CHAPTER 2

TUMOR INFILTRATING MACROPHAGES REDUCE DEVELOPMENT OF PERITONEAL COLORECTAL CARCINOMA METASTASES
Chapter 2

Tumour infiltrating macrophages reduce development of peritoneal colorectal carcinoma metastases

Abstract

Macrophages generally constitute a major component of tumor stroma, and possess either tumor growth promoting or inhibiting capabilities. Classically activated macrophages exert cytotoxicity and produce inflammatory cytokines, which limits tumor growth. By contrast, alternatively activated or M2 macrophages induce tumor progression by stimulating angiogenesis and proliferation. Previously we showed that resident macrophages control metastatic spread of colon carcinoma cells in liver and peritoneal tumor models. However, it is proposed that newly recruited macrophages develop into tumor-associated M2 macrophages, as they are exposed to a microenvironment that favors alternative activation. Previously we showed that monocyte migration was diminished after flavonoid treatment in an experimental autoimmune encephalomyelitis animal model. In the present study we investigated the role of newly recruited macrophages in colon carcinoma development, by using the flavonoids rutin and luteolin were used to reduce monocyte migration into peritoneal tumors. Increased tumor development was observed in animals that were treated with rutin and luteolin. Immunohistochemical analyses showed that the number of ED2+ resident macrophages was normal in tumors of animals that received rutin and luteolin treatment. However, the number of ED1+ cells (marker immature macrophages) was reduced, indicating decreased macrophage recruitment. Thus, inhibition of monocyte migration promotes tumor growth, supporting that not only resident, but also newly recruited macrophages limit peritoneal colon carcinoma metastases development.

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Introduction

Most tumors contain a large stromal compartment, which consists of fibroblasts, endothelial cells, immune cells and extracellular matrix (ECM). It is becoming increasingly clear that not only tumor cells themselves, but also stroma plays an important role in cancer development and progression. Within the tumor microenvironment mediators such as growth factors, pro- and anti-inflammatory cytokines as well as proteolytic enzymes are present that can either stimulate or inhibit metastases growth. For instance, immune reactions that have been evoked against the tumor can lead to a microenvironment in which tumor cells are killed and tumor growth is limited. In contrast, stromal cells like fibroblasts and endothelial cells frequently produce pro-angiogenic mediators, thereby assisting tumor development.

A large fraction of the stromal compartment is comprised of macrophages, which have a complicated dual role in tumor development. Traditionally, macrophages have been regarded as cells that produce pro-inflammatory mediators and exert cellular cytotoxicity. Macrophages with these characteristics are referred to as classically activated (or M1) macrophages and are commonly found during infections and inflammation. In the context of cancer, M1 macrophages inhibit tumor growth, as they can eradicate tumor cells and stimulate immune responses. Conversely, a different subset of macrophages, called alternatively activated (or M2) macrophages, is reported to contribute to tumor progression by producing pro-angiogenic and anti-inflammatory mediators, as well as growth factors and proteases. Macrophages frequently accumulate into malignant tissue since a large number of tumors produce mediators, such as colony-stimulating factor 1 (CSF-1 or macrophage-colony stimulating factor) and macrophage chemotactic protein 1 (MCP-1 or C-C chemokine ligand 2), which attract monocytes. Local alternative activation of macrophages may be induced by mediators like interleukin (IL) 4, IL-10 and IL-13, which are reported to be present in tumors. In line with these findings it has even been proposed that infiltrating monocytes are instructed by the local tumor environment to differentiate into alternative macrophages and as such assist tumor progression.

Conflicting reports exist on the exact role of macrophages in tumor progression. In various types of cancer, including breast, bladder and prostate carcinomas, macrophage presence is associated with unfavorable prognosis, supporting a tumor-promoting role for macrophages. However, macrophage infiltration in colorectal carcinomas seems to benefit patient outcome. Previously we showed in a rat model that tissue macrophages such as peritoneal macrophages and Kupffer cells protect against colorectal carcinoma (CRC) metastases outgrowth. Elimination of these macrophages before tumor inoculation led to significantly increased tumor load and shortened survival, hereby strongly supporting the clinical data that indicate that macrophages have anti-tumor properties in CRC. Paradoxically, tumors that developed in the absence of macrophages were morphologically much better differentiated than control tumors containing macrophages. The latter were undifferentiated with a prominent desmoplastic stroma reaction, and displayed hallmark characteristics of malignancy such as increased angiogenesis. These data suggested that subsets of macrophages influence tumor growth differently. Whereas tissue macrophages inhibited metastases outgrowth initially, newly recruited alternatively activated macrophages may stimulate new vessel formation and tumor progression.
Introduction

We previously showed that flavonoids inhibit monocyte migration in an experimental autoimmune encephalomyelitis model by interfering with reactive oxygen species (ROS) production and small GTPase activity \(^2\). To elucidate the role of macrophages in peritoneal CRC metastases, we used these plant derived polyphenolic compounds to inhibit monocyte migration into tumors. Additionally, liposome-encapsulated clodronate was used for elimination of peritoneal macrophages to study the effects of flavonoids in the absence of residential macrophages. In this way, we were able to investigate the role of tumor infiltrating macrophages in peritoneal CRC metastases development.
Materials and Methods

Antibodies
Monoclonal antibodies (mAbs) against ED1 (pan-monocyte/macrophage marker) and ED2 (mature tissue macrophages) were purchased at Serotec, Oxford, UK. As second antibodies Alexa488-labeled goat anti-mouse antibodies (Molecular Probes Inc., Eugene, OR) were used.

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

Cell culture
The colon carcinoma cell line CC531s is a well-characterized, moderately differentiated and weakly immunogenic syngeneic rat cell line. CC531s cells were cultured under standard incubator conditions in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Irvine, UK), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (200 mM) (further referred to as complete RPMI). Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution, and contained single tumor cells as well as small clusters (2-8 cells). Viability was assessed by trypan blue exclusion and routinely exceeded 95%.

Peritoneal macrophages were obtained by performing peritoneal lavages of sacrificed rats with 10 ml Hank’s balanced salt solution (HBSS). Cells were cultured overnight in complete RPMI with or without 100 ng/ml LPS (phenol extracted lipopolysaccharide from Escherichia coli 0111:B4, Sigma, St Louis, MO). Peritoneal macrophages were prepared for experiments by enzymatic detachment using trypsin-EDTA solution.

Monocyte migration
The capacity of monocytes to cross a monolayer of endothelial cells was determined using time-lapse video-microscopy, as described previously. Briefly, isolated rat monocytes were added to 96-wells plates containing endothelial cell monolayers. Monocyte migration was conducted for 4 hours in the presence of autologous serum of either control or flavonoid-treated rats. Subsequently, monocytes were monitored with an inverted phase-contrast microscope (Nikon Eclipse TE300) for every 10 minutes in randomly selected fields at 37°C with 5% CO₂. Tapes were analyzed by enumerating the number of monocytes that had migrated trough the monolayer.

Macrophage activity
ROS production of monocytes from control or flavonoid-treated animals was determined using the dihydorhodamine (DHR)-assay. DHR can react with ROS, including H₂O₂, superoxide and nitric oxide (NO), resulting in fluorescent DHR-123 (1 μM, BioChemika Fluka, Buchs, Switzerland), which was measured with flow cytometry (Becton Dickinson,
Materials and Methods

Mountain View, NJ). Control or phorbol myristate acetate (PMA)-stimulated (1 μg/ml overnight) peritoneal macrophages were incubated for 24 hours in the absence or presence of increasing concentrations of rutin and luteolin dissolved in dimethyl sulfoxide (DMSO), after which H₂O₂ production was measured with the Amplex™ Red Hydrogen Peroxide Assay Kit (Molecular Probes Inc, Eugene, OR) 24. Briefly, peritoneal macrophages were incubated with 100 μl hepes+ buffer (132 mM NaCl, 20mM Hapes, 6 mM KCl, 1 mM MgSO₄·7H₂O, 1.2 mM K₂HPO₄·3H₂O, 1 mM CaCl₂, 0.5% BSA, 1 mg/ml glucose) with or without (as negative control) 50 μl Amplex Red reaction mix (1x Hapes+ buffer containing 200 μM Amplex Red reagent and 4 μg/ml 12-myristate-13-acetate). Fluorescence of the produced resorufin was measured every 2 minutes for 1 hour at 37°C in a fluorimeter (Galaxy Fluorstar, BMG Labtechnologies, Offenburg, Germany) with an excitation of 550 nm and an emission of 590 nm. A standard curve of H₂O₂ in hepes+ buffer was used as standard measure. The Griess-assay was used for NO determination. In short, 100 μl supernatant was incubated for 5 minutes with 100 μl Griess reagentia. Optical densities were measured at 550 nm using a microplate reader (Ultramark Microplate Imaging System, Bio-Rad, Hercules, CA). Proliferation was evaluated using a MTT assay as previously described by Heuff et al 25.

Cytotoxicity

CC531s cells (5x10⁶ cells/ml) or peritoneal macrophages (5x10⁶ cells/ml) were incubated in complete RPMI containing 0.05 μg/ml 3,3′-dioctadecyloxacarbocyanine perchlorate (DIO) (Molecular Probes Inc., Eugene, OR) or 0.05 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich, St Louis, MO) respectively for 30 minutes at 37°C and subsequently washed with HBSS, according to the manufacturer’s instructions. After 24 hours of co-culture (E:T ratio 5:1), the amount of phagocytosed tumor cells was determined using a FACScan flow cytometer (BD).

Animal model

To induce peritoneal metastases, rats (n = 10 per group) were injected i.p. with 0.5 x 10⁶ CC531s cells at day 0. On day 3 to 14, rats received a daily oral gavage of either an isotonic NaCl solution containing 200 mg/kg rutin and 200 mg/kg luteolin (98% pure, Synorx, San Clemente, CA) or an isotonic NaCl solution alone. Animals were sacrificed on day 14 and number and diameter of tumors were scored. Tumors were snap-frozen in liquid nitrogen and stored at −80°C. The effect of rutin and luteolin treatment on tumor development in the absence of peritoneal and omental macrophages was examined by injection of rats at day −5 and −2 with 0.5 ml of liposome-encapsulated clodronate in order to deplete macrophages 26, 27.

High performance liquid chromatography (HPLC) assay

Luteolin concentrations in plasma (n=3 per group) were determined by HPLC analysis, as described previously.28 In brief, plasma was acidified with equal volumes of 0.01M oxalic acid. Acidified plasma samples were applied to Sep-Pak C₁₈ cartridges (Waters Coop, Milford, MA), washed with 0.01 M oxalic acid (6ml), methanol/H₂O/0.5 M oxalic acid (25:73:2 v/v, 6ml) and H₂O (6ml) and eluted with 4 ml methanol. After drying in a speedvac
Materials and Methods

concentrator (Savant instruments inc., Farmingdale, NY) the residue was dissolved in 100µl methanol. 5 µl of the samples and 0-20 µl (0-200 pmol) of standard concentrations of luteolin in methanol were analyzed by a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific, Waltham, MA) using a Nucleosil 100-5-C18 reverse phase column (5 µm 4mm x 250 mm, Agilent Technologies, Santa Clara, CA). The mobile phase consisted of the following: Solvent A: 1% acetic acid in distilled water; Solvent B: 100% acetonitril. The flow rate was held constant at 1000 µl/min. Quantification of luteolin was done by relating the peak areas to standard luteolin concentrations.

Immunohistochemistry
Cryostat tissue sections of 5 μm were fixed for 10 minutes in acetone and air-dried. Slides were incubated for 1 hour with primary mAbs (10 µg/ml) at room temperature in a humidified tissue chamber. After washing, visualization was achieved by incubating with goat anti-mouse-Alexa<sub>488</sub> antibodies (1:400). Nuclei were stained with Hoechst (10 µg/ml, Molecular Probes, OR). Sections were washed, mounted in vinol and examined with a Leica DM6000B fluorescence microscope (Leica Microsystems, Heidelberg, Germany). ED2<sup>+</sup> cells were counted in tumor nodules (8 animals per group). As large accumulations of ED1<sup>+</sup> cells were observed, ED1<sup>+</sup> areas per photo was determined in 5 photos from different tumor nodules by means of a constant predefined threshold for color components (8 animals per group). For both ED2<sup>+</sup> and ED1<sup>+</sup> cell analysis the digital image analysis program AnalySIS (Soft Imaging System GmbH, Münster, Germany) was used.

Statistical analysis
For comparisons between two groups the Mann-Whitney U test was used. Comparisons between multiple groups (>2) were performed with the Kruskal Wallis test and an additional Mann-Whitney U test. Normally distributed data was analyzed by performing an ANOVA and subsequently by analyzing contrasts (control vs. experimental groups). Statistical significance was accepted at p<0.05.
Results

Serum levels of luteolin
To investigate serum concentrations of luteolin after treatment, flavonoids were orally administered to rats and serum levels were determined by HPLC. First, binding kinetics of methanol-dissolved luteolin was analyzed, which showed that luteolin was released at 17.7 minutes (Fig 1a). After three daily oral gifts of flavonoids, concentration of luteolin in serum was determined by HPLC. The concentration of luteolin in serum was roughly 1.2 μM. In vitro addition of rutin to luteolin did not alter the peak pattern (data not shown). However, it has been reported that rutin enhances the uptake of luteolin in vivo. As such serum from animals that received both flavonoids was analyzed. Addition of rutin increased concentration of luteolin to 2μM (Fig. 1b). Monocytes from animals that received flavonoids showed a decreased ROS production compared to monocytes from control animals, as indicated by diminished fluorescence in a DHR assay. (Fig. 1c) Interestingly however, ROS production of peritoneal macrophages from these animals was not affected. (Fig. 1d). Additionally, monocytes from animals that received flavonoids showed decreased capacity to migrate over an endothelial monolayer. (Fig 1e.) These data indicate that serum flavonoid levels were sufficient to affect monocyte migration and ROS production.

![Figure 1](image_url)
Figure 1 Serum concentrations of luteolin after 3 consecutive daily oral gifts of flavonoids. (a) HPLC of different concentrations of luteolin in DMSO. (b) HPLC of serum from animals (n=3 per group) that received luteolin, rutin and luteolin or vehicle alone. DHR assay, which reflects ROS production, of monocytes (c) and peritoneal macrophages (d) from control animals or from animals that received flavonoids (c). ROS production of monocytes/peritoneal macrophages from control animals was set at 100%. (e). Migration of monocytes from animals that received either flavonoids or vehicle alone. Monocyte migration of control animals was set as 100%. Data are expressed as mean +/- SD and analyzed with the Mann-Whitney U test.
Results

Rutin and luteolin administration enhances peritoneal metastases development and reduces intratumoral macrophage recruitment

To investigate effect of flavonoid treatment on tumor development, rutin and luteolin was given daily starting 3 days after intraperitoneal injection of CC531s cells. Fourteen days after tumor inoculation all animals were sacrificed and number and size of tumors were scored. Animals that received flavonoids had increased total tumor load compared to control animals of respectively 176 +/- 11.1 versus 79 +/- 42.8 mm (Fig. 2a-c). Both number of tumor nodules (63 +/- 25.0 versus 111 +/- 13.6) and average tumor diameter (1.3 +/- 0.10 to 1.6 +/- 0.14 mm) were increased after rutin and luteolin administration (Fig. 2d-e). Next, the effects of flavonoids were determined after depletion of peritoneal macrophages by i.p. injection of clodronate liposomes. In all animals without peritoneal macrophages tumor load was increased. However, no differences in tumor load were observed between flavonoid-treated and untreated animals when peritoneal macrophages were depleted during the experiment (Fig. 2f). These data suggest that rutin and luteolin exert their effect through macrophages.

Figure 2 Peritoneal metastases outgrowth after flavonoid treatment.

Tumor outgrowth on omenta 14 days after injection of tumor cells in (a) control animals (n=9) and (b) animals that received daily rutin and luteolin starting 72 hours after tumor cell administration (n=7). (Arrows indicate CC531s tumor nodules) (c-e) Quantitative analysis of tumor load of control and experimental groups. (c) Tumor score, which is defined as sum of diameters of all tumors within one animal. (d) The amount of individual tumor nodules and (e) average tumor size. (f) Tumor score of macrophage-depleted animals that received either vehicle alone (n=8) or flavonoids (n=10). Data are analyzed with Kruskal Wallis test and/or with the Mann-Whitney U test.

Since rutin and luteolin decrease monocyte migration, influx of monocytes and macrophages into tumors was determined. Mature ED2⁺ macrophages were present mainly at the outer margin of tumor nodules. Flavonoid treatment did not result in altered numbers of...
Chapter 2 Tumour infiltrating macrophages reduce development of peritoneal colorectal carcinoma metastases

Results

ED2⁺ cells (Fig. 3a-c). Immature monocytes/macrophages were present throughout the tumor nodules, as indicated by ED1 staining. In contrast to ED2⁺ cells, the number of ED1⁺ macrophages was diminished upon administration of rutin and luteolin, indicating decreased monocyte/macrophage recruitment (Fig. 3d-f).

Figure 3 Effect of rutin and luteolin treatment on macrophage presence in peritoneal metastases. Immunofluorescence images of ED2⁺ macrophages in tumors from (a) control or (b) flavonoid treated groups. (c) Quantification of ED2⁺ mature macrophages in tumors. Presence of newly recruited macrophages in tumors from (d) control of (e) flavonoid treated animals was visualized by ED1 staining. (f) Quantification of ED1⁺ area in tumors (pictures from 5 separate tumor nodules per rat, 7 rats per group) by digital analysis using proAnalysis software. Data are analyzed with the Mann-Whitney U test.

Effect of rutin and luteolin on proliferation of CC531s cells

Flavonoids have also been reported to affect tumor cell growth and as such the effect of increasing concentrations of rutin and luteolin (0-50 μM) on CC531s cell proliferation was studied. Incubation with 50 and 25 μM concentrations of rutin and luteolin inhibited CC531s proliferation by respectively 35 +/- 3.87 and 70 +/- 2.14 %. Furthermore, co-culture with 10 or 5 μM flavonoids resulted in a small, but statistically different, decrease in proliferation. However, incubation with 5 2 μM flavonoids did not affect CC531s proliferation (Fig. 4).

Figure 4 Effect of rutin and luteolin on in vitro cellular proliferation. (a) Cellular proliferation of CC531s cells after 24 hours of incubation with increasing concentrations of rutin and luteolin. Percentage of proliferation compared to control cells is shown (* p < 0.001). Data are expressed as mean +/- SD and analyzed by performing an ANOVA and subsequently by analyzing contrasts (control vs. 2, 5, 10, 25 or 50 μM) Data is representative of tree separate experiments.

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Modification of NO and H$_2$O$_2$ production and cytotoxic potential of macrophages after incubation with rutin and luteolin

To study whether reduced macrophage activity underlies the increased outgrowth of peritoneal metastases, the effect of rutin and luteolin incubation on NO and H$_2$O$_2$ production by peritoneal macrophages was analyzed. Stimulation with 50 and 25 μM rutin and luteolin reverted NO production to the same level of that of unstimulated macrophages, whereas incubation with 10 μM concentrations led to a 24 +/- 2.94 % inhibition of NO production. Macrophage stimulation with 5 and 2 μM flavonoids did not affect NO production (Fig. 5a) Incubation with 50 and 25 μM rutin and luteolin reduced macrophage H$_2$O$_2$ production from 1522 +/- 356 mM to respectively 799 +/- 110 and 134 +/- 29 mM. However, no changes were observed after incubation with 10, 5 or 2 μM of flavonoids (Fig. 5b).

Next, the effect of rutin and luteolin on cytotoxic properties of peritoneal macrophages was investigated. 90% of CC531s cells were phagocytosed by macrophages that were not stimulated by flavonoids. Tumor cell phagocytosis by macrophages was reduced to respectively 52 +/- 3.79 and 22 +/- 2.53 % after incubation with 50 and 25 μM flavonoids. In addition, in the presence of 10 or 5 μM rutin and luteolin phagocytosis was slightly decreased, but phagocytosis was not affected after incubation with 2 μM flavonoids (Fig. 6).

Figure 5 ROS production of peritoneal macrophages after 24-hour incubation with rutin and luteolin. (a) NO production of unstimulated (grey bars) or LPS-stimulated (black bars) peritoneal macrophages after incubation with increasing concentrations of rutin and luteolin. NO production of stimulated macrophages without incubation with flavonoids was set at 100%. (b) Effect of rutin and luteolin on H$_2$O$_2$ production by PMA stimulated (black bars) and unstimulated (grey bars) macrophages (* p < 0.001). Data are expressed as mean +/- SD and analyzed by performing an ANOVA and subsequently by analyzing contrasts (control vs. 2, 5, 10, 25 or 50 μM) Data is representative of tree separate experiments.

Figure 6 Effect of rutin and luteolin on cytotoxicity of peritoneal macrophages. LPS-stimulated macrophages were labeled with DiI and co-cultured with DiO-labeled CC531s cells in the presence of different concentrations of flavonoids. After 24 hours percentages of phagocytosed cells (double positive cells) were determined (* p < 0.05). Data are expressed as mean +/- SD and analyzed by performing an ANOVA and subsequently by analyzing contrasts (control vs. 2, 5, 10, 25 or 50 μM) Data is representative of tree separate experiments.
Discussion

Both tumor-promoting and tumor-inhibiting roles have been attributed to macrophages, which has made their influence on tumor development subject of debate. Macrophage-mediated tumor cytotoxicity and induction of adaptive immune responses may limit tumor growth, whereas induction of angiogenesis and cell proliferation may stimulate cancer progression. At present, mostly tumor-promoting roles are attributed to macrophages. As macrophage function is strongly influenced by the microenvironment, it is postulated that factors produced within tumors (e.g. IL-4, IL-10) favor differentiation of tumor-promoting macrophages. We previously showed in an animal model that macrophage presence in CRC metastases was correlated with poor differentiation, as indicated by irregular basement membranes, a substantial desmoplastic stroma reaction and increased angiogenesis, which supported a tumor promoting role of macrophages. However, removal of macrophages prior to tumor cell injection led to increased tumor load and shortened survival, implicating that cytotoxic capacities of macrophages were predominant. Immunohistochemical analyses demonstrated the presence of two macrophage populations, namely ED2+ tissue macrophages at the border of tumor nodules and newly recruited ED1+ macrophages that were scattered throughout the tumors. This suggested that residential macrophages are classically activated (M1) and aim to contain tumor growth, whereas newly recruited macrophages are alternatively activated (M2) and may produce (angiogenic) factors that stimulate tumor progression. In order to test this hypothesis, we studied peritoneal C57Bl6 development in animals that were treated with flavonoids, which led to reduced monocyte/macrophage migration. Impairment of macrophage migration by oral administration of rutin and luteolin resulted in a 2-fold increase in metastases development after 14 days. Immunohistochemical analysis revealed no difference in the number of ED2+ cells, but a strongly reduced presence of newly recruited ED1+ macrophages in tumors from flavonoid treated groups was observed. This suggests that newly recruited macrophages, like residential macrophages, play an important role in reducing outgrowth of peritoneal CRC metastases.

However, rutin and luteolin treatment might affect metastases growth in other ways as well. Various flavonoids are able to reduce proliferation of tumor cells in vitro, and in accordance with this we found that incubation with high concentrations of rutin and luteolin inhibited C57Bl6 cell proliferation in vitro. However, as we observed increased tumor growth after in vivo flavonoid administration, induction of tumor cell death is unlikely to occur in our model. This can be explained by the low in vivo concentration of luteolin after oral administration (2μM), which is not sufficient to affect tumor cell proliferation in vitro. In addition, no difference in tumor development was observed between rutin/ luteolin treated or untreated animals if macrophages had been depleted, suggesting that flavonoids exert their effect through macrophages.

In addition to reducing monocyte migration, flavonoids are able to decrease the activation of macrophages and monocytes. It has been reported that flavonoids can reduce ROS production. Additionally, luteolin is known to inhibit nuclear factor kappa B signaling and cyclooxygenase-2 expression. Although very little is known about rutin and luteolin metabolism, reports exist showing that biotransformation of flavonoids gives rise to metabolites that have effects on the immune system in vivo. However, this seems not
Discussion

to be the case for luteolin, as metabolites of luteolin are reported to be less biologically active than untransformed luteolin. The anti-inflammatory effects of flavonoids may hamper both innate and adaptive immune responses and thereby facilitate tumor growth. For instance, residential peritoneal macrophages protect against tumor outgrowth by eradicating disseminated tumor cells, which takes place shortly after injection of tumor cells. In vitro ROS production and tumor cell phagocytosis was affected after incubation with high concentrations of flavonoids. However, incubation with concentrations up to 10 μM flavonoids did not alter macrophage function. Since the in vivo concentration of flavonoids was 2μM, effects of flavonoid treatment on peritoneal macrophage function seem unlikely in our model. This was supported by the observation that ROS activity of peritoneal macrophages from rats that received flavonoids was unaltered. Furthermore, to fully exclude effects on residential macrophages in the first phase of metastases outgrowth, treatment of rutin and luteolin was started 3 days after injection of tumor cells at which time point no free-floating tumor cells are present anymore in the peritoneal cavity.

Taken together, our data strongly suggest that rutin and luteolin exert their effect by diminishing macrophage influx into tumors. More importantly, we demonstrate here that not only residential macrophages, but also newly recruited macrophages play an important role in reducing CRC metastases development. Since clinical studies showed that macrophage presence in primary CRC is correlated with better patient outcome, recruitment of monocytes and macrophage maturation in primary CRC is likely beneficial as well. However, as in other malignancies like breast and prostate cancer macrophage presence is linked to poor prognosis, our findings may specifically apply to CRC. It has previously been suggested that prevention of macrophage influx into tumors might increase patient survival. While possibly beneficial for some types of cancer (e.g. breast cancer, in which high macrophage presence is correlated with poor patient outcome), our data indicates that inhibition of macrophage influx into CRC metastases should be avoided. Rather, enhancing presence of classically activated (M1) macrophages by administration of mediators like granulocyte macrophage-colony stimulating factor or interferon-γ should be explored for therapeutic potential.
Chapter 2 Tumour infiltrating macrophages reduce development of peritoneal colorectal carcinoma metastases