CARCINOMA ORIGIN DICTATES DIFFERENTIAL SKEWING OF MONOCYTE FUNCTION

CHAPTER 3
Abstract

Macrophages are versatile cells, which phenotype is profoundly influenced by their environment. Pro-inflammatory classically activated or M1 macrophages, and anti-inflammatory alternatively-activated or M2 macrophages represent two extremes of a continuum of functional states. Consequently, macrophages that are present in tumours can exert tumour-promoting and tumour-suppressing activity, depending on the tumour milieu. In this study we investigated how human monocytes – the precursors of macrophages – are influenced by carcinoma cells of different origin. We demonstrate that monocytes, stimulated with breast cancer supernatant, showed increased expression of interleukin (IL)-10, IL-8, and chemokines CCL17 and CCL22, which are associated with an alternatively-activated phenotype. By contrast, monocytes that were cultured in supernatants of colon cancer cells produced more pro-inflammatory cytokines (e.g. IL-12 and TNF-α) and reactive oxygen species. Secretome analysis revealed differential secretion of proteins by colon and breast cancer cell lines, of which the proteoglycan versican was exclusively secreted by colon carcinoma cell lines. Reducing active versican by blocking with monoclonal antibodies or shRNA diminished pro-inflammatory cytokine production by monocytes. Thus, colon carcinoma cells polarize monocytes towards a more classically-activated anti-tumourigenic phenotype, whereas breast carcinomas predispose monocytes towards an alternatively activated phenotype. Interestingly, presence of macrophages in breast or colon carcinomas correlates with poor or good prognosis in patients, respectively. The observed discrepancy in macrophage activation by either colon or breast carcinoma cells may therefore explain the dichotomy between patient prognosis and macrophage presence in these different tumours. Designing new therapies, directing development of monocytes towards M1 activated tumour macrophages in cancer patients, may have great clinical benefits.
Solid tumours not only consist of malignant cells, but also contain non-malignant stromal cells like fibroblasts and endothelial cells as well as a variety of haematopoietic immune cells. Over the years evidence has accumulated from clinical and experimental studies supporting that tumour behaviour is strongly influenced by the infiltrating immune cell populations.\(^1\) Especially tumour associated macrophages (TAM) comprise a large fraction of the immune infiltrate in tumours, and are thought to play a major role in tumour development.

TAMs originate from monocytes, which enter the tumour via the vasculature, and develop into mature macrophages in the tumour tissue. Macrophages are, however, versatile cells, which phenotype is profoundly influenced by their environment.\(^2,3\) In the presence of microbial products like lipopolysaccharide (LPS) or pro-inflammatory cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)), monocytes develop into inflammatory macrophages that produce high amounts of nitric oxide, reactive oxygen species (ROS), interleukin (IL)-12 and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)). This type of macrophage, referred to as classically activated or M1, has cytotoxic ability, and is able to induce Th1 adaptive immune responses.\(^4\) Alternatively-activated or M2 macrophages have completely different functions that include production of growth and angiogenic factors, as well as release of metalloproteases (MMP) to promote tissue remodelling and wound healing. M2 macrophages can dampen inflammatory responses\(^5\) and are involved in immunoregulation and Th2 responses. Importantly, it has become clear that the distinction between M1 or M2 macrophages is oversimplified, as subtleties of macrophage activation have been described that result in different functional characteristics. As such, classification of macrophages should be regarded as a conceptual view of a wide range of diverse macrophage subtypes with M1 and M2 phenotypes as extremes in a continuum of various activational states.

Nonetheless, as macrophages constitute a major component of the inflammatory infiltrate of many tumours they can profoundly influence tumour development, depending on their phenotype.\(^4,6,7\) Monocytes are recruited into the tumour via a number of chemokines, including colony-stimulating factor-1 (CSF-1 or M-CSF), CCL2 (MCP-1), CCL3 or CCL4.\(^6,8-10\) Once infiltrated, monocytes differentiate into mature tumour macrophages where they can play a complicated dual role in tumour development. Pro-inflammatory classically activated or M1 macrophages have cytotoxic properties, and secrete TNF-\(\alpha\), NO and ROS\(^2,3\), whereas M2 macrophages can promote tumour cell growth and invasion by secreting growth factors, cytokines, angiogenic factors and MMP.\(^9\) The evidence that macrophages are influenced by tumour cells, and subsequently contribute to tumour behaviour - hereby significantly affecting clinical prognosis of cancer patients - is increasing.\(^11\) It is nowadays well established that in patients with e.g. breast, endometrial, or bladder cancer, presence of macrophages in or around the tumour is associated with disease progression and poor prognosis.\(^5\) TAMs that are isolated from these tumours generally have an alternatively activated M2 phenotype.\(^6\) Interestingly, increased macrophage presence in tumours of patients with colon carcinoma was shown to correlate with improved prognosis.\(^12\) In agreement with this finding, we previously demonstrated that macrophage-depleted rats had increased colon carcinoma metastases development and poorer survival\(^13\), indicating a crucial role for macrophages in clearance of colon
cancer cells. By contrast, breast carcinoma metastases development was decreased in macrophage deficient mice, which supported that macrophages were involved in tumour progression.\textsuperscript{14}

In this study we investigated why macrophages in breast carcinoma may behave differently from macrophages in colon carcinoma. We demonstrate that colon and breast cancer cells themselves influence monocyte skewing, which subsequently may predispose macrophage phenotype. This supports that malignant cells, by changing their micro-milieu, can directly manipulate macrophage behaviour.
Results

Monocytes are recruited towards carcinoma supernatants

It has previously been proposed that tumour cells themselves can recruit monocytes from the blood circulation into the tumour where monocytes differentiate into TAM. To investigate whether the ability to recruit monocytes differs between colon and breast cancer cells, a chemotactic assay was performed. Supernatants of colon and breast carcinoma cells were loaded into the bottom wells of blind well chemotaxis chambers, and recruitment of human peripheral blood monocytes, loaded into upper wells, was quantified by counting migrated cells. Supernatant of the breast carcinoma cell-line SKBR3 showed high chemotactic potential, which was comparable to the positive controls (containing either MCP-1 or fMLP) (Figure 1A). However, supernatants of either other breast carcinoma showed minimally enhanced chemotactic potential. Additionally, of 5 different colon carcinoma cells tested, two colon carcinoma supernatants (HCT116 & SW948) showed amplified monocyte recruitment, two supernatants (RKO and HT29) induced minimal enhanced chemotaxis and SW620 did not contain chemotactic stimuli (Figure 1A). Thus, overall, no significant difference in monocyte recruitment towards either breast or colon carcinoma supernatant was observed.

Figure 1 Monocyte migration towards carcinoma supernatants and monocyte activity

A, Migration of monocytes towards different breast (dark grey) or colon (black) carcinoma supernatants. fMLP and MCP-1 were used as positive controls (white), whereas DMEM (light grey) served as negative control. B+C, Monocytes were pre-incubated with DMEM (control), breast or colon carcinoma supernatant for B) 24 h or C) 96h and activity and viability was measured after additional stimulation with DMEM (control), LPS or LPS/IFN-γ. D, Monocytes were pre-incubated with DMEM (control), breast or colon carcinoma supernatant and H2O2 production in time after PMA stimulation was measured. ***p<0.001. Experiments were repeated three times.
Monocyte activity and viability do not differ after incubation with colon or breast carcinoma supernatants

To study whether incubation with colon or breast carcinoma cell supernatants influenced monocyte metabolic activity and viability, an MTT assay was performed. Incubation with carcinoma cell supernatants for 24 hours showed increased formazan production by monocytes compared to incubation with complete medium (negative control), indicating an increased activity of monocytes due to carcinoma cell supernatant incubation (Figure 1B). Monocyte activity was further increased after stimulation with either LPS or a combination of LPS and IFN-γ. However, no overall differences in activity were observed between incubation of monocytes with either breast or colon carcinoma cell supernatant. Similar results were observed when monocytes where incubated with carcinoma supernatant for 96 hours (Figure 1C). Monocytes that had been cultured with supernatant of either breast or colon cancer cells were viable, had adhered and were spread (indicative of development into macrophages) (data not shown). However, no overall difference was observed between cells that had been grown in supernatant of either breast or colon carcinoma supernatants. Less monocytes were observed after 96 hours when they had been grown in culture medium alone (without carcinoma supernatant). Moreover, remaining cells were round (Figure 1C, and data not shown). Thus, carcinoma cells produced growth factors, supporting differentiation into macrophages. As such, it was investigated whether breast or colon cancer cell lines produced the prototypic macrophage growth factor CSF-1 or granulocyte/macrophage-colony stimulating factor (GM-CSF). However, minimal production of either growth factor was present in carcinoma cells as shown by mRNA as well as secretome analyses. Moreover, no difference was observed between breast and colon cancer cell lines (data not shown).

Induction of \( \text{H}_2\text{O}_2 \) production by carcinoma supernatants

As neither monocyte chemotaxis nor activation and viability were different after incubation with colon versus breast carcinoma supernatants, we next investigated whether carcinomas would induce distinct functional phenotypes. ROS production is one of the key features of classically activated M1 macrophages. As such, \( \text{H}_2\text{O}_2 \) production by monocytes was studied after incubation with supernatants of either breast or colon carcinoma and PMA stimulation, which is generally used to stimulate ROS production.\(^{15, 16}\) Control monocytes, which were incubated with complete DMEM medium showed an average \( \text{H}_2\text{O}_2 \) production of 40.2 ± 2.7 nmol/min after PMA stimulation (Figure 1C). PMA stimulation of monocytes that had been incubated with supernatants of colon carcinoma cells led to a significant increase in \( \text{H}_2\text{O}_2 \) production (average \( \text{H}_2\text{O}_2 \) production of 54.7 ± 12.17 nmol/min) compared to control monocytes. By contrast, monocytes, which were first incubated with breast carcinoma supernatants had a lower average \( \text{H}_2\text{O}_2 \) production of 20.5 ± 13.8 nmol/min) (p<0.001) (Figure 2). Incubation of monocytes with supernatants of 2 out of 3 breast carcinoma cells (SKBR3 and MCF-7) led to an \( \text{H}_2\text{O}_2 \) production lower than control monocytes, whereas incubation with ZR-75-1 supernatant resulted in \( \text{H}_2\text{O}_2 \) production similar to control cells (Figure 1C).
Tumour cell supernatants alter monocyte cytokine production

Cytokine profiles represent major characteristics of distinct macrophage functional phenotypes as well, and were investigated next. Human peripheral blood monocytes were first stimulated with supernatants of different colon or breast carcinoma cell lines. After 24 h, monocytes were stimulated with LPS alone or a combination of LPS/IFN-γ for 24 h. Changes in proteins levels of different M1 and M2 cytokines were measured in monocyte supernatants. No IL-6, IL-12p40, IL-10, IL-8 or TNF-α production was observed in either colon or breast carcinoma supernatants (data not shown). Without LPS or LPS/IFN-γ stimulation, monocytes produced only very low levels of cytokines, irrespectively of prior carcinoma cell supernatant incubation (Figure 2A-C). Furthermore, stimulation of control monocytes with LPS or LPS/IFN-g (incubated in medium alone) increased cytokine production only marginally. However, a 24 h pre-incubation of monocytes with supernatants of different colon or breast carcinoma cell lines, led to a subsequent increase of 2 to 250 fold in IL-6, IL-12p40 and TNF-α production after LPS stimulation (Figure 2A-C). Importantly, pre-incubation with colon carcinoma supernatant resulted in higher production of IL-6, IL-12, and TNF-α, compared to pre-incubation with breast carcinoma supernatant. Stimulation of monocytes with LPS/IFN-γ after pre-incubation with either colon or breast supernatant led to an even further increase in cytokine production, which was most pronounced when monocytes had been pre-incubated with colon carcinoma supernatant. Thus, all individual colon carcinoma cell lines consistently secreted factors that stimulated higher production of pro-inflammatory cytokines by monocytes, compared to all individual breast cancer cell lines.

As such, further experiments were performed with mixtures of either all colon carcinoma supernatants or all breast carcinoma cell lines as a model system. First, IL-6, IL-12p40, or TNF-α was measured to check whether mixing of the carcinoma cell supernatants would indeed result in comparable data. Similar to pre-incubation with individual cell lines, we observed a significant increase in TNF-α, IL-6 and IL-12p40 production after pre-incubation of monocytes with a mixture of supernatants of colon carcinoma cells and LPS or LPS/IFN-γ stimulation compared to monocytes, which were pre-incubated with a mixture of breast carcinoma supernatants (Figure 2D-F). Next, production of the M2 macrophage cytokine IL-10 and the angiogenic cytokine IL-8 were studied. Incubation with breast carcinoma supernatants led to a minor production of the anti-inflammatory cytokine IL-10 (5.3 ± 0.6 pg/ml), compared to monocytes pre-incubated with control medium (2.5 ± 0.2 pg/ml) or colon carcinoma cell supernatant without LPS or LPS/IFN-γ stimulation (2.7 ± 0.3 pg/ml) (Figure 2G). Stimulation with LPS or LPS/IFN-γ led to an increase in IL-10 production in all monocytes (Figure 2G). However, pre-incubation of monocytes with breast carcinoma cell supernatant resulted in enhanced IL-10 production (62.8 ± 6.1 pg/ml), compared to incubation with control medium alone (39.9 ± 2.1 pg/ml), which was not observed when monocytes had been pre-incubated with colon carcinoma supernatant (43.7 ± 0.9 pg/ml (Figure 2G). Pre-incubation with breast carcinoma cell supernatant led overall to an increase in IL-8 production by monocytes as well, compared to incubation with control medium or colon carcinoma cell supernatant, albeit less pronounced than IL-10 production (Figure 2H).
**Results**

Incubation with tumour supernatants alters monocyte gene profile

To further investigate M1 or M2 phenotype, we performed real-time RT-PCR analyses of gene expression of monocytes that had been stimulated with carcinoma supernatant. Monocytes were challenged for 4 hour with either LPS or LPS/IFN-γ. mRNA expression of the pro-inflammatory cytokine genes IL-6, IL-12p40, IL-12p35 and TNF-α was markedly decreased...
in monocytes pre-incubated with breast carcinoma supernatant and stimulated with LPS/IFN-γ (Figure 3A-D). Additionally, pre-incubation with colon carcinoma supernatant resulted in an increased gene expression of the chemokine ligand CXCL13 (Figure 3E), which is upregulated during inflammation and associated with classically activated macrophages.18 By contrast, gene expression profiles of the anti-inflammatory cytokine IL-10 and the pro-angiogenic cytokine IL-8 were reduced in monocytes that had been pre-incubated with colon carcinoma supernatant (Figure 3F-G).

Figure 3 Monocyte mRNA expression is differentially altered after incubation with carcinoma supernatants. Monocytes were pre-incubated with DMEM (white bars) or mixtures of breast (grey bars) or colon (black bars) carcinoma cell supernatants for 24 hours. mRNA levels of A) IL-6, B) IL-12p40, C) IL-12p35, D) TNF-α, E) CXCL13, F) IL-10, G) IL-8, H) CCL17, I) CCL22, J) MR1 or K) CCL2 were measured after stimulation with DMEM (control), LPS or LPS/IFN-γ, and correlated to mRNA GAPDH expression levels. *p<0.05, **p<0.01. The experiment was repeated three times with similar results.

Additionally, expression of the M2- associated chemokines CCL17 and CCL22 was higher in monocytes that had been pre-incubated with breast carcinoma supernatant, compared to monocytes which had been stimulated with colon carcinoma cell supernatant (Figure 3H-I). Monocytes cultured with breast carcinoma cell supernatant also showed increased mannose receptor 1 (MR1) mRNA expression (Figure 3J), which is a distinctive marker for IL-4 activated alternative macrophages.19 mRNA expression of the chemokine CCL2 (MCP-1) was not different between monocytes incubated with either colon or breast carcinoma cell supernatants (Figure 3K), which is in agreement with our finding that there is no difference
in monocyte recruitment towards either colon or breast carcinoma cell supernatants (Figure 1A). Thus taken together, incubation with colon carcinoma cell supernatants overall favoured production of M1-associated factors, whereas pre-incubation with breast carcinoma supernatants resulted in production of factors that are more correlated with an alternative-activated phenotype of macrophages.

Role of Versican in directing monocyte phenotype
To investigate which factors were secreted by either colon or breast carcinoma cells that could influence macrophage phenotype, protein contents of carcinoma secretomes were analyzed by in-depth proteomics (GeLC/MS/MS). Extensive analyses of differential protein expression, confirmation by RT-PCR and an extended literature search on relevance in macrophage biology, identified the proteoglycan versican (VCAN) as candidate target molecule. VCAN mRNA was expressed in all colon carcinoma cell lines, whereas no expression was seen in breast carcinoma cells (Figure 4A).

Figure 4 Blocking VCAN in colon carcinoma supernatants decreases pro-inflammatory cytokine production in monocytes
A) VCAN mRNA expression of and B) VCAN protein secretion by different colon or breast carcinoma cell lines. C-E) Monocytes were pre-incubated with mixture of colon carcinoma cell supernatants together with 10 µg/ml blocking antibodies against VCAN (grey bars) or isotype IgGs (black bars) for 24 hours. Production of C) IL-6, D) IL-12p40, or E) TNF-α was measured after stimulation with LPS or LPS/IFN-γ for 24h. *=p<0.05. The experiment was repeated three times with similar results.
Results

Furthermore, VCAN protein secretion was confirmed by ELISA in supernatants of all colon carcinoma cell lines. Because both mRNA and VCAN protein were absent in breast carcinoma cells and supernatants (Figure 4A/B), further experiments to investigate whether secreted VCAN influenced macrophage phenotype were performed with colon carcinoma cell lines. First, VCAN in conditioned medium of colon carcinoma cells was blocked with anti-VCAN mAb, which reduced IL-6, IL-12 and TNF-α production by monocytes after stimulation with LPS/IFN-γ (Figure 4C-E). Incubation with colon carcinoma supernatant in which an isotype control mAb had been added, did not influence cytokine production. Second, as HCT116 and HT29 showed highest VCAN mRNA expression and protein secretion (Figure 4A/B), cells were transduced with lentivirus containing specific shRNA targeting VCAN. VCAN-B11 and VCAN-B12 shRNAs reduced VCAN mRNA expression approximately 5 fold compared to transduction with a-specific scrambled shRNA (Figure 5A).

Figure 5 Downregulation of VCAN in colon carcinoma cell lines leads to decreased pro-inflammatory cytokine production in monocytes
A) VCAN mRNA relative to GAPDH expression and B) VCAN protein secretion after transduction of the colon carcinoma cell lines HCT116 and HT29 with lentiviral particles containing irrelevant scrambled shRNAs or specific VCAN shRNAs. C+D) Monocytes were pre-incubated with supernatants of VCAN shRNA (grey bars) or scrambled shRNA (black bars) transduced C) HCT116 or D) HT29 cells for 24 or 72 hours. Production of IL-6, TNF-α and IL-12 was measured after stimulation with LPS or LPS/IFN-γ for 24h. *=p<0.05, **=p<0.01, ***=p<0.001.

VCAN protein secretion by HCT116 and HT29 cells was reduced with 30-50% (Figure 5B). Furthermore, incubation of human monocytes with conditioned medium of HCT116 or HT29 cells that had been transduced with VCAN shRNA for 24h or 72h resulted in decreased secretion of pro-inflammatory cytokines IL-6, TNF-α and IL-12 after subsequent LPS or LPS/IFNy stimulation (Figure 5 C/D), compared to monocytes that had been incubated with conditioned medium of scrambled shRNA transduced HCT116 or HT29 cells as a control (Figure 5 C/D).
Discussion

The current consensus proposes that TAMs promote tumour development and have an alternatively activated or M2 phenotype.\textsuperscript{21, 22} However, we now demonstrate that whereas breast carcinoma cells drive differentiation of monocytes towards a more alternative activation state, colon carcinoma cells direct monocytes toward an inflammatory M1 phenotype with elevated levels of ROS and pro-inflammatory cytokines. For instance, stimulation with breast carcinoma cell supernatant led to diminished IL-12 production, which is a hallmark for alternatively activated macrophages.\textsuperscript{4} Studies, in which naïve macrophages were stimulated with supernatant of ovarian cancer cells, showed a likewise polarization of macrophages towards an alternative phenotype.\textsuperscript{23} It was furthermore demonstrated that alternatively activated TAM in human ovarian cancer had defective production of IL-12. Concurringly, presence of macrophages in ovarian cancer is a prognostic factor for poor survival.\textsuperscript{24, 25} TAM seem to have a growth promoting or alternative M2 phenotype in many different kinds of tumours, and especially in breast carcinoma.\textsuperscript{26-28} The presence of increased numbers of alternatively activated TAM, - which produce growth factors like fibroblast growth factor, epidermal growth factor and pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8,\textsuperscript{4, 11} strongly correlates with poor outcome in patients.\textsuperscript{29, 30} By contrast, in several clinical studies it was demonstrated that increased presence of macrophages in colorectal cancer was correlated with good prognosis.\textsuperscript{12, 31-35} It has been postulated that tumours actively recruit monocytes, after which they differentiate into alternatively activated macrophages.\textsuperscript{9} It was shown that breast epithelial cells and breast carcinoma cells are able to produce high amounts of the chemokines CCL2, CCL5 or RANTES. This can lead to up-regulation of production of VEGF, IL-8 and chemokines like CCL17 and CCL22 by monocytes in breast tumours.\textsuperscript{36-38} Both CCL17 and CCL22 react with the receptor CCR4 on CD4\textsuperscript{+} T-cells, which leads to a Th2 mediated immune response.\textsuperscript{39} As such, CCL17 and CCL22 are considered markers for alternative activation of macrophages. In agreement, we show upregulation of CCL17 and CCL22 mRNA by monocytes that had been incubated with breast carcinoma, compared to colon carcinoma supernatant-stimulated monocytes. However, we did not observe an overall difference between breast versus colon carcinoma supernatant in the ability to recruit monocytes. Thus, the inconsistency between prognosis and macrophage presence in different colon versus breast carcinoma is presumably not dependent on differences in the level of monocyte recruitment. Moreover, we previously demonstrated ample monocyte recruitment into colon carcinoma metastases in rats. However, since inhibition in monocyte recruitment led to increased tumour growth,\textsuperscript{16} we postulate that monocytes in colon carcinomas develop into cytotoxic M1 macrophages instead of pro-tumourigenic alternative macrophages. In contrast, removal of macrophages in a mouse breast carcinoma model resulted in decreased tumourigenicity, supporting a more M2 phenotype.\textsuperscript{14} We now show that stimulation of monocytes by colon or breast carcinoma cells resulted in an activation state resembling the more classically activated M1 or alternatively activated M2 phenotype, respectively. Thus, tumour cells themselves may initiate the differentiation of infiltrated monocytes towards mature tumour macrophages with different functional phenotypes. This transpired in a cell-cell contact independent manner, supporting that tumour cells release factors in their microenvironment, which determine the skewing of
Discussion

monocytes, and ultimately may direct macrophage polarization. This was consistently observed when different cell lines from similar origin were used. For instance, all colon carcinoma cell lines induced high IL-6, IL-12 and TNF-α production by monocytes, which was not observed when different breast carcinomas were used. One difference between breast and colon carcinomas, which may explain the dissimilarity in behaviour, is their origin from distinct locations in the body. Although both types of carcinomas are derived from epithelial tissues, colon epithelial cells are continuously exposed to microorganisms and able to induce a pro-inflammatory signal cascade. Breast epithelial cells however reside in a sterile environment, and both breast epithelial cells and breast carcinomas were shown to up-regulate VEGF and IL-8 by monocytes.

Comparative secretome analyses of different colon and breast carcinoma cell lines suggested that the chondroitin sulphate proteoglycan VCAN may be involved in directing monocyte differentiation, because it was uniquely secreted by colon carcinoma cell lines, but not by breast carcinoma cells. VCAN was previously shown to activate myeloid cells through binding of toll-like receptor 2, which enhanced both IL-6 and TNF-α production in mouse macrophages. We observed that inhibition of VCAN secretion by colon carcinoma cells downregulated release of IL-6 and TNF-α in human monocytes. The role of VCAN in tumour progression is however controversial. VCAN was shown to play a role in cell proliferation, migration, and inhibition of apoptosis. Elevated VCAN levels have furthermore been found in tumour-stroma of different malignant tumours, where it is produced by fibroblasts. VCAN expression in tumour stroma has been correlated with a poor prognosis in ovarian cancer, oral squamous cell carcinoma and breast carcinoma. However, only high VCAN presence in ovarian tumour stroma was clearly associated with decreased progression free and overall patient survival. In contrast, expression of VCAN in epithelial ovarian carcinoma cells correlated with improved patient outcome. Interestingly, stromal VCAN presence was not associated with survival in patients with colorectal cancer, but VCAN expression by epithelial cells in the periphery of the tumour was correlated with a longer disease free survival in a cohort of stage II and stage III patients (M. de Wit and RJ Fijneman, submitted for publication). As such, we hypothesize that VCAN contributes to monocyte differentiation into cytotoxic M1 macrophages, which may explain why high macrophage number in colorectal cancer correlates with enhanced overall survival of patients.

Reducing the tumour-promoting and/or enhancing the tumouricidal activity of macrophages in the tumour may represent an elegant way to use tumour macrophages in a therapeutic setting. Because VCAN appears to have opposing effects, depending on its location in stroma or epithelial cells, it may not represent the most suitable candidate for clinical applications. However, several mechanisms to turn pro-tumourigenic macrophages into anti-tumourigenic macrophages have been proposed. For instance, treatment of ovarian tumour bearing mice with IL-12 induced tumour regression. When TAM from a mouse lung carcinoma model were treated with IL-12 production of anti-inflammatory cytokines like IL-10, transforming growth factor-β and migration inhibitory factor was reduced, whereas pro-inflammatory factors such as TNF-α, IL-15 and IL-18 were upregulated, which indicated re-polarization of macrophages into classically activated anti-tumourigenic macrophages. Saccani et al. demonstrated that M2 TAM from murine fibrosarcomas, which have defective production of IL-12, IL-6 and TNF-α due to p50 NF-κB overexpression, could be re-educated
towards M1-like macrophages with restored expression of pro-inflammatory cytokines, resulting in reduced tumour growth. Thus, directing tumour macrophages to a more M1 phenotype with high IL-12 and TNF-α expression promoted anti-tumour responses. 

Treating mice with for example bacteriophages, TLR9 ligands or IL-12, led to induction of M1 phenotype in originally M2 macrophages, with concomitant regression in tumour growth. Similarly, treatment of breast carcinoma bearing mice with GM-CSF led to an increase in tumour macrophages with decreased angiogenic ability and an anti-tumour M1 phenotype with increased iNOS and decreased arginase I expression. This resulted into slowed tumour growth and reduced metastases development. GM-CSF treatment furthermore led to an increase in soluble VEGF (sVEGF), which inhibited VEGF production by alternatively activated tumour macrophages. Interestingly, upregulation of sVEGF was also observed in patients with colorectal cancer, where it is associated with good prognosis.

In conclusion, we propose that colon carcinoma cancer cells secrete factors (including VCAN), in their micro-milieu which renders monocytes more prone for development into M1 macrophages. By contrast, breast carcinoma cells release factors, which inhibit the development of anti-tumour macrophages, but supports the skewing into tumour-supporting macrophages. A better understanding of how tumour cells influence functional monocyte/macrophage phenotype, and how these processes can be manipulated will be crucial to develop therapeutic strategies that target macrophages to eradicate the tumour.
Materials and Methods

Cell lines and monocyte isolation
Human breast carcinoma cell lines SKBR3, MCF7 and ZR-75-1 and colon carcinoma cell lines HT29, HCT116, RKO, SW620 and SW948 were cultured in complete DMEM medium (Invitrogen, 41966052) containing 10% FCS (Lonza, DE14-801F), 1% L-glutamine, penicillin and streptomycin. Cell suspensions were prepared by enzymatic detachment using trypsin-EDTA solution. Viability was assessed by trypan blue exclusion and always exceeded 95%. All experiments were performed in complete DMEM.

Human monocytes where isolated from whole human blood (buffycoats < 24h after blood collection (Sanquin, The Netherlands), according to guidelines 2005/61/EG, 2004/33/EG, 2002/98/EG and 2005/62/EG of the EU and the Helsinki Declaration. All donors gave informed consent. Whole blood was separated on a lymphoprep gradient and Peripheral Blood Mononuclear Cells (PBMCs) were extracted from the interphase. PBMC fraction was incubated with magnetic CD14-positive beads (Miltenyi Biotec, M5021) and CD14 positive monocytes were trapped using a magnet and an LS-positive selection column (Miltenyi Biotec, 130-042-401). After washing of the column, monocytes were extracted from the column and extensively washed in complete medium. Monocytes were seeded in 96 wells plates at a concentration of 8 x 10^4 cells/well.

Production of conditioned medium
To produce conditioned medium carcinoma cells were seeded (1.5x10^4 cells/cm^2) in 25 cm^2 culture flasks, led to adhere overnight, extensively washed and incubated for 24h with fresh complete DMEM. Conditioned media were collected, centrifuged at 4750 x g and filtered using a 0.2 µm filter (Millipore, Billerica, MA). For blocking experiments 10 µg/ml anti-versican (VCAN) (Abcam, ab19345) or 10 µg/ml a-specific isotype polyclonal rabbit control antibody (antibodies-online.com, ABIN467272) were added to conditioned media prior to monocyte incubation.

Generation of VCAN shRNA virus particles
At day -1 1.3x10^6 HEK293T cells were seeded into T25 culture flasks. Cells were transfected at day 0 with plasmids of the third generation lentiviral packaging constructs together with shRNA containing plasmids (kindly provided by Dr. H.A.M. Geerts, Amsterdam Medical Center, The Netherlands) using calcium-phosphate transfection kit (clontech, cat. 631312). Twenty hours post transfection cells were washed twice with PBS, after which 4ml complete DMEM was added to the cells. After 48 hours supernatants containing live lentiviruses were harvested, centrifuged for 10min at 1500g and stored in aliquots at -80°C.

Generation of VCAN knockdown cell lines
HCT-116 and HT-29 were seeded at a density of 1x10^5 cells per well in a 24wells cell-culture plate and led to adhere for 24 hours. Virus containing supernatant (hoeveel?) was added to wells and incubated overnight. Cells were washed twice with PBS, after which complete DMEM was added. Twenty-four hours post transduction culture medium was replaced by selection medium containing puromycin (Sigma, P7255).
Materials and Methods

VCAN ELISA
Human carcinoma cell lines were seeded at a density of 1.5x10^6 cells in T25 culture flasks and led to adhere for 24 hours, after which medium was removed, cells were washed twice with complete DMEM and incubated for 24 hours with 4ml complete DMEM. After 24 hours conditioned medium was collected, centrifuged for 25min at 4°C, 4500 x g and filtered (0.2µm filter). VCAN ELISA was performed according to manufacturer’s protocol (Cusabio Biotech co., LTD, CSB-E11884h).

Chemotaxis
Chemotaxis assays were essentially performed as described, modified for use in a 48 well Neuroprobe blind well chemotaxis chamber (Gaithersburg, BW25). Briefly, bottom wells were filled with supernatants of either colon or breast carcinoma cell-line supernatants (25 ml), and covered with a 5 mm pore polyvinylpyrrolidone (PVP) free polycarbonate filter (Neuroprobe, PFB5). Purified FMLP (N-formyl-L-methionyl-L-leucyl-phenylalanine) (10^-8 M.) or MCP-1 (30 ng/ml) were used as positive controls. Top wells were filled with 50 ml human peripheral blood monocytes (8x10^5/ml). After a 90 minutes incubation period at 37°C, non migrated cells were scraped off and migrated cells, which adhered to the membrane, were stained with Coomassie stain (2.5% Coomassie Brilliant Blue R-250 (Sigma, B0149), 45% methanol, 7,5% acetic acid) and quantified with a microscope.

Monocyte stimulation and monocyte activity
Monocytes, seeded in 96 wells culture plates (8x10^4 cells/well), were incubated in freshly isolated conditioned medium of either colon or breast carcinoma cell lines or complete DMEM alone as control. After 24h -96h of incubation, monocytes were stimulated for 4 or 24h with complete DMEM supplemented with either 50 ng/ml LPS or 50 ng/ml LPS (Sigma, L4391) and 500 U/ml human IFNγ (U-CyTech, CT280). Cell activity and viability of stimulated monocytes after 24h or 96h was measured by MTT assays as described.

ROS production
H_2O_2 production was measured with Amplex™ Red Hydrogen Peroxide Assay Kits (Invitrogen, A-12221) as described. Briefly, monocytes were incubated with 100 µl Hepes’ buffer (132 mM NaCl, 20 mM hepes, 6 mM KCl, 1 mM MgSO_4·7H_2O, 1.2 mM K_2HPO_4·3H_2O, 1 mM CaCl_2, 0.5% BSA, 1 mg/ml glucose) supplemented with 50 µl Amplex red reaction mix (200 µM Amplex red reagent and 4 U/ml horse radish peroxidase in 1× Hepes’ buffer). Monocytes were stimulated with 4 µg/ml 12-myristate-13-acetate of buffer alone. Fluorescence of the produced resorufin was measured every 1 min for 1 h 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 H_2O_2 in Hepes’ buffer was used as standard measure.
Cytokine detection
Human IL-6, IL-12p40, TNF-α, IL-8 and IL-10 were measured by ELISA according to the manufacturer’s instructions (Invitrogen, (IL-6, CHC1263), (IL-12p40+p70, Diiacalone, Sanquin, M950075192), (TNF-α, CHC1753), (IL-8, CHC1303), (IL-10, AHC8102 & AHC7109).

mRNA isolation, cDNA production and semi-quantitative real time PCR
mRNA was isolated from monocytes using mRNA capture kits (Roche, 11787896001) according to the manufacturer’s instructions. mRNA was directly used for cDNA synthesis using Promega cDNA synthesis kits (Promega, a3500). For semi-quantitative real time PCR, 2 µl of diluted cDNA was mixed with 4 µl SYBR-green (Applied Biosystems, 4385614) and 0.5 μmol/l primer mix. Gene expression was semi-quantitatively measured by performing 40 real time PCR cycles in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) using the primers shown in Table 1.

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<th>Protein</th>
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<td>gcaggttctggaggtgtaa</td>
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<tr>
<td>GAPDH</td>
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<td>ggtgctagtctgtgtagtg</td>
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</table>

Table 1: Primers sequences

Statistical analysis.
Data was analyzed using student T-tests (2 groups) or ANOVA followed by Bonferroni post-hoc tests (multiple groups). Significance was accepted at p<0.05.