneic metastasis models for colon, breast and non-small cell lung cancer in mice, through a DNA vaccine, inducing an immune response against legumain, resulted in a dramatic reduction of tumour growth and metastasis. Decreased tumour development was correlated with a marked decline in pro-angiogenic factors like TGF-β, VEGF and matrix metalloproteinase-9 (MMP9) that were released by tumour infiltrating macrophages, and concomitant suppressed tumour angiogenesis. As other studies however indicate that besides macrophages, tumour cells themselves and also endothelial cells also can overexpress legumain, which is correlated with invasion and metastasis, caution needs to be taken in interpreting results of anti-legumain DNA vaccine treatment. In the K14-HPV16 epidermal squamous cell carcinoma mouse model it was furthermore demonstrated that tumour infiltrating macrophages produced MMP9, which degrades extracellular matrix (ECM) and renders angiogenic factors like VEGF more available to their receptors on endothelial cells. K14-HPV16 mice that were crossed with MMP9−/− mice exhibit delayed invasive carcinoma development and diminished angiogenesis, whereas adoptive transfer of wild type bone marrow - containing MMP9 expressing macrophage precursor cells - into tumour bearing K14-HPV16xMMP9−/− mice restored the malignant progression. Similar results were observed when cervical cancer-bearing mice were treated with an MMP9 inhibitor, which prevented its expression by tumour infiltrating macrophages. In this model MMP9 inhibition blocked the angiogenic switch, hereby impairing the transition of pre-invasive lesions to malignant tumours. Thus, infiltrating macrophages clearly play an important role in tumour vascularisation as inhibition their ligands, removal of angiogenic receptors on tumour infiltrating macrophages or blocking recruitment of tumour infiltrating macrophages reduces angiogenesis and decreases tumour size and malignancy.

Recently a new subset of tumour infiltrating macrophages was identified, expressing the angiopoietin receptor Tie2, which was previously thought to be restricted mainly to endothelial and hematopoietic stem cells. Tie2 expressing macrophages or TEMs have been shown to promote tumour angiogenesis in various murine tumour models and depletion of TEMs in e.g. mouse mammary tumours results in smaller and less vascularised tumours. Interestingly, depletion of TEMs in the tumour did not affect the total number of tumour infiltrated macrophages, indicating the presence of different macrophage subsets in tumours.

Macrophages and metastasis & invasion

Invasion into surrounding tissues and extravasation into the bloodstream, which subsequently leads to metastasis of tumour cells requires the controlled degradation of ECM components. It was shown that tumour infiltrating macrophages can produce different ECM-degrading proteases including MMP-1, -2, -7, -9, -12 and -19 together with urokinase-type plasminogen activator (uPA), plasmin, tissue type plasminogen activator (tPA) and uPA receptor (uPAR). ECM-degrading proteases are not only important for infiltration of endothelial cells into tissues and subsequent induction of
angiogenesis but also enable tumour cells to escape into the surrounding tissues and vasculature. Expression of proteases in tumours of human patients is correlated with tumour grade, invasion and poor prognosis in e.g. bladder, breast or ovarian cancer. Furthermore, MMP9 was upregulated in tumour infiltrated macrophages in a model for invasive epidermal cancer in mice, whereas transgenic mice carrying a homozygous disruption of the MMP-9 gene showed decreased incidence of invasive tumors. Breast carcinoma cells produce chemokines like CCL2 and CCL5 which attract monocytes into tumours and furthermore did co-culturing macrophages with mamma carcinoma cells or stimulation of macrophages with the chemokines CCL2 or CCL5 induces the release of MMPs, which supports tumour cell invasion in vitro. Macrophage derived TNFα was demonstrated to enhance expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and macrophage inhibitory factor (MIF) in ovarian and breast cancer cell lines, which led to further induction of MMP-2 and -9 expression in macrophages. Additionally, tumour infiltrating macrophages displayed increased expression of MMP7 in regions of tumour hypoxia in a mouse model for prostate cancer, which converted receptor-activator of nuclear-factor-κB (RANKL) - a potent pro-metastatic factor - into its active form. Activation of the Inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) by RANKL and subsequent activation of nuclear-factor-κB (NFκB) pathways, inhibits the metastasis suppressor Maspin and it was shown that the amount of active IKKα in mouse or human prostate cancer correlates with metastatic progression of tumours. The cysteine protease cathepsin B (CTSB) that is expressed by tumour cells and upregulated in tumour infiltrating macrophages, furthermore enhanced metastasis of mouse PyMT-mammary cancer towards the lung. Injection of CTSB+/PyMT-mammary cancer cells into CTSB−/− mice, without CTSB expressing macrophages, showed delayed tumour growth and diminished metastasis. Tumour infiltrating macrophages may also promote tumour cell invasion through the non-canonical signalling pathway via macrophage-derived Wnt5a, which acts via the JNK/c-jun pathway in human breast carcinoma cell lines, and induces invasiveness of tumour cells. Importantly, in an elegant intravital imaging mouse model for mamma carcinoma it was shown that extravasation and metastasis of tumour cells into the hematogenous or lymphatic circulation occurred more frequently when mammary tumour cells were in the vicinity of tumour infiltrated macrophages, strongly supporting the role of macrophages in tumour metastasis.

Macrophages and Immune suppression

Another mechanism how tumour infiltrated macrophages can promote tumour progression is via suppression of adaptive immune responses. Tumour macrophages modulate host immune responses by secreting cytokines, chemokines and enzymes that influence the function of antigen-presenting cells and B- and T-cells. One of the most well-known anti-inflammatory cytokines is IL10, which, produced by tumour associated macrophages, can prevent secretion of pro-inflammatory cytokine IL12 by neighbouring tumour infiltrated macrophages. As IL12 initiates T-cell and natural killer cell action against tumours, IL10 production by tumour infiltrating macrophages results in decreased tumour cytotoxicity. Signal transducer and activator of transcription molecule 3 (STAT3) is upregulated after IL10 stimulation of macrophages, whereas IL4 and IL13 (produced by Th2 cells) enhance
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STAT6, which leads to an alternative anti-inflammatory macrophage phenotype. Depletion of either STAT3 or STAT6 activity in myeloid cells in mouse tumours was shown to diminish numbers and activity of myeloid suppressor cells (MDC), leading to weakened suppression of CD8+ cytotoxic T-cells.

Ablation of STAT activity also resulted in increased M1 polarization of tumour infiltrating macrophages, with increased antigen presenting ability, amplified NO production, diminished arginase activity and improved cytotoxicity towards tumour cells, leading to delayed tumour growth in murine mammary carcinoma \(^{114,115}\). Anti-inflammatory cytokines influence macrophage function via the NFκB pathway as well. It was shown that anti-inflammatory macrophages had increased nuclear translocation of p50 NFκB inhibitory homodimers and a deficiency in their ability to form active NFκB P50/P65 heterodimers \(^{116}\). By deletion of the inhibitor of NFκB kinase subunit beta (IKKβ), IKKβ can not phosphorylate the IκB proteins, which subsequently are not marked for degradation via the ubiquitination route. This leaves NFκB in the inactive form furthermore leading to inhibition of NFκB activity and upregulation of the transcription factor STAT1. IKKβ deletion in tumour infiltrated macrophages subsequently resulted in increased IL12 and iNOS production and consecutive regression of advanced mouse ovarian carcinoma tumours \(^{117}\).

Depletion of the aminoacid l-arginine, which is important for normal proliferation and cytokine production of T-cells, from the local microenvironment leads to induction of T-cell apoptosis and subsequent inhibition of T-cell mediated immune responses. Upregulation of e.g. arginase-I in tumour infiltrating macrophages, via the STAT1 and 3 pathway, can lead to diminished l-arginine availability for infiltrated T-cells in the tumour \(^{118}\). Recently a new population of immunosuppressive monocytes, referred to as myeloid-derived suppressor cells (MDSCs), was described. Accumulation of MDSCs in different mouse models was shown to block CD4+ and CD8+ T-cell activation. Furthermore, increased IL10 and reduced IL-12 production by tumour infiltrated macrophages was observed, thereby promoting a type 2 anti-inflammatory response \(^{119}\).

It is however important to note that the majority of evidence on immune-regulation by tumour infiltrating macrophages has been generated in experimental mouse-models, which differ from the human situation. Unlike their mouse counterpart do human macrophages, not express arginase I. Additionally, T-cell immunosuppression by macrophages, which had been isolated from human ovarian cancer patient ascites, was mediated through IL10-induced expression of e.g. macrophage T-cell co-inhibitory molecules B7-H1 and B7-H4 \(^{120}\).

In human ovarian tumours the expression of B7-H4 was associated with regulatory T cell (Tregs) number in tumours and negatively predicted ovarian cancer patient survival \(^{120}\). Moreover, co-culture of macrophages with tumour derived Tregs enhanced IL10 and IL6 release by macrophages, which increased B7-H4 expression of macrophages in a autocrine manner \(^{120}\). Similarly was an increase in B7-H4 expressing macrophages found in peripheral blood of lung cancer patients, which may favour tumour progression \(^{121}\). Macrophages, isolated from human ovarian cancer ascites express CCL22, a Treg chemokine and ligand for Th2 T-cell associated CCR4 receptor \(^{122}\). By producing CCL22, tumour associated macrophages attract high numbers of Tregs, which subsequently blocked tumor-specific T cell immunity in vivo \(^{122}\). In ovarian cancer patients, increased numbers of Tregs \(^{122}\) are associated with decreased survival.
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So by influencing different parts of the host immune system, tumour associated macrophages can decrease specific anti-tumour immunity in many different ways, subsequently leading to enhanced tumour growth and a decrease in patient survival.

Macrophages and tumour inhibition
In addition to the well described tumour promoting properties are macrophages also capable of recognizing and killing tumour cells. Macrophages are a vital component of the innate immune system, and play an important role in scavenging and killing microorganisms, virus infected cells and other foreign debris. After phagocytosing, macrophages kill micro-organisms through lysosomal enzymes and by releasing large quantities of NO, and ROS. Furthermore, pro-inflammatory cytokines like IL-1, TNFo, IL-6 and IL-12 are produced. By presenting antigens on their MHCI molecules together with co-stimulatory CD40 receptors macrophages can stimulate CD4 Th1 T-cells. Macrophages can recognize tumour cells by their altered membrane composition, like increased phosphatidylserine molecules or altered carbohydrate structures on their surface. In vitro macrophages, which are activated with e.g. microbial substances, are able to induce tumour cell killing by producing large quantities of TNFα and NO. TNFα induces a macrophage-mediated release of IL-1, which has both cytotoxic and growth inhibitory effects on tumour cells. The toxic effects of NO include the inhibition of DNA-binding activity of zinc finger type transcription factors and destroying mitochondrial membrane potential leading to cell death.

In vivo evidence for an important cytotoxic role of macrophages was previously demonstrated in an animal model for peritoneal growth of colon carcinoma metastases in rats, in which all peritoneal macrophages had been deleted before inoculation with syngeneic tumour cells. Interestingly, macrophage-depleted tumours were better differentiated with decreased vascularisation and IL4 expression in the tumours, which supported a tumour-promoting role of tumour infiltrated M2 macrophages. Nonetheless, these rats had a significant increase in tumour nodule number and in tumour mass, which was correlated with a major decrease in survival. Similar results were obtained in a model for growth of colon carcinoma metastases in the liver, in which all Kupffer Cells (KC) – the resident macrophages of the liver - had been deleted. This indicates that both peritoneal macrophages and KC played a crucial role in arresting and killing free tumour cells, preventing metastasis formation. However, it was shown that inhibiting recruitment of new macrophages into colon carcinoma metastasis with flavonoids led to a significant increase in tumour load, supporting that also infiltrating macrophages into colon carcinoma are able to suppress tumour growth. By contrast, in a mouse model for mamma carcinoma influx of new macrophages by overexpression of CCL2 is associated with enhanced tumour progression. Infiltrating monocytes are therefore primed in the tumour micromilieu to exert either anti or pro-tumourigenic characteristics. Recently we demonstrated that tumour cells themselves secreted factors into their surroundings, which influence monocyte priming. Incubation of naive monocytes with colon carcinoma cell supernatants led to increased secretion of M1 associated cytokines IL12, IL6 and TNFo as well as enhanced production of reactive oxygen species. Incubation with mamma carcinoma cell supernatants led to opposite effects and enhanced secretion of tumour promoting M2 cytokines like IL10 and...
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IL8 (Bögels et al. OncoImmunology 2012, In Press).
Additionally, in animal experiments in which macrophage recruitment into tumours was inhibited by blocking antibodies against CCL2, tumour growth of human pancreatic or pharyngeal carcinomas in SCID mice was accelerated. High overexpression of CCL2 in a mouse melanoma model led to massive macrophage infiltration and destruction of the tumour. Low levels of CCL2 resulted in modest monocyte infiltration, which subsequently resulted in increased tumour formation. Thus, although macrophages can play important roles in anti-tumour defence there is a fine balance between the pro- and anti-tumour effects of macrophages during tumour development.
**CCL2/MCP-1**

The idea that the tumoricidal activity of macrophages can be used as target for immunotherapy against tumours already arose in the 1960s. Intraperitoneal injection with ascitis of tumour bearing mice into naïve tumour bearing mice led to influx and activation of peritoneal "large macrophages" and subsequent tumour cell destruction. This effect was initially attributed to cytotoxic substances in the ascitis fluid, which would result in tumour cell damage. Nowadays it has become clear that immune- and tumour cell derived chemokines in the ascites induced massive influx of macrophages leading to ablation of tumour growth. Chemokines, like CCL2, are small 8-11 kDa molecules, secreted to regulate migration of leukocytes like macrophages, NK cells and T-lymphocytes in response to inflammation and immune responses. The finding that overexpression of CCL2 by tumour cells increased influx of monocytes and macrophages into the tumour, led to different experiments interfering with this chemokine. Animal experiments aimed to enhance or block CCL2-induced macrophage infiltration into tumours however, gave conflicting results. Overexpression of CCL2 in e.g. mouse colon carcinoma cells, mouse fibroblasts in renal carcinoma tumours or in Chinese hamster ovary (CHO) cells suppressed tumour formation and growth and diminished the metastatic potential of tumours. Furthermore, CCL2 increased tumoricidal activity of LPS activated macrophages, resulting in killing of non-transfected tumour cells as well, in vitro. Similarly, vaccination of rats with irradiated tumour cells that had been transduced to express CCL2 had a protective effect against intradermal tumour challenge in a gliosarcoma model. This was presumably due to activation of attracted monocytes and macrophages, which were better capable of presenting tumour antigens. However in many human cancers and animal cancer models an increase in macrophage infiltration into the tumour is associated with worsened prognosis and survival. The ruling theory proposes that infiltrated monocytes will differentiate into alternative macrophages with increased pro-tumorigenic characteristics. Mice deficient for CCL2 were unable to mount Th2 responses, suggesting that CCL2 is involved in Th2 polarization, which is a characteristic of alternatively activated macrophages. One model overexpressing CCL2 in a mouse colon carcinoma model showed increased angiogenesis in the primary tumour and enhanced lung metastasis formation, whereas others showed regression of tumour growth and decreased metastasis in the same model. Overexpression of CCL2 in CHO cells led in one experiment to tumour suppression, whereas in another it had no effect on tumour formation and growth compared to wild type cells. An explanation for these conflicting results is probably found in the amount of CCL2 expressed in the tumour milieu. High levels of CCL2 lead to massive intra-tumoural influx of monocytes and macrophages, which then come into contact with the tumour cells, leading to tumour cell cytotoxicity and resulting in destruction of the tumour. Low levels of CCL2 however attract predominately alternatively activated macrophages into the vicinity of the tumours where they produce factors sustaining the tumour mass, leading to enhanced angiogenesis, tumour growth and metastasis formation. Daily intraperitoneal treatment with a neutralizing antibody directed against CCL2 almost completely inhibited tumour growth of low-level CCL2 secreting melanoma cells, whereas high-level secreting melanoma cells led to massive intra-tumoural macrophages infiltration and subsequent destruction of the tumours. Hence, caution should be taken in interfering with CCL2 mediated macrophage infiltration in patients because there is a delicate, concentration-dependent balance which determines the effect of CCL2; either leading to tumour progression or tumour destruction by infiltrating macrophages.

Therapeutic potential of macrophages
Macrophage activation

LPS and muramyl peptides
The finding that massive influx of macrophages can destroy tumours suggests that aspecific activation of macrophages may enhance tumoricidal activity of macrophages. Already in the 18th century physicians noticed that introducing an infection by injecting pus into the leg of a patient with inoperable breast cancer sometimes led to regression of the cancer. Starting in the 1970s studies showed that activation of macrophages with bacterial adjuvant like LPS, living BCG (Bacillus Calmette-Guérin (Mycobacterium bovis)) or other bacterial extracts led to non-specifically tumour cytotoxicity \textit{in vitro} 140. Nowadays immunotherapy with BCG is an approved and widely used treatment for patients with bladder carcinoma 141. Clinical application of BCG in treatment of patients with colorectal carcinomas is under investigation and until now is the most effective treatment for non-muscle invasive bladder cancer 142-144. Although the direct mode of action of BCG vaccination on tumour regression is not completely clarified, it is thought to involve a-specific activation of the immune response towards the tumours. Treating patients with BCG enhances production of macrophage derived pro-inflammatory cytokines such as TNFα, IL6 and IL12 and \textit{in vitro} stimulation of macrophages with BCG confirm these results 143.

The best known and one of the most potent activators of macrophages is LPS, originating from the outer membrane of gram-negative bacteria. The application of LPS in \textit{in vivo} mouse models and patient studies is however hindered by its toxicity and severe side effects, like hypotension, fever, disseminated blood clotting and lethal shock. Therefore, other microbial molecules, like muramyl peptides, which are a group of peptidoglycans derived from the cell wall of gram-positive bacteria have been investigated, as they have similar properties as LPS but less severe side effects. Experiments with liposome-encapsulated muramyl-tripeptide phosphatidyl-ethanolamine (MTP-PE) in rats led to increased tumoricidal activity of KC against tumour cells \textit{in vitro} 145. Moreover, injections with MTP-PE containing liposomes resulted in regression of human bladder carcinoma 146 in nude mice and prevented of development of liver metastases in a mouse model for melanoma 147. Phase II clinical trials, in which treatment of osteosarcoma patients who have pulmonary metastases with muramyl tripeptides or Mifamurtide demonstrated increased time to relapse in patients 148. As such, Mifamurtide is now an approved treatment of newly diagnosed osteosarcoma patients in combination with chemotherapy 149.

GcMAF
GcMAF or vitamin D-binding protein-derived macrophage-activating factor is a potent macrophage activating factor, which induces no side effects in humans. \textit{In vivo} GcMAF is produced in inflamed tissues in a process involving inflammation primed B and T cells. Serum vitamin D₃ binding protein (Gc protein) is converted by inflammation primed B and T-cells into GcMAF, which enhances phagocytic activity of mouse macrophages \textit{in vitro} 150. Tumour cells can produce different enzymes, which renders Gc protein inert to conversion into MAF, resulting in immunosuppression in cancerous tissues 151. \textit{Ex vivo} blocking of the tumour derived enzymes bypasses inactivation of GcMAF precursor and generates active GcMAF, which enhances phagocytic activity of mouse macrophages \textit{in vitro} 150. Treating a human pancreatic tumour model in immune compromised mice with increasing doses of mouse homologue of GcMAF (DBP-MAF) inhibited growth and caused tumour
regression with increased survival time. Treatment led to increased number of infiltrating macrophages and diminished microvessel density compared to untreated tumours. Similar results were obtained by treating Ehrlich Ascites tumour or human hepatocellular carcinoma (HCC) bearing mice with DBP-MAF. Although DBP-MAF therapy alone showed no effect on growth of SCCVII (squamous cell carcinoma) tumours in mice, it greatly enhanced efficiency of photodynamic therapy in combination therapy by reducing tumour induced immunosuppression. Immunotherapy with GcMAF of patients seem promising as GcMAF treatment of small cohorts of patients with prostate cancer, metastatic breast cancer or metastatic colorectal cancer leads to reduced cancer recurrence up to 7 years after treatment. GcMAF treatment leads to decreased serum Nagalase levels, which are proportional to tumour burden, indicating that treatment with activated GcMAF reduces tumour induced immune suppression. Although promising, GcMAF treatment of cancer patients needs to be confirmed in larger phase II/III multicenter trials to shows its effect on recurrence free and overall survival of patients. Besides directly infusing activated GcMAF into patients, future therapies may involve ex vivo stimulating autologous monocyte-derived macrophages with GcMAF and removal of Nagalase from serum to eradicate tumours (US patent: US 2011/0123591 A1).

IFN-γ
Another easy way of enhancing the cytotoxicity of macrophages toward tumorcells is by activation of macrophages by IFN-γ. Already in the 1970s it was shown that injection of mouse macrophages, which were firstly ex vivo activated by rat lymphocytes, led to suppression of pulmonary metastases in a B16 melanoma mouse model. Experiments using in vitro activation of rat Kupffer Cells (KC) with IFN-γ, showed that IFN-γ activated macrophages to become more cytotoxic against different syngeneic and xenogeneic colon carcinoma tumour cells. Injection of IFN-γ prior to injection of syngeneic colon carcinoma cells in a rat model for liver metastases did not have a complete curative effect but led to a significant reduction in metastases outgrowth in the liver. Injection of IFN-γ 3 days after injection of the tumourcells resulted in less tumour inhibition but still led to a small decrease in tumour burden compared to control animals. Similar results were shown when pre-treating a mouse model for colon carcinoma liver metastases with high doses of IFN-γ. Another elegant way to locally activate the cytotoxic capacity of macrophages is by transfecting macrophages ex vivo with genes encoding endogenous IFN-γ under a hypoxia induced promoter. Macrophages are known to migrate and accumulate in hypoxic areas of solid tumours, where they can promote tumour growth and suppress the anti-tumour immune-response. By using this local environmental hypoxia as a signal, engineered macrophages are able to upregulate IFN-γ production, which leads to increased production of NO and enhanced phagocytosis by these macrophages in vitro. The overall enhancement of anti-tumour activity of macrophages after IFN-γ activation led to the start of different clinical trials with mixed results. Early phase I trials showed that patients with advanced malignancies could tolerate high dose treatments with IFN-γ, but did show only limited clinical respons. IFN-γ treatment in a clinical trial to prevent tumour relapse following curative surgery in patients with primary colon cancer not
significant enhancement in overall patient survival. However an upregulation of HLA-DR and Fc receptor expression on peripheral blood monocytes indicated an enhancement of the immune response against the tumour \textsuperscript{165}. Similar upregulation in immune response was seen trial in metastatic malignant melanoma patients after IFN-γ treatment but the effects on patient survival are still unknown \textsuperscript{166}.

Nowadays IFN-γ treatment is still extensively studied in different clinical settings, for example combining the IFN-γ treatment with another immune regulatory cytokine like IL2 in a whole cell cancer vaccine strategy against prostate cancer. Treatment leads to an increased response against tumour antigens, however no data are yet available for long time survival in these patients \textsuperscript{167}. Another approach to treat malignancies is by \textit{ex vivo} activating autologous macrophages with IFN-γ, so called monocyte-derived activated killer cells (MAK). MAK cells show increased cytotoxicity against different tumour cell lines \textit{in vitro} and enhanced ADCC towards tumour cells in combination with specific anti-tumour antibodies \textsuperscript{168,169}. Treatment of small numbers of malignant pleural mesothelioma, superficial bladder cancer or ovarian cancer patients with MAK showed this treatment to be safe and somewhat promising preliminary results on tumour recurrence and \textit{in vivo} anti-tumoural activity were seen in treated patients \textsuperscript{170-172}. In a clinical phase II trial, MAK treatment together with anti-CD20 mAbs of 10 patients with chronic lymphocytic leukaemia showed enhanced eradication of minimal residual disease \textsuperscript{173}. Disappointing results however, were seen after treating non-muscle invasive bladder cancer patients with MAKs \textsuperscript{174}, indicating that even though treatment of malignancies with MAK is promising, further research to improve efficiency is needed. Although IFN-γ treatment alone or treatment with MAK does not seem adequate to improve patient survival yet, a combination of IFN-γ treatment of cancers together with current carcinostatics may be more promising. In a randomized phase III trial, subsequent IFN-γ treatment above normal carcinostatic treatment of ovarian cancer patients led to improved progression-free survival and improved overall survival over patients who received normal treatment only \textsuperscript{175}. Clinical data about the exact role macrophages play in this enhancement of cancer therapy are still scarce, but overall it seems that IFN-γ stimulation of macrophages is beneficial in treatment of some types of cancer, but further study is needed to improve its advantageous effect in combination therapies.
Tumour cells in solid tumours secrete different chemokines like CCL2, which attract monocytes into the tumour mass. By secreting different proteins do tumour cells themselves influence macrophage differentiation. Enhancing M2 characteristics of macrophages favours the formation of a pro-tumourigenic microenvironment. M2 macrophages secrete different growth factors and angiogenic factors which enhance both tumour growth and tumour vascularisation. By producing anti-inflammatory cytokines and enhancing the influx of regulatory T-cells (Tregs), M2 macrophages can inhibit a successful inflammatory response against the tumour. A high incidence of M2 macrophages in tumours therefore is associated with poor patient prognosis (for example in breast carcinoma patients). Inhibiting the influx of monocytes and subsequently inhibiting the formation of M2 macrophages in tumours is one possible therapeutic approach, which may be beneficial in, for example, breast carcinoma patients. In other tumours however, monocytes are differentiated into more M1 like macrophages. These macrophages can induce an inflammatory response by producing pro-inflammatory cytokines and activating both CD4 T-helper cells and Natural Killer (NK) cells. By producing high amounts of reactive oxygen species (ROS) and nitric oxide (NO), M1 macrophages can enhance tumour cell killing. Presence of high numbers of M1 macrophages, in for example colon carcinoma tumours, is therefore associated with a good patient prognosis. Contrary to breast carcinoma tumours, can enhancing monocyte infiltration into colon carcinoma tumours therefore lead to more M1 like macrophages and an enhanced anti-tumour response. Activating macrophages with for example different bacterial products like LPS or muramyl peptides, IFNy or GcMAF can both enhance the anti-tumourigenic characteristics of macrophages and inhibits its pro-tumourigenic activity.
In conclusion, macrophages are one of the most prominent leukocyte populations present within tumours and they profoundly influence tumour behavior. Depending on the microenvironment of the tumour, macrophages can exert both inhibitory and promoting effects on tumour growth and development. Tumour masses secrete factors, which recruit naïve monocytes into the malignant tumours, where they can differentiate into macrophages with a plethora of different functions and characteristics. Exactly how macrophages in tumours are educated to become either anti- or pro-tumourigenic is still unknown, however tumour cells themselves produce factors that change the micromilieu in which macrophages reside and can therefore directly influence macrophage polarization. Further knowledge on the synergistic relationship between tumour cells and macrophages is needed to unravel the mechanisms behind macrophage polarization. Because tumour macrophages are extremely important in tumour progression and patients’ survival, they may be ideal candidates for therapeutic strategies. Diminishing pro-tumour properties or re-educating macrophages to become anti-tumourigenic may be an elegant approach to reduce tumour development. Caution should however be urged when inhibition of macrophage influx into tumours is used as another therapeutic approach as tumour infiltrating macrophages can also have cytotoxic capabilities against tumour cells. Stimulation of the anti-tumour properties of macrophages by different microbial products or pro-inflammatory cytokines therefore seems to be the most elegant way to use macrophages as a therapeutic target against tumours. Extensive research is therefore needed to elucidate exactly how we can tip the scale and change or re-educate macrophages in the tumour to become anti-tumourigenic and improve patient survival.


References


Reference List


