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Carcinoma origin dictates differential skewing of monocyte function

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Abbreviations: IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MR1, mannose receptor 1; MMP, metalloproteases; NO, nitric oxide; ROS, reactive oxygen species; shRNA, short hairpin RNA; TAM, tumour associated macrophage; TNFα, tumour necrosis factor α; VCAN, versican

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 tumors can exert tumor-promoting and tumor-suppressing activity, depending on the tumor 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 TNFα) 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 toward a more classically-activated anti-tumorigenic phenotype, whereas breast carcinomas predispose monocytes toward 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 tumors. Designing new therapies, directing development of monocytes toward M1 activated tumor macrophages in cancer patients, may have great clinical benefits.

Introduction

Solid tumors not only consist of malignant cells, but also contain non-malignant stromal cells like fibroblasts and endothelial cells as well as a variety of hematopoietic immune cells. Over the years evidence has accumulated from clinical and experimental studies supporting that tumor behavior is strongly influenced by the infiltrating immune cell populations.[1] Especially tumor associated macrophages (TAM) comprise a large fraction of the immune infiltrate in tumors, and are thought to play a major role in tumor development.

TAMs originate from monocytes, which enter the tumor via the vasculature, and develop into mature macrophages in the tumor 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 γ (IFNγ), monocytes develop into inflammatory macrophages that produce high amounts of nitric oxide, reactive oxygen species (ROS), interleukin (IL)-12 and tumor necrosis factor α (TNFα). 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

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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 tumors they can profoundly influence tumor development, depending on their phenotype.[4,6,7] Monocytes are recruited into the tumor 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 tumor macrophages where they can play a complicated dual role in tumor development. Pro-inflammatory classically activated or M1 macrophages have cytotoxic properties, and secrete TNFα, NO and ROS,[2,3] whereas M2 macrophages can promote tumor cell growth and invasion by secreting growth factors, cytokines, angiogenic factors and MMP.[9]

The evidence that macrophages are influenced by tumor cells, and subsequently contribute to tumor behavior—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 tumor is associated with disease progression and poor prognosis.[9] TAMs that are isolated from these tumors generally have an alternatively activated M2 phenotype.[6] Interestingly, increased macrophage presence in tumors 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 carcinoma cells. By contrast, breast carcinoma metastases development was decreased in macrophage deficient mice, which supported that macrophages were involved in tumor progression.[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 behavior.

Results

Monocytes are recruited toward carinoma supernatants. It has previously been proposed that tumor cells themselves can recruit monocytes from the blood circulation into the tumor where monocytes differentiate into TAM.[9] 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) (**Fig. 1A**). However, supernatants of either other breast carcinoma showed minimally enhanced chemotactic potential. Additionally, of five different colon carcinoma cells tested, two colon carcinoma supernatants (HCT116 and SW948) showed amplified monocyte recruitment, two supernatants (RKO and HT29) induced minimal enhanced chemotaxis and SW620 did not contain chemotactic stimuli (**Fig. 1A**). Thus, overall, no significant difference in monocyte recruitment toward either breast or colon carcinoma supernatant was observed.

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, a MTT assay was performed. Incubation with carcinoma cell supernatants for 24 h showed increased formazan production by monocytes compared with incubation with complete medium (negative control), indicating an increased activity of monocytes due to carcinoma cell supernatant incubation (**Fig. 1B**). Monocyte activity was further increased after stimulation with either LPS or a combination of LPS and IFNγ. 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 h (**Fig. 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 h when they had been grown in culture medium alone (without carcinoma supernatant). Moreover, remaining cells were round (**Fig. 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 H_2O_2 production by carcinoma supernatants. As neither monocyte chemotaxis nor activation and viability were different after incubation with colon vs. 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, H_2O_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 H_2O_2 production of 40.2 ± 2.7 nmol/min after PMA stimulation (**Fig. 1C**). PMA stimulation of monocytes that had been incubated with supernatants of colon carcinoma cells led to a significant increase in

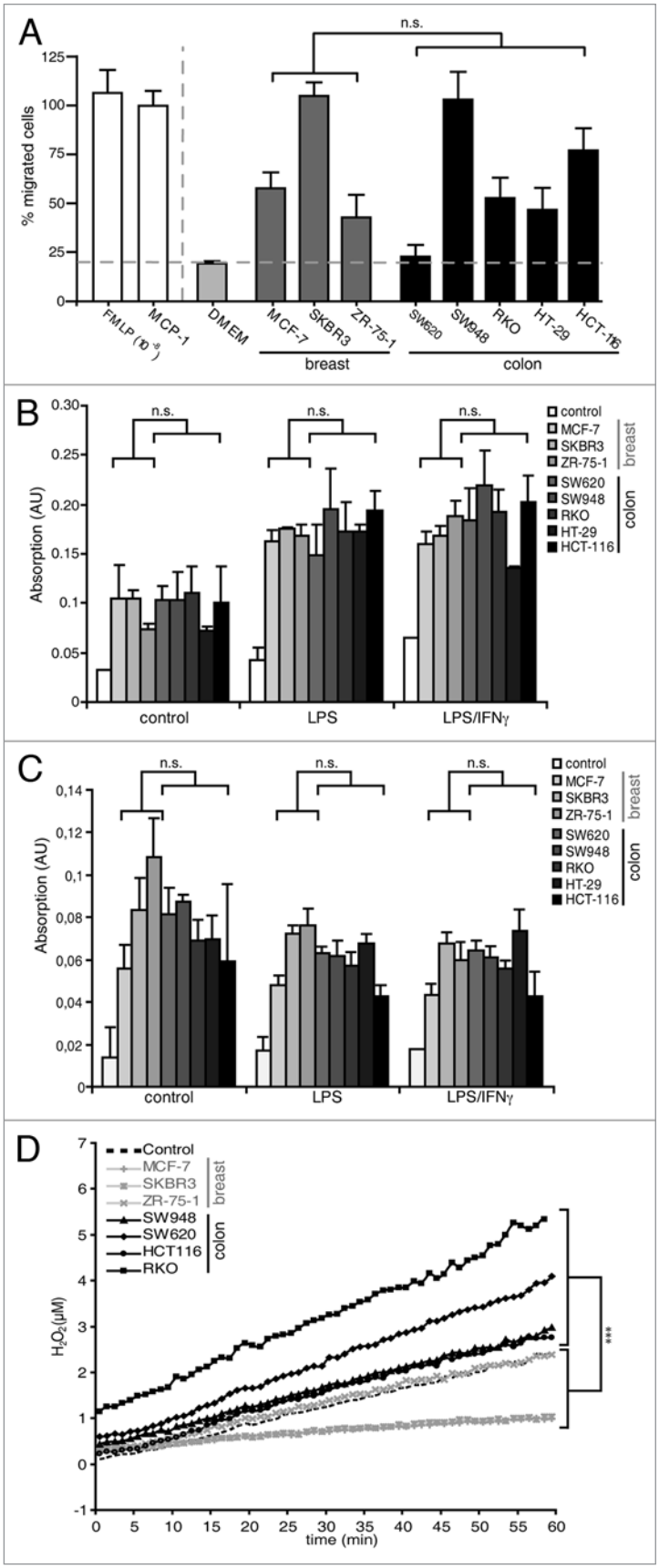

Figure 1. Monocyte migration toward carcinoma supernatants and monocyte activity. (**A**) Migration of monocytes toward different breast (dark gray) or colon (black) carcinoma supernatants. FMLP and MCP-1 were used as positive controls (white), whereas DMEM (light gray) served as negative control. (**B and C**) Monocytes were pre-incubated with DMEM (control), breast or colon carcinoma supernatant for (**B**) 24 h or (**C**) 96 h and activity and viability was measured after additional stimulation with DMEM (control), LPS or LPS/IFNγ. (**D**) Monocytes were pre-incubated with DMEM (control), breast or colon carcinoma supernatant and H_2O_2 production in time after PMA stimulation was measured. ***p < 0.001. Experiments were repeated three times.

H_2O_2 production (average H_2O_2 production of 54.7 ± 12.17 nmol/min) compared with control monocytes. By contrast, monocytes, which were first incubated with breast carcinoma supernatants had a lower average H_2O_2 production of 20.5 ± 13.8 nmol/min ($p < 0.001$) (**Fig. 2**). Incubation of monocytes with supernatants of 2 out of 3 breast carcinoma cells (SKBR3 and MCF-7) led to an H_2O_2 production lower than control monocytes, whereas incubation with ZR-75-1 supernatant resulted in H_2O_2 production similar to control cells (**Fig. 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/IFNγ 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 TNFα production was observed in either colon or breast carcinoma supernatants (data not shown). Without LPS or LPS/IFNγ stimulation, monocytes produced only very low levels of cytokines, irrespectively of prior carcinoma cell supernatant incubation (**Fig. 2A–C**). Furthermore, stimulation of control monocytes with LPS or LPS/IFNγ (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 TNFα production after LPS stimulation (**Fig. 2A–C**). Importantly, pre-incubation with colon carcinoma supernatant resulted in higher production of IL-6, IL-12 and TNFα, compared with pre-incubation with breast carcinoma supernatant. Stimulation of monocytes with LPS/IFNγ 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 with all individual breast cancer cell lines.

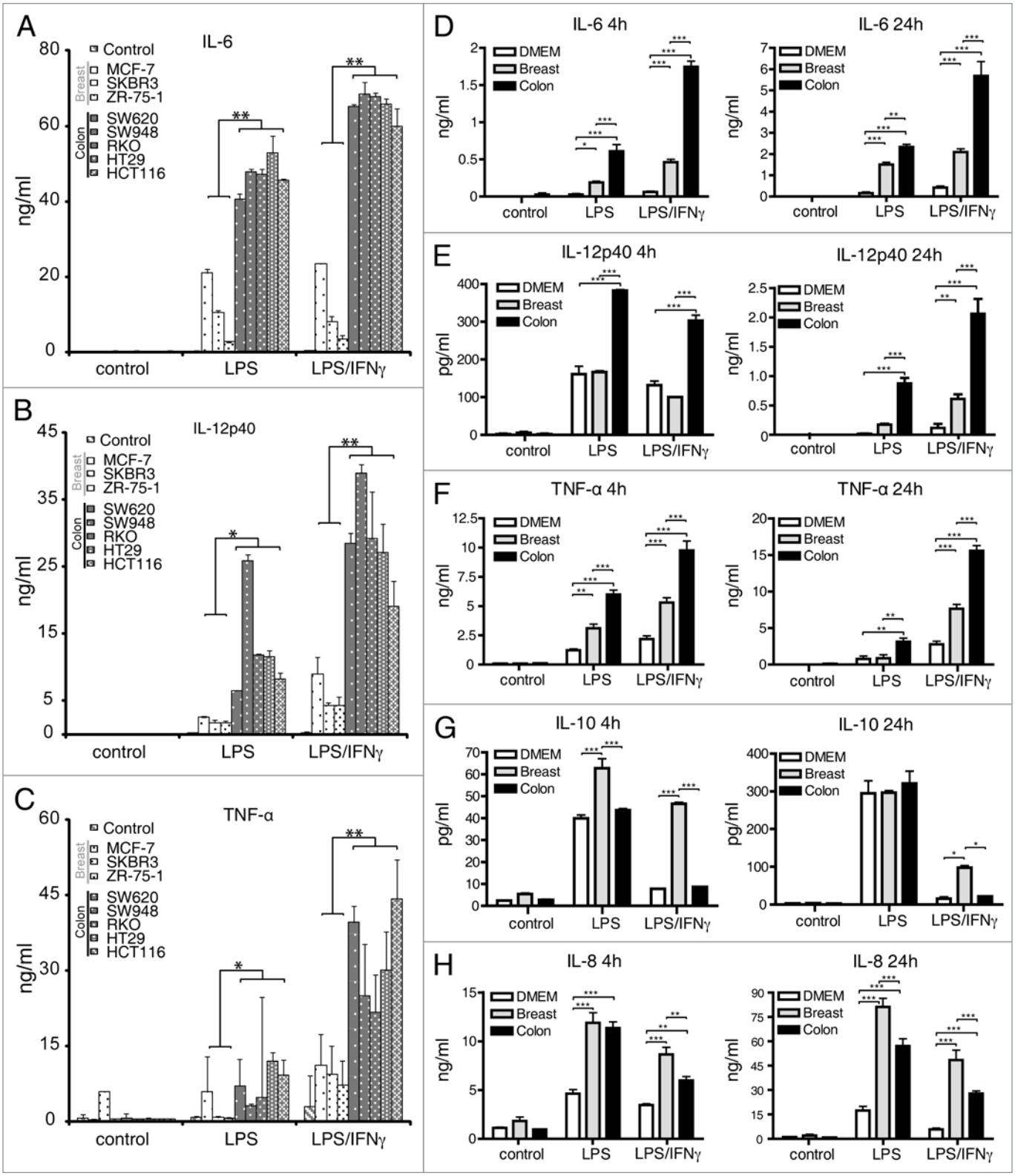

Figure 2. Incubation with carcinoma supernatant directs monocyte cytokine production. (**A–C**) Monocytes were pre-incubated with DMEM or individual colon or breast carcinoma cell supernatants for 24 h. Production of (**A**) IL-6, (**B**) IL-12p40 or (**C**) TNFα was measured after stimulation with DMEM (control), LPS or LPS/IFNγ. (**D–H**) Production of (**D**) IL-6, (**E**) IL-12p40, (**F**) TNFα, (**G**) IL-10 or (**H**) IL-8 by monocytes with have been pre-incubated with a mixture of breast (gray bars) or colon (black bars) carcinoma supernatants after stimulation with DMEM (control), LPS or LPS/IFNγ for 4 (left parts) or 24 h (right parts). White bars represent monocytes, which had been pre-incubated with DMEM. *p < 0.05, **p < 0.01, ***p < 0.001. A representative experiment out of 5 is shown.

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 TNFα 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 TNFα, IL-6 and IL-12p40 production after pre-incubation of monocytes with a mixture of supernatants of colon carcinoma cells and LPS or LPS/IFNγ stimulation compared with monocytes, which were pre-incubated with a mixture of breast carcinoma supernatants (**Fig. 2D–F**). Next, production of the M2 macrophage cytokine IL-10 [17] 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 with monocytes pre-incubated with control medium (2.5 ± 0.2 pg/ml) or colon carcinoma cell supernatant without LPS or LPS/IFNγ stimulation (2.7 ± 0.3 pg/ml) (**Fig. 2G**). Stimulation with LPS or LPS/IFNγ led to an increase in IL-10 production in all monocytes (**Fig. 2G**). However, pre-incubation of monocytes with breast carcinoma cell supernatant resulted in enhanced IL-10 production (62.8 ± 6.1 pg/ml), compared with 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) (**Fig. 2G**). Pre-incubation with breast carcinoma cell supernatant led overall to an increase in IL-8 production by monocytes as well, compared with incubation with control medium or colon carcinoma cell supernatant, albeit less pronounced than IL-10 production (**Fig. 2H**).

Incubation with tumor 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 for 24 h. Monocytes were challenged for 4 h with either LPS or LPS/IFNγ. mRNA expression of the pro-inflammatory cytokine genes IL-6, IL-12p40, IL-12p35 and TNFα was markedly decreased in monocytes pre-incubated with breast carcinoma supernatant and stimulated with LPS/IFNγ (**Fig. 3A–D**). Additionally, pre-incubation with colon carcinoma supernatant resulted in an increased gene expression of the chemokine ligand CXCL13 (**Fig. 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 (**Fig. 3F and G**). Additionally, expression of the M2- associated chemokines CCL17 and CCL22 was higher in monocytes that had been pre-incubated with breast carcinoma supernatant, compared with monocytes which had been stimulated with colon carcinoma cell supernatant (**Fig. 3H and I**). Monocytes cultured with breast carcinoma cell supernatant also showed increased mannose receptor 1 (MR1) mRNA expression (**Fig. 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 (**Fig. 3K**), which is in agreement with our finding that there is no difference in monocyte recruitment toward either colon or breast carcinoma cell supernatants (**Fig. 1A**). Thus taken together, incubation with colon carcinoma cell supernatants overall favored 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).[20] 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 (**Fig. 4A**). 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 (**Fig. 4A and 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 TNFα production by monocytes after stimulation with LPS/IFNγ (**Fig. 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 (**Fig. 4A and 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 with transduction with a-specific scrambled shRNA (**Fig. 5A**). VCAN protein secretion by HCT116 and HT29 cells was reduced with 30–50% (**Fig. 5B**). Furthermore, incubation of human monocytes with conditioned medium of HCT116 or HT29 cells that had been transduced with VCAN shRNA for 24 h or 72 h resulted in decreased secretion of pro-inflammatory cytokines IL-6, TNFα and IL-12 after subsequent LPS or LPS/IFNγ stimulation (**Fig. 5C and D**), compared with monocytes that had been incubated with conditioned medium of scrambled shRNA transduced HCT116 or HT29 cells as a control (**Fig. 5C and D**).

Discussion

The current consensus proposes that TAMs promote tumor development and have an alternatively activated or M2 phenotype.[21,22] However, we now demonstrate that whereas breast carcinoma cells drive differentiation of monocytes toward 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

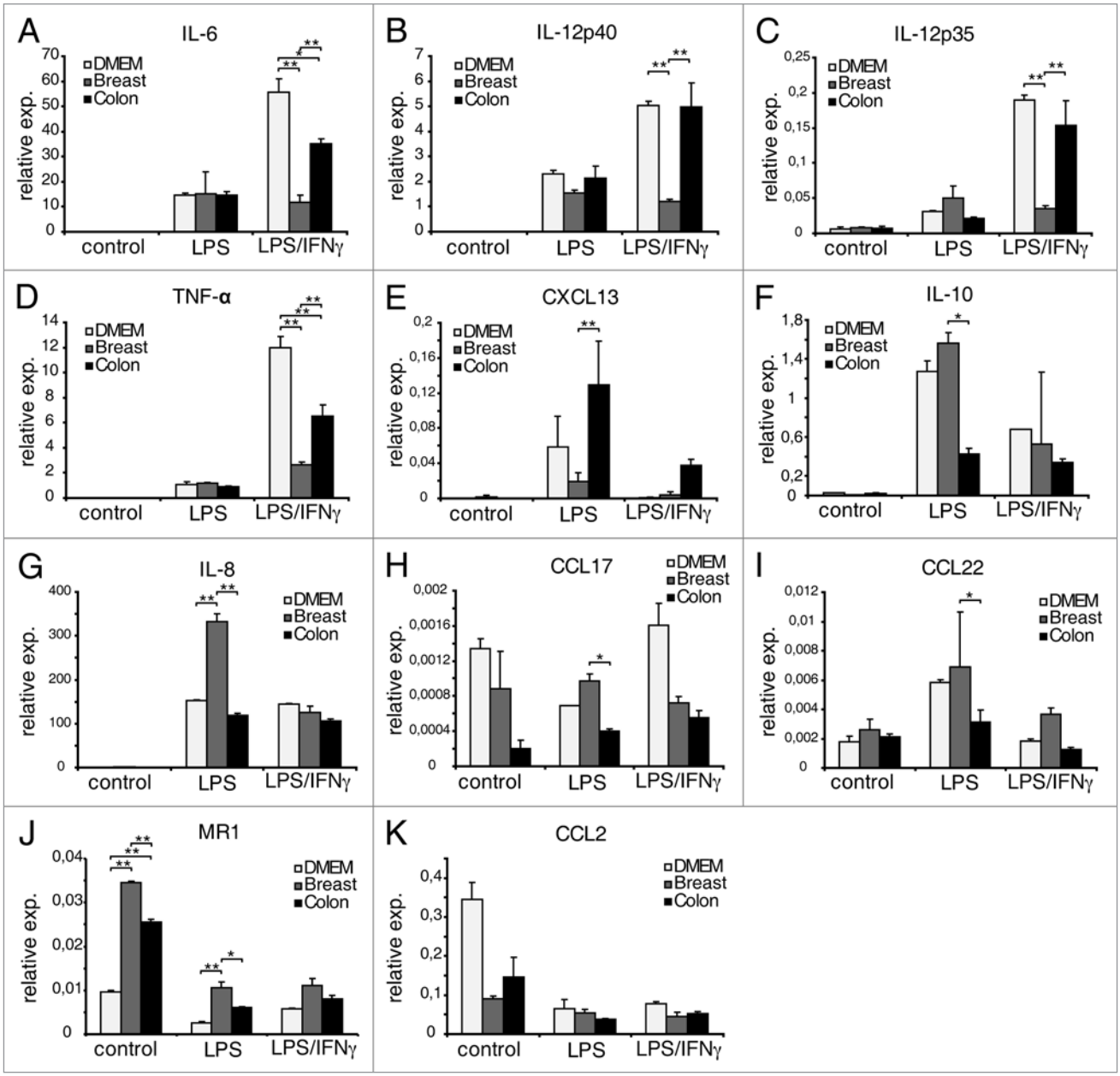

Figure 3. Monocyte mRNA expression is differentially altered after incubation with carcinoma supernatants. Monocytes were pre-incubated with DMEM (white bars) or mixtures of breast (gray bars) or colon (black bars) carcinoma cell supernatants for 24 h. mRNA levels of (**A**) IL-6, (**B**) IL-12p40, (**C**) IL-12p35, (**D**) TNFα, (**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/IFNγ and correlated with mRNA GAPDH expression levels. *p < 0.05, **p < 0.01. The experiment was repeated three times with similar results.

macrophages.[4] Studies, in which naïve macrophages were stimulated with supernatant of ovarian cancer cells, showed a likewise polarization of macrophages toward an alternative phenotype.[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.[24,25] TAM seem to have a growth promoting or alternative M2 phenotype in many different kinds of tumors, and especially in breast carcinoma.[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[4,11]—strongly correlates with poor outcome in patients.[29,30] By contrast, in several clinical studies it was demonstrated that increased presence of macrophages in colorectal cancer was correlated with good prognosis.[12,31-35]

It has been postulated that tumors actively recruit monocytes, after which they differentiate into alternatively activated macrophages.[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 upregulation

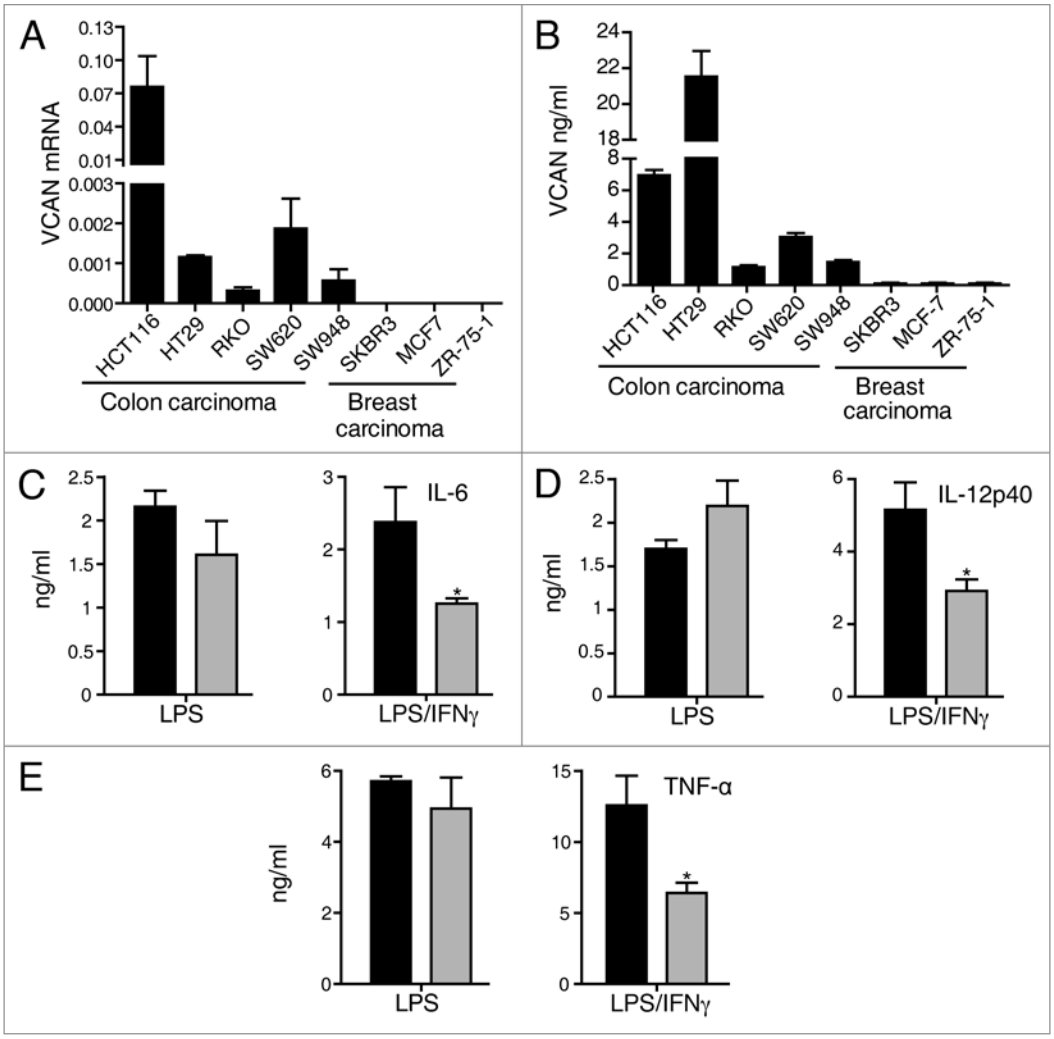

Figure 4. Blocking VCAN in colon carcinoma supernatants decreases pro-inflammatory cytokine production in monocytes. (**A**) VCAN mRNA expression of and (**B**) VCAN protein secretion by different colon or breast carcinoma cell lines. (**C–E**) Monocytes were pre-incubated with mixture of colon carcinoma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

of production of VEGF, IL-8 and chemokines like CCL17 and CCL22 by monocytes in breast tumors.[36-38] Both CCL17 and CCL22 react with the receptor CCR4 on $CD4^+$ T cells, which leads to a Th2 mediated immune response.[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 with colon carcinoma supernatant-stimulated monocytes. However, we did not observe an overall difference between breast vs. colon carcinoma supernatant in the ability to recruit monocytes. Thus, the inconsistency between prognosis and macrophage presence in different colon vs. 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 tumor growth,[16] we postulate that monocytes in colon carcinomas develop into cytotoxic M1 macrophages instead of pro-tumorigenic alternative macrophages. In contrast, removal of macrophages in a mouse breast carcinoma model resulted in decreased tumorigenicity, supporting a more M2 phenotype.[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, tumor cells themselves may initiate the differentiation of infiltrated monocytes toward mature tumor macrophages with different functional phenotypes. This transpired in a cell-cell contact independent manner, supporting that tumor cells release factors in their microenvironment, which determine the skewing of 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 TNFα production by monocytes, which was not observed when

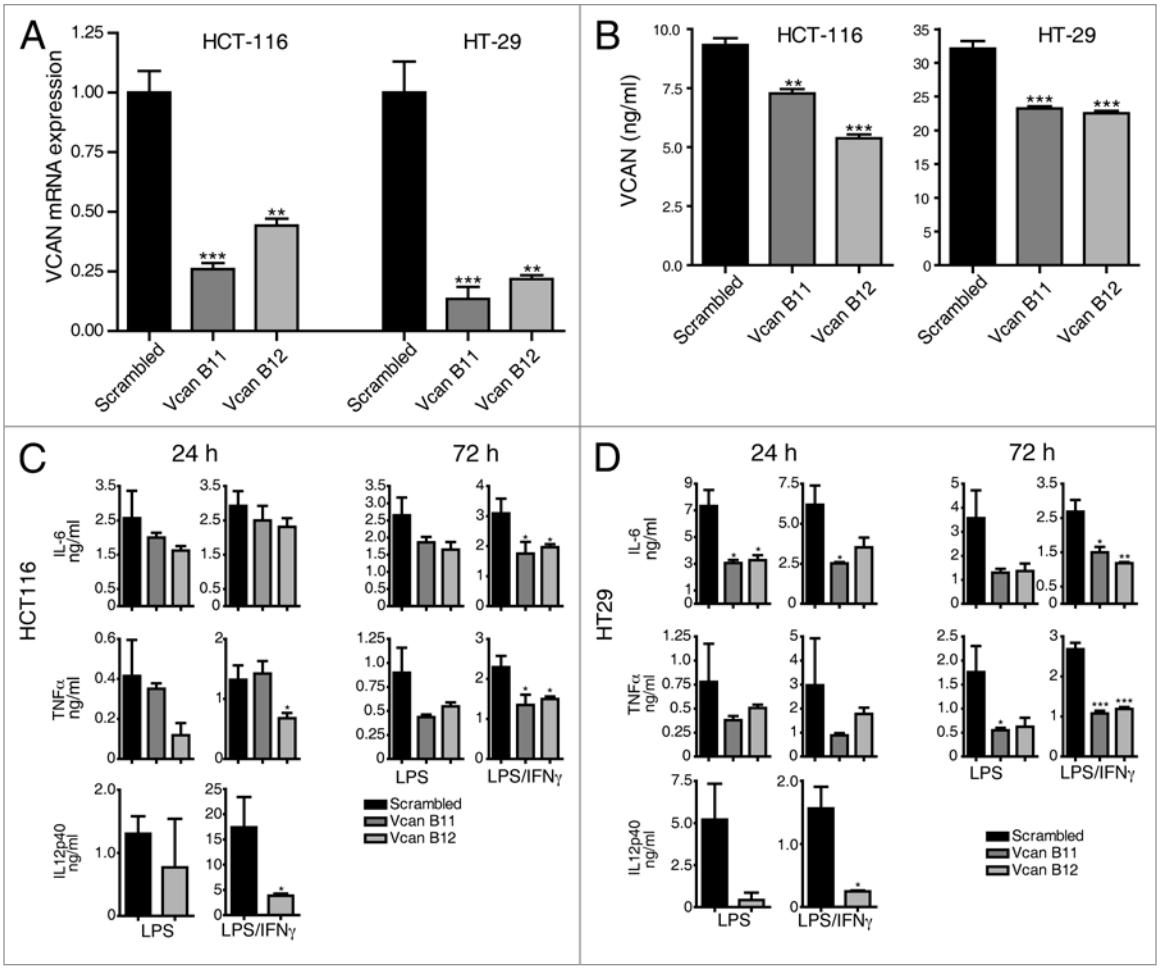

Figure 5. Downregulation of VCAN in colon carcinoma cell lines leads to decreased pro-inflammatory cytokine production in monocytes. (**A**) VCAN mRNA relative to GAPDH expression and (**B**) VCAN protein secretion after transduction of the colon carcinoma cell lines HCT116 and HT29 with lentiviral particles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

different breast carcinomas were used. One difference between breast and colon carcinomas, which may explain the dissimilarity in behavior, 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.[40] Breast epithelial cells however reside in a sterile environment, and both breast epithelial cells and breast carcinomas were shown to upregulate VEGF and IL-8 by monocytes.[36-38]

Comparative secretome analyses of different colon and breast carcinoma cell lines suggested that the chondroitin sulfate 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 TNFα production in mouse macrophages.[41] We observed that inhibition of VCAN secretion by colon carcinoma cells downregulated release of IL-6 and TNFα in human monocytes. The role of VCAN in tumor progression is however controversial. VCAN was shown to play a role in cell proliferation, migration and inhibition of apoptosis.[42-44] Elevated VCAN levels have furthermore been found in tumor-stroma of different malignant tumors, where it is produced by fibroblasts.[45] VCAN expression in tumor stroma has been correlated with a poor prognosis in ovarian cancer, oral squamous cell carcinoma and breast carcinoma.[45-50] However, only high VCAN presence in ovarian tumor 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.[47,51] 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 tumor was correlated with a longer disease free survival in a cohort of Stage II and Stage III patients (de Wit M and Fijneman RJ, 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 tumor-promoting and/or enhancing the tumoricidal activity of macrophages in the tumor may represent an elegant way to use tumor 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-tumorigenic macrophages into anti-tumorigenic macrophages have been proposed. For instance, treatment of ovarian tumor bearing mice with IL-12 induced tumor regression.[52] 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 TNFα, IL-15 and IL-18 were upregulated,[52] which indicated re-polarization of macrophages into classically activated anti-tumorigenic macrophages. Saccani et al. demonstrated that M2 TAM from murine fibrosarcomas, which have defective production of IL-12, IL-6 and TNFα due to p50 NFκB overexpression, could be re-educated toward M1-like macrophages with restored expression of pro-inflammatory cytokines, resulting in reduced tumor growth.[53] Thus, directing tumor macrophages to a more M1 phenotype with high IL-12 and TNFα expression promoted anti-tumor responses.[54-56] Treating mice with for example bacteriophages,[57] TLR9 ligands[58] or IL-12,[52,59] led to induction of M1 phenotype in originally M2 macrophages, with concomitant regression in tumor growth. Similarly, treatment of breast carcinoma bearing mice with GM-CSF led to an increase in tumor macrophages with decreased angiogenetic ability and an anti-tumor M1 phenotype with increased iNOS and decreased arginase I expression. This resulted into slowed tumor growth and reduced metastases development.[30,60,61] GM-CSF treatment furthermore led to an increase in soluble VEGF (sVEGF), which inhibited VEGF production by alternatively activated tumor macrophages. Interestingly, upregulation of sVEGF was also observed in patients with colorectal cancer, where it is associated with good prognosis.[62]

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-tumor macrophages, but supports the skewing into tumor-supporting macrophages. A better understanding of how tumor 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 tumor.

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 < 24 h 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 lymfoprep 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 well plates at a concentration of 8×10^4 cells/well.

Production of conditioned medium. To produce conditioned medium carcinoma cells were seeded (1.5×10^4 cells/cm^2) in 25 cm^2 culture flasks, led to adhere overnight, extensively washed and incubated for 24 h with fresh complete DMEM. Conditioned media were collected, centrifuged at 4,750× g and filtered using a 0.2 μm filter (Millipore). 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 1.3×10^6 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, Academic Medical Center, The Netherlands) using calcium-phosphate transfection kit (clontech, cat. 631312). Twenty hours post transfection cells were washed twice with PBS, after which 4 ml complete DMEM was added to the cells. After 48 h supernatants containing live lentiviruses were harvested, centrifuged for 10 min at 1,500 g and stored in aliquots at -80°C.

Generation of VCAN knockdown cell lines. HCT-116 and HT-29 were seeded at a density of 1×10^5 cells per well in a 24 wells cell-culture plate and led to adhere for 24 h. Virus containing supernatant 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).

VCAN ELISA. Human carcinoma cell lines were seeded at a density of 1.5×10^6 cells in T25 culture flasks and led to adhere for 24 h, after which medium was removed, cells were washed twice with complete DMEM and incubated for 24 h with 4 ml complete DMEM. After 24 h conditioned medium was collected, centrifuged for 25 min at 4°C, 4,500× g and filtered (0.2 μm filter). VCAN ELISA was performed according to manufacturer's protocol (Cusabio Biotech Co., Ltd., CSB-E11884h).

Chemotaxis. Chemotaxis assays were essentially performed as described in reference 63, modified for use in a 48 well Neuroprobe

Table 1. Primers sequences

Protein	5' primer	3' primer
IL-6	TGC AAT AAC CAC CCC TGA CC	TGC GCA GAA TGA GAT GAG TTG
IL-8	TGA GAG TGG ACC ACA CTG CG	TCT CCA CAA CCC TCT GCA CC
IL-10	GAG GCT ACG GCG CTG TCA T	CCA CGG CCT TGC TCT TGT T
IL-12p35	CCA CTC CAG ACC CAG GAA TGT	GCA GGT TTT GGG AGT GGT GA
IL-12p40	CCA GAG CAG TGA GGT CTT AGG C	TGT GAA GCA GCA GGA GCG
TNF-α	GCC CAG GCA GTC AGA TCA TC	TGG GCT ACA GGC TTG TCA CTC
CCL2	GCG TTT AAT CAC ATT CGA GTG TTT	CCA CTG GCA AAT TAG GGA ACA A
CCL17	AGG GAC CTG CAC ACA GAG AC	CTC GAG CTG CGT GGA TGT GC
CCL22	ATG GCT CGC CTA CAG ACT GCA CTC	CAC GGC AGC AGA CGC TGT CTT CCA
CXCL13	TGT GTG TGT GGA CCC TCA AG	CAG AGC AGG GAT AAG GGA AG
MRC1	GTC TTG GGC CAC AGG TGA A	AAG GCG TTT GGA TAG CCA CA
VCAN	TCA ACG TCA CCT TCC AAC TAT C	AGT CCT TTG GTA TGC AGA TGG
GAPDH	CCA TGT TCG TCA TGG GTG TG	GGT GCT AAG CAG TTG GTG GTG

blind well chemotaxis chamber (Gaithersburg, BW25). Briefly, bottom wells were filled with supernatants of either colon or breast carcinoma cell-line supernatants (25 μl), and covered with a 5 μm 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 μl human peripheral blood monocytes (8×10^5/ml). After a 90 min 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 (8×10^4 cells/well), were incubated in freshly isolated conditioned medium of either colon or breast carcinoma cell lines or complete DMEM alone as control. After 24 h–96 h of incubation, monocytes were stimulated for 4 or 24 h 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 24 h or 96 h was measured by MTT assays as described in reference 16.

ROS production. H_2O_2 production was measured with Amplex™ Red Hydrogen Peroxide Assay Kits (Invitrogen, A-12221) as described in reference 16. Briefly, monocytes were incubated with 100 μl Hepes$^+$ buffer (132 nM NaCl, 20 mM hepes, 6 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $K_2HPO_4 \cdot 3H_2O$, 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) 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, TNFα, IL-8 and IL-10 were measured by ELISA according to the manufacturer's instructions (Invitrogen, (IL-6, CHC1263), (IL12p40, Diaclone, Sanquin, M851880020), (TNFα, CHC1753), (IL-8, CHC1303), (IL-10, AHC8102 and AHC7109)).

mRNA isolation, cDNA production and semiquantitative 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) using the primers shown in **Table 1**.

Statistical analysis. Data was analyzed using Student's t-tests (2 groups) or ANOVA followed by Bonferroni post-hoc tests (multiple groups). Significance was accepted at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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