

Human mast cells promote colon cancer growth via bidirectional crosstalk: studies in 2D and 3D coculture models

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ABSTRACT

Chronic inflammation drives the development of colorectal cancer (CRC), where tumor-infiltrating immune cells interact with cancer cells in a dynamic crosstalk. Mast cells (MC), one of earliest recruited immune cells, accumulate in CRC tissues and their density is correlated with cancer progression. However, the exact contribution of MC in CRC and their interaction with colon cancer cells is poorly understood. Here, we investigated the impact of primary human MC and their mediators on colon cancer growth using 2D and 3D coculture models. Primary human MC were generated from peripheral CD34⁺ stem cells. Transwell chambers were used to analyze MC chemotaxis to colon cancer. Colon cancer cells HT29 and Caco2 differentially recruited MC by releasing CCL15 or SCF, respectively. Using BrdU proliferation assays, we demonstrated that MC can directly support colon cancer proliferation and this effect was mediated by their cellular crosstalk. 3D coculture models with cancer spheroids further confirmed the pro-tumor effect of MC on colon cancer growth, where direct cell-cell contact is dispensable and increased production of multiple soluble mediators was detected. Moreover, TLR2 stimulation of MC promoted stronger growth of colon cancer spheroids. By examining the transcriptome profile of colon cancer-cocultured MC versus control MC, we identified several MC marker genes, which were deregulated in expression. Our study provides an advanced in vitro model to investigate the role of human MC in cancer. Our data support the detrimental role of MC in CRC development and provide a molecular insight into the cellular crosstalk between MC and colon cancer cells.

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer among both men and women.¹ Between 35–50% of those diagnosed will die from CRC, making it the second leading cause of cancer deaths affecting both sexes.¹ Recent development of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) targeted inhibitors has prolonged the overall survival in patients with metastatic CRC.² However, not all patients respond to these targeted inhibitors and this might be due to the heterogeneous nature of colon cancer cells as well as the complexity of tumor microenvironment.^{2–4}


Inflammation is an integral feature of tumor microenvironment.^{5,6} It is driven by the accumulation of various immune and inflammatory cells and soluble inflammatory mediators, such as cytokines, chemokines, growth factors, lipid molecules, reactive oxygen (ROS), and nitrogen species.⁵ The interaction between these immune and inflammatory cells and mediators leads to generation of autocrine and paracrine signals that foster cancer growth and metastases.⁵ Hence, to understand the relationship between these immune cells and colon cancer cells is key to prevent inflammation-driven cancer progression.

Mast cells (MC), one of earliest recruited immune cells during tumorigenesis, can be activated by immunologic and non-immunologic factors present in tumor microenvironment.^{7,8} Upon activation, they release a plethora of pro-tumorigenic mediators, such as IL-8, VEGF, matrix metalloproteinases and proteases.^{7,9} Angiogenesis factors (e.g., IL-8) and growth factors (e.g., VEGF) facilitate tumor growth and angiogenesis. Matrix metalloproteinases (e.g., MMP-9) and proteases (tryptase and chymase) degrade components of the extracellular matrix (ECM) and thus favoring the implantation of tumor cells. In addition, MC release TGF- β and adenosine, which subsequently suppress protective responses in tumor microenvironment. On the other hand, MC can also exhibit anti-tumor activity through direct tumor cell cytotoxicity mediated by ROS and TNF- α or indirectly through the release of heparin, IL-9 and stimulation of dendritic cell maturation, whereas in some cases MC are merely an innocent bystander.⁷

MC accumulate in CRC tissues,^{10–17} but their exact role is still controversial in cancer progression. Particularly, there are discrepancies regarding the relationship between MC density and tumor progression and clinical outcomes in patients with

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CRC.⁷ Some studies suggested MC number is positively associated with a poor prognosis for CRC,^{10–13,15,17} while other studies had contrasting findings.^{14,16} This suggests that simply assessing MC number does not provide insight into MC contribution in CRC progression. By using 2D and 3D coculture models, our aim is to investigate the impact of human MC on colon cancer growth and delineate the molecular mechanism of MC-colon cancer cell interaction. Our results support pro-tumor effects of MC on colon cancer growth, where the bidirectional crosstalk between MC and colon cancer cells is responsible for MC recruitment and enhanced cancer growth.

Results

Validation of CD34⁺ derived human MC

Primary human MC were generated from CD34⁺ purified peripheral stem cells in the presence of IL-3 and SCF. Cells gradually acquired histochemical markers of differentiated MC including metachromatic cytoplasmic granules (Figure 1(a)). To exclude the influence of contaminating cells on our study, we removed dead cells and further purified the live cell fraction with CD117 positive selection. The purity of MC (CD117⁺, FcεRIa⁺) was up to 90% after purification

(Figure 1(b)). MC functionality was assessed by measuring FcεRI-mediated degranulation, as indicated by β-hex release. CD34⁺ derived human MC showed a dose-response to stimulation of anti-FcεRI MAb and the percentage of β-hex release reached up to 50% (Figure 1(c)).

Colon cancer cells recruit human MC by releasing SCF and CCL15

We investigated MC migration to chemoattractants released by colon cancer cells using 5-μm pore size transwell chambers. CD34⁺ derived human MC were loaded in the top chamber and the conditioned medium (CM) from HT29 or Caco2 was placed in the bottom chamber. Both HT29 and Caco2 CM induced a significant MC migration ($P < 0.0001$) (Figure 2(a)). We further investigated which chemoattractant was responsible for MC migration towards colon cancer cells. SCF is a well-known chemoattractant for MC migration in the context of cancer.¹⁸ Therefore, we added various concentrations of SCF blocking antibody in the migration assay. Inhibition of SCF resulted in up to 65% reduction of MC recruitment to Caco2, but had no effect on that of HT29 (Figure 2(b)). As HT29 constitutively express CCL15 (cytokine arrays in Figure 6(a)) and its

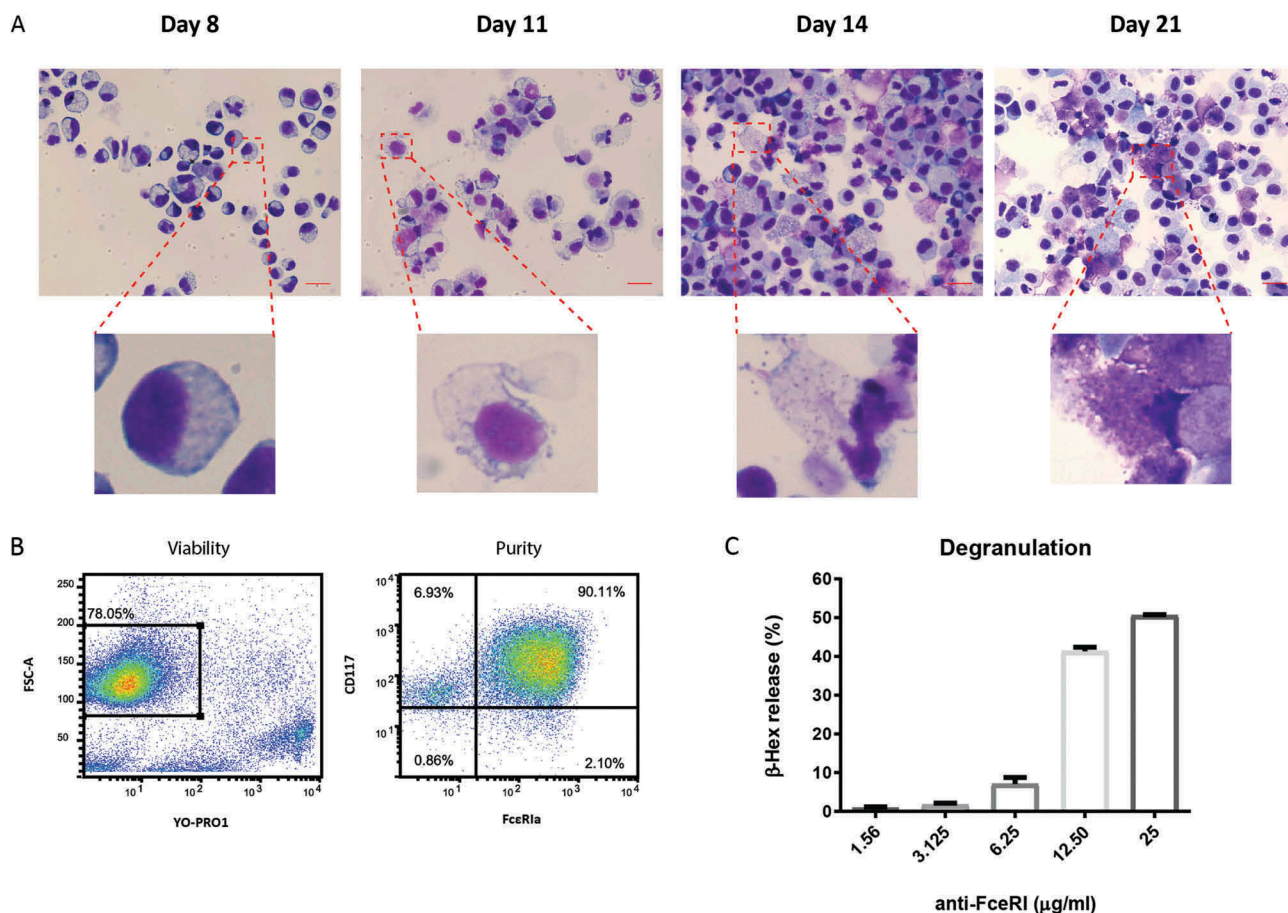


Figure 1. Generation of primary human MC.

(a) Granule development in CD34⁺ derived human MC during the course of culture (scale bar, 20μm). (b) Viability and purity of human MC followed by dead cell removal and CD117⁺ positive selection. (c) β-hexosaminidase (β-hex) release by human MC in response to FcεRI-mediated activation. Bars represent mean value ± SEM. Data shown are a representative of three independent experiments.

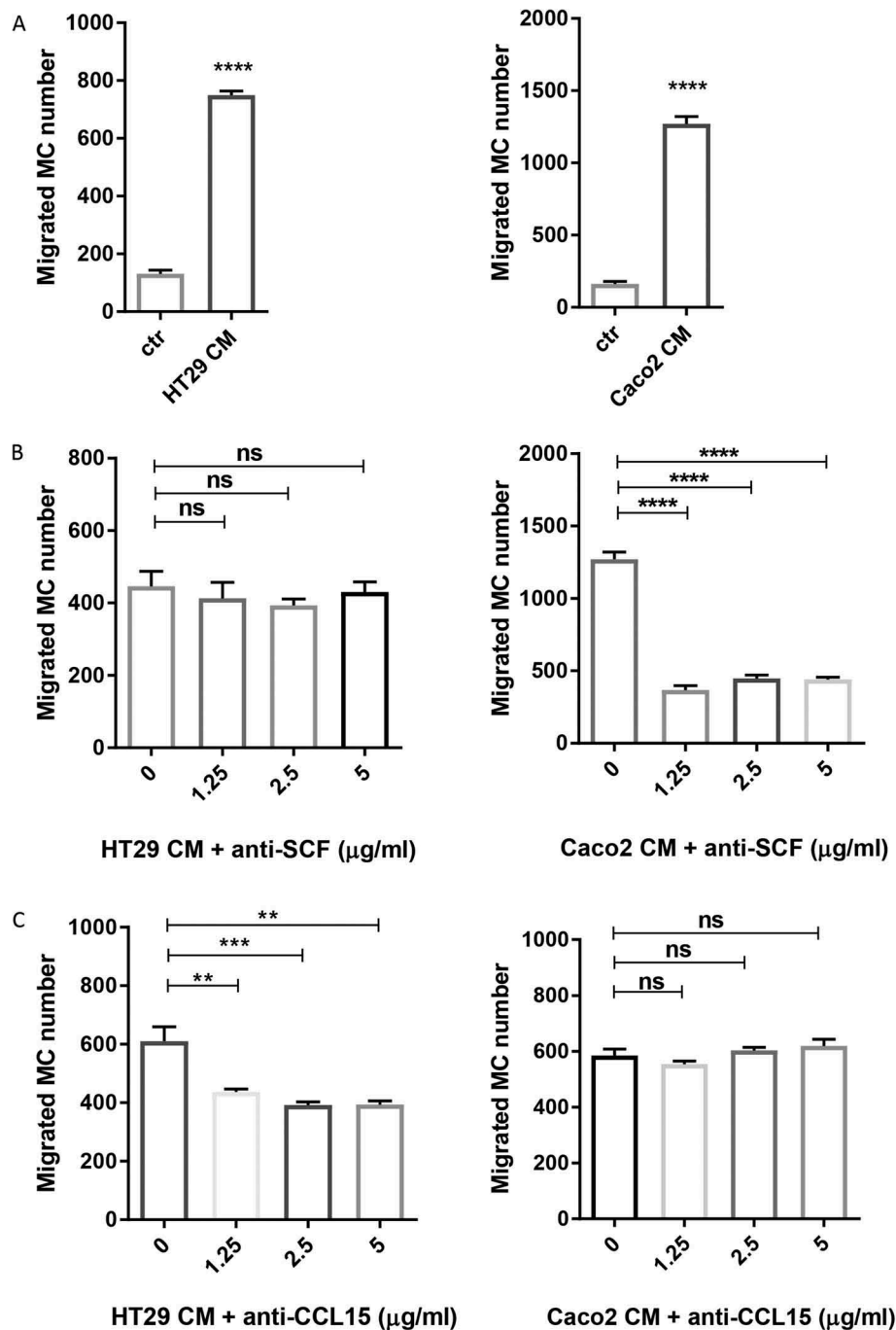


Figure 2. Colon cancer cells recruit human MC via the release of soluble mediators.

(a) MC chemotaxis to conditioned medium (CM) of human colon cancer cell lines HT29 and Caco2. (b) MC migration induced by HT29 CM and Caco2 CM in the presence of SCF blocking antibody. (c) MC migration induced by HT29 CM and Caco2 CM in the presence of CCL15 blocking antibody. Bars represent mean value \pm SEM. Data shown are a representative of three independent experiments. ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$, assessed by unpaired Student's t test or one-way ANOVA.

receptors (CCR1 and CCR3) are expressed by human MC,¹⁹ we next tested if CCL15 was responsible for MC migration toward HT29. Interestingly, we observed 34% reduction in MC migration toward HT29 in the presence of CCL15 blocking antibody (Figure 2(c)). By contrast, we observed no inhibition of blocking CCL15 in Caco2-induced MC recruitment (Figure 2(c)). Our data indicate that colon cancer cells differentially recruit MC by releasing soluble mediators, such as SCF and CCL15.

Crosstalk between human MC and colon cancer cells is necessary for cancer proliferation

After establishing that MC accumulate in colon cancer in response to mediators secreted by colon cancer cells, we evaluated the effect of CD34⁺-derived human MC on colon cancer cell proliferation. To test this, we cocultured HT29 or Caco2 colon cancer cells with MC at different ratios in a serum-free medium. After 72 hr, cancer proliferation was determined using a BrdU uptake assay. MC can directly

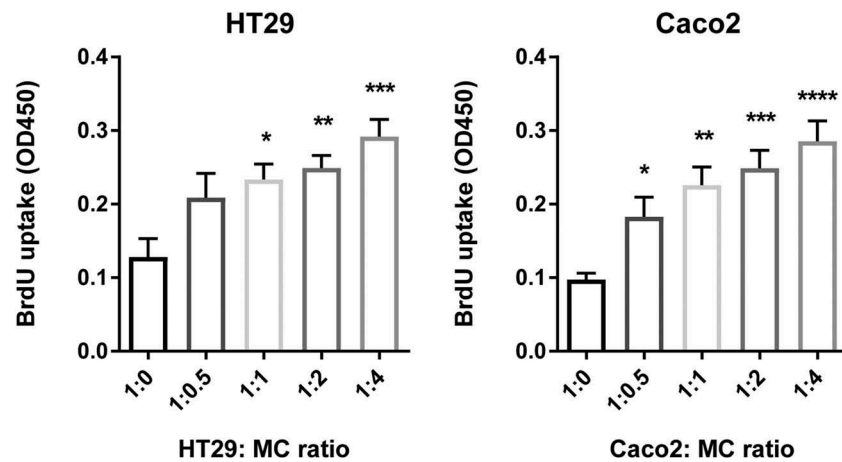


Figure 3. Human MC directly promote colon cancer proliferation.

Proliferation of colon cancer cells (HT29 and Caco2), as measured by BrdU uptake, cultured with different ratios of human MC (1:0, 1:0.5, 1:1, 1:2, 1:4) in a serum-free medium for 72 hr. Data are displayed as mean \pm SEM of three independent experiments ($n=3$). * $P < 0.05$; ** $P < 0.005$; **** $P < 0.001$, assessed by one-way ANOVA.

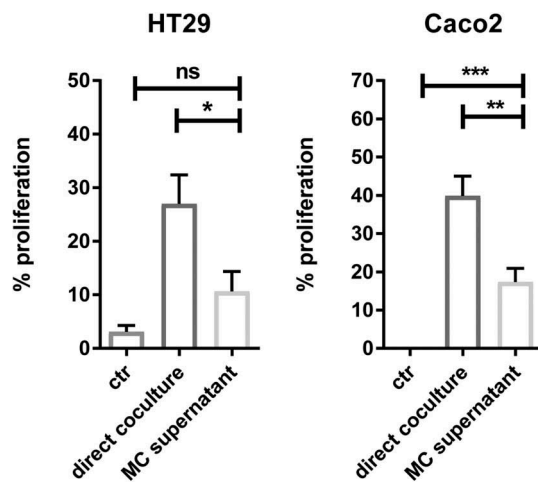


Figure 4. Crosstalk between human MC and colon cancer cells is necessary for cancer proliferation.

Proliferation percentage (% proliferation) of colon cancer cells (HT29 and Caco2) cultured with MC directly or MC supernatant alone in a serum-free medium for 72 hr. The proliferation of cancer cells cultured in serum-free medium alone serves as a positive control (POS). Proliferation percentage was calculated as follows: BrdU uptake [(treated group - NEG)/(POS - NEG)] \times 100%. Data are displayed as mean \pm SEM of three independent experiments ($n=3$). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$, assessed by one-way ANOVA.

promote colon cancer cell proliferation (HT29 alone = 0.12, HT29 + MC (1:1) = 0.23, $P < 0.05$; Caco2 alone = 0.09, Caco2 + MC (1:0.5) = 0.18, $P < 0.05$) and there was a dose-dependent increase in cancer proliferation in response to increasing ratios of MC (Figure 3). To evaluate if this effect is dependent on the bidirectional crosstalk between MC and colon cancer cells, colon cancer cells were cultured with MC-conditioned supernatant alone. Surprisingly, in the absence of MC, the proliferation ratio of HT29 was significantly reduced by 60% ($P < 0.05$) and this of Caco2 reduced from 40% to 17% ($P < 0.01$) (Figure 4). Collectively, our data indicate that cellular crosstalk between MC and colon cancer cells is needed for the pro-tumorigenic effect.

TLR2-stimulated MC induce stronger growth of colon cancer in a 3D spheroid model

As cancer spheroids are more likely to mirror the 3D cellular context and therapeutically relevant pathophysiological gradients of *in vivo* tumors,^{20,21} we further tested if human MC could also promote the growth of colon cancer spheroids. To answer this, we developed a coculture model of HT29 spheroids and human MC embedded in an extracellular matrix (ECM). Confocal imaging of HT29 spheroids revealed a considerable increase in tumor size when spheroids were cultured in medium containing FCS for 6 days (Figure 5(a)). Moreover, HT29 spheroids exhibited an aggressive phenotype in FCS-containing medium, as indicated by their irregular border and protrusion into ECM (Figure 5(a)). Similar to the finding in 2D models (Figure 3), culturing HT29 spheroids with human MC (MC NS) led to an increase in tumor size compared with the negative control (HT29 BSA = 0.15 ± 0.08 mm², HT29 + MC NS = 0.23 ± 0.05 mm², $P = 0.058$) (Figures 5(a,b)).

Chronic inflammation is an integral feature of many cancers, but the drivers of inflammation remain obscure.²² Evidence points to the importance of endogenous “danger signals” released from stressed and dying cancer cells.^{23–25} They can bind and activate TLRs, frequently TLR2 and TLR4, to trigger immune response.²⁶ Furthermore, TLR2-mediated MC activation has been exploited in the context of a mouse cancer model.²⁷ Therefore, we next asked if the presence of TLR2 agonists could influence human MC-induced colon cancer growth. To test this, we pre-stimulated human MC with TLR2 agonist FSL-1 and cocultured them with HT29 spheroids for 6 days. Interestingly, compared with non-stimulated MC (MC NS), FSL-1 stimulated MC (MC S) induced a markedly stronger growth of HT29 spheroids (HT29 + MC NS = 0.23 ± 0.05 mm², HT29 + MC S = 0.34 ± 0.12 mm², $P < 0.005$) (Figure 5(a,b)). We also observed a similar trend when using Pam3CSK4-stimulated MC (data not shown). Moreover, this effect is not caused by the direct action of FSL-1 on HT29 spheroids (Supplemental Figure 1A). Our data suggest that MC induce stronger colon

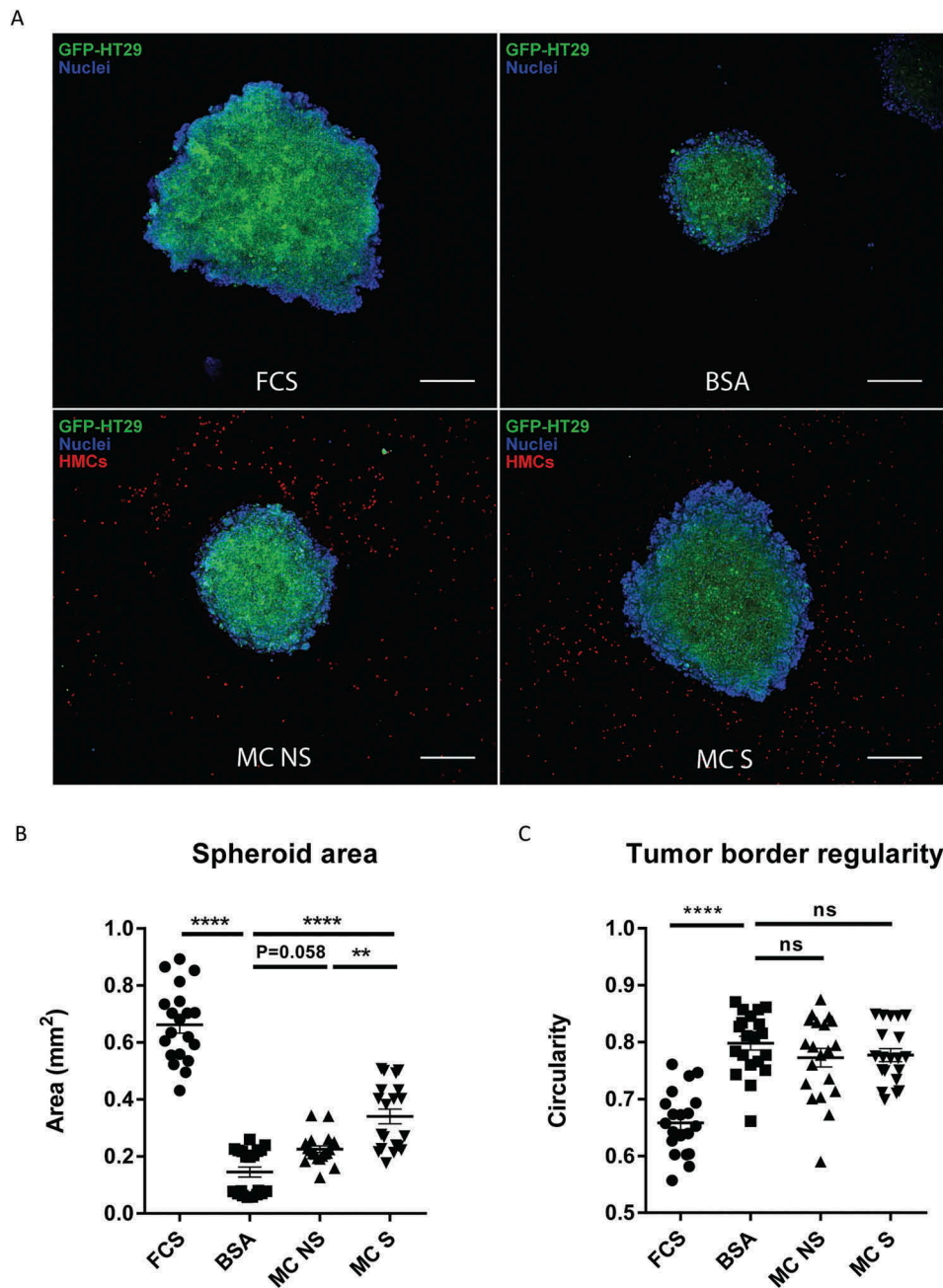


Figure 5. TLR2-stimulated MC induce stronger growth of colon cancer in a 3D spheroid model.

(a) Representative confocal images of HT29 cancer spheroids cultured in 10% FCS medium, 1% BSA medium or 1% BSA medium in presence of non-stimulated human MC (MC NS) or FSL-1 stimulated human MC (MC S) for 6 days. GFP transfected HT29 cells are shown in green, cell nuclei are shown in blue and CMTPX-labeled MC in red. (Scale bar, 200µm). (b) Spheroid area of HT29 as an indicator of cancer growth. (c) Border regularity of HT29 spheroid as an indicator of cancer invasiveness. Border regularity was calculated in a formula: 4π (spheroid area/spheroid perimeter²). Data are displayed as mean \pm SEM of two independent experiments (n=2). **P < 0.005; **** P < 0.0001, assessed by one-way ANOVA, Tukey's multiple comparisons.

cancer growth when stimulated by an inflammatory signal, such as TLR2 ligands.

The major characteristic to define malignant cancers is their invasiveness. In order to evaluate the invasion capacity of HT29 spheroids, we calculated their circularity and a lower value (0–1) predicts a better invasiveness ability. HT29 spheroids exhibited an invasive phenotype in 10% FCS medium, while a smooth border remained in 1% BSA medium (HT29 FCS = 0.66, HT29 BSA = 0.80, P < 0.0001) (Figure 5(c)). Nevertheless, we observed no significant difference in

circularity between spheroids cocultured with MC and control spheroids (Figure 5(c)), indicating that in this setting MC have no direct effect on the invasiveness of colon cancer cells. To investigate whether cell-cell contact is necessary for MC-induced colon cancer growth, we reconstructed a 3D view of the spacious relationship between human MC and HT29 spheroid by the end of coculture (Supplemental video). From every direction of the coculture model, no direct cell-cell contact was observed between HT29 and human MC. This indicates that cell-cell contact with cancer cells is not

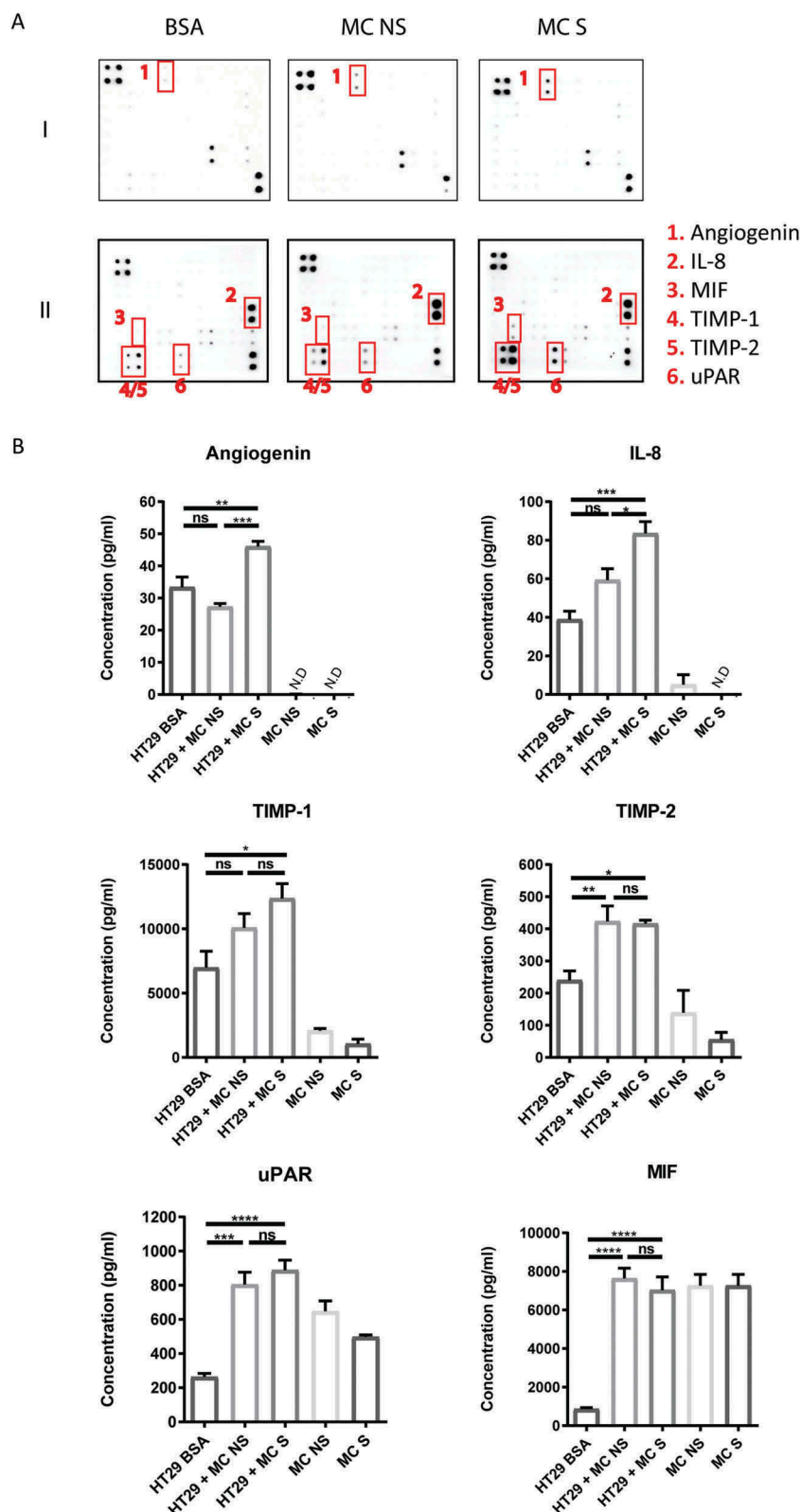


Figure 6. Coculture of colon cancer spheroids with MC induces production of various cytokines.

(a) Human antibody cytokine arrays to assess mediators secreted from HT29 spheroids cultured in 1% BSA medium with or without non-stimulated human MC (MC NS) or FSL-1 stimulated human MC (MC S) for 6 days. Increased levels of angiogenin, IL-8, MIF, TIMP-1, TIMP-2 and uPAR were detected in human MC-HT29 coculture compared with HT29 spheroids alone (BSA). MIF: macrophage migration inhibitory factor; TIMP-1/2: tissue inhibitor of metalloproteinases-1/2; uPAR: urokinase receptor. (b) ELISA assays to quantify the concentration of angiogenin, IL-8, MIF, TIMP-1, TIMP-2 and uPAR secreted from HT29 spheroids alone, human MC alone and human MC-HT29 coculture. Data are displayed as mean \pm SEM of two independent experiments (n=2). *P < 0.05; **P < 0.005; ***P < 0.001, ****P < 0.0001, assessed by one-way ANOVA.

needed for MC to promote tumor growth and other mechanism, such as secreted mediators, may be involved in the cellular communication.

Cytokines produced in 3D MC-colon cancer spheroid coculture

To dissect the mediator profile responsible for MC-HT29 crosstalk, we measured 108 cytokines/chemokines in the supernatant of 3D coculture by antibody arrays. Increased levels of angiogenin, IL-8, MIF (macrophage migration inhibitory factor), TIMP-1/2 (tissue inhibitor of metalloproteinases) and uPAR (urokinase receptor) were detected in the coculture compared with HT29 alone (Figure 6(a)). ELISA assays were then carried out to quantify the concentration and assess the source of these mediators (Figure 6(b)).

Stimulation of MC with FSL-1 led to higher levels of angiogenin and IL-8 released from the coculture with HT29 ($P < 0.005$), while there was no significant difference observed between HT29 alone and HT29 cocultured with non-stimulated MC (Figure 6(b)). Only little angiogenin and IL-8 was released by human MC alone, suggesting that the source of these mediators was mainly HT29 (Figure 6(b)). Furthermore, the increase of angiogenin and IL-8 was not due to the direct action of FSL-1 on HT29 (Supplemental Figure 2).

Coculture with MC led to higher levels of secreted TIMP-1 and TIMP-2 (Figure 6(b)). Notably, human MC themselves were able to secrete measurable levels of TIMP-1 (2×10^5 cells – $2,078 \pm 181.6$ pg/ml) and TIMP-2 (2×10^5 cells – 139.3 ± 69.31 pg/ml) (Figure 6(b)), implying that levels of TIMP-1/2 in the coculture were contributed by both HT29 and MC. Similarly, 3-fold higher levels of uPAR were detected in the coculture and human MC constitutively secreted uPAR (2×10^5 cells – 647.3 ± 61.69 pg/ml) (Figure 6(b)). Interestingly, HT29 themselves secreted low amounts of MIF (Figure 6(b)). High levels of MIF in the coculture may be contributed by MC, as they constitutively secreted high amounts of MIF (2×10^5 cells – $7,263 \pm 583.5$ pg/ml) (Figure 6(b)).

Deregulated genes in colon cancer-cocultured MC

Transcriptome analysis can provide further molecular insight into how colon cancer cells may educate MC as a pro-tumor player. Therefore, we compared the transcriptomic signature of MC cocultured with HT29 spheroids versus MC cultured only in ECM without HT29. Log₂-transformed gene expression fold change (log₂FC) in HT29-cocultured MC (CCS) was calculated over control MC (CTR). At the specified significance level of corrected P value < 0.05 and log₂FC > 0.2 , expression of 281 genes was found deregulated in CCS compared with that in CTR (Figure 7(a)). Notably, 5 upregulated genes (NOTCH1, PTGS2, PTGER4, VEGFA, MMP2) (Figure 7(b)) and 1 downregulated gene (ITGA3) (Figure 7(b)) were predicted as possible MC marker genes associated with HT29 spheroid coculture (Supplemental table 2).

We identified various canonical pathways which are enriched for these MC marker genes. Intriguingly, the Notch1 – NF- κ B – COX2 canonical pathway was found enriched in CCS (Supplemental Figure 3). RelA, a nuclear

factor NF- κ B subunit, was upregulated in the gene expression analysis (Figure 7(b)) and its activity can be triggered by Notch1 signaling, leading to upregulated PTGS2 (also known as COX-2, prostaglandin-endoperoxide synthase 2) (Figure 7(b)). PTGER4 (prostaglandin E receptor 4), a gene encoding EP4, was also upregulated (Figure 7(b)), which supports an increased activity of PGE₂-EP4 interaction. HT29 coculture induced upregulated VEGFA in MC (Figure 7(b)), which could be a downstream product of Notch1- NF- κ B canonical pathway (Supplemental Figure 3). In addition, HT29 promoted gene expression of PDGFA (platelet derived growth factor subunit a) and MMP2 (matrix metalloproteinase 2) in MC (Figure 7(b)), which are the factors supporting tumor growth and metastasis. Another pathway, integrin-Rho-Citron, was found enriched, as supported by downregulation of ITGA2/3 (integrin alpha-2/3), RHOB (ras-related homology B) and CIT (citron) (Figure 7(b)). The downregulation of integrin-Rho-Citron pathway indicates lower level of cytokinesis in HT29-associated MC (Supplemental Figure 4).

Discussion

In this study, we have used coculture models of primary human MC and colon epithelial cancer cells HT29 and Caco-2 to investigate the effect of MC on colon cancer development. To investigate the spatial interaction and molecular mechanism of MC-colon cancer communication, we developed an advanced *in vitro* 3D coculture model. Our experiments demonstrate a bidirectional communication between MC and colon cancer cells leading to MC attraction toward cancer cells, release of pro-tumorigenic mediators and increased cancer cell proliferation.

Prolonged low-grade inflammation is a hallmark of cancer and MC play a central role in the inflammatory response associated with cancer.⁷ MC density in CRC tissues is positively associated with poor clinical outcomes in patients^{10–13,15,17} and decreased intestinal tumor growth has been observed in MC-deficient mice.^{28,29} Our study further provides experimental evidence that primary human MC promote colon cancer growth by stimulating production of pro-tumorigenic mediators in a bidirectional manner. Thus far, most studies in human MC-cancer interaction were using MC cell lines (LAD2 and HMC-1)^{30–35} with specific limitations that these cell lines are leukemia-derived (e.g. obtain a nature of cancer cells) and hardly express MC proteases or MC-specific receptors.³⁶ 77% of infiltrating MC in human CRC tissues are found to express both trypsin and chymase, which are considered as MC_{TC}.¹⁶ Moreover, Khan *et al* have observed that submucosal MC, but not mucosal MC, increased as the disease progressed from colitis to invasive cancer in patients.³⁵ In the current study, we used primary human MC derived from peripheral CD34⁺ cells, which may better represent qualities of CRC-infiltrating MC subtype (MC_{TC} or submucosal MC) including the expression of Fc ϵ RI, trypsin and chymase.³⁷

We show that mediators released from colon cancer cells (Caco2 and HT29) induced human MC chemotaxis. These findings are consistent with clinical observations, where high

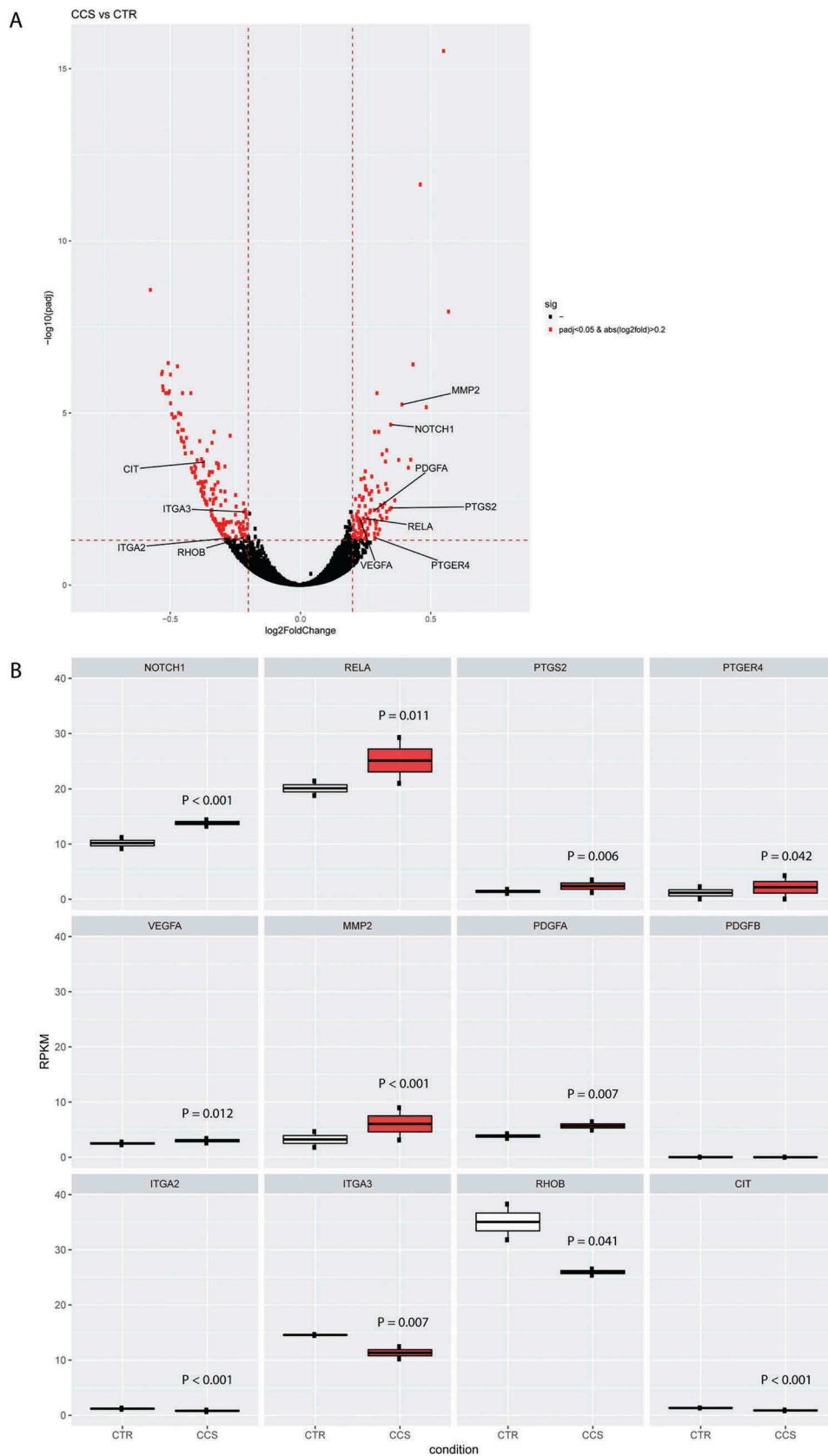


Figure 7. Deregulated genes in MC cocultured with colon cancer spheroids.

(a) Differentially expressed genes in MC cocultured with HT29 spheroids (CCS) versus MC alone (CTR). Genes labeled in red were significantly differentially up or down regulated, respectively. MMP2: matrix metalloproteinase 2; PDGFA: platelet derived growth factor subunit a; PTGS2: prostaglandin-endoperoxide synthase 2; RELA: NF- κ B subunit P65; PTGER4: prostaglandin E receptor 4; VEGFA: vascular endothelial growth factor A; ITGA2/3: integrin alpha-2/3; CIT: citron; RHOB: ras-related homology B. The P values were computed by Wald test and corrected by the Benjamini-Hochberg procedure. The horizontal dash-line denotes the significance level of $\alpha = 0.05$ ($n=2$). The vertical dash-line denotes the value of \log_2 -transformed gene expression fold change (\log_2FC) = 0.2. (b) Gene expression (Reads Per Kilobase of transcript, per Million mapped reads, RPKM) of MC associated genes were compared between CTR and CCS. The P values were computed by Wald test and corrected by the Benjamini-Hochberg procedure ($n=2$).

infiltration of MC has been observed in human colon cancer.^{10–13,15–17} MC migration toward Caco2 was observed solely SCF-dependent. SCF is an important growth factor for MC, but also induces chemotaxis and survival.³⁸ The importance of SCF in attracting MC into tumor environment was previously demonstrated by the migration of mouse primary MC toward mouse H22 hepatocarcinoma cells.¹⁸ Surprisingly, HT29 colon cancer cells induced MC chemotaxis partly via the secretion of CCL15, while no SCF-induced migration was involved. This may reflect the intrinsic biological difference among individual colon cancers. HT29, but not Caco2, is a SMAD4-deficient colon cancer cell and loss of SMAD4 correlates with elevated CCL15 expression.^{39,40} This is of particular interest, because in a mouse xenograft model, CCL15 secreted from SMAD4-deficient colon cancer cells recruited CCR1⁺ cells, resulting in aggressive tumor growth and metastasis. In the same study, it was found that CCL15 positively correlated with a worse survival in patients with CRC.^{39,40} Human MC express surface receptors for CCL15, which are CCR1 and CCR3.¹⁹ Therefore, based on our current data it is reasonable to speculate that MC may be among those infiltrating CCR1⁺ cells and subsequently contribute to colon cancer growth. This notion is consistent with a study in APC^{Δ8,41} mouse model, where they suggest MC represent CCR1+ immature myeloid cells in the tumor invasive front.²⁸ Subsequent RNA sequence analysis revealed a less proliferative profile of MC when cocultured with colon cancer cells (Supplemental Figures 4 and 5), implying the increased MC density in colorectal cancer tissues might be due to migration rather than local proliferation.

It has been shown that murine MC promote colon cancer growth, as illustrated by MC-deficient mouse models^{28,29} and the coculture of primary murine MC and mouse colon cancer CT26.¹⁷ We present for the first time that primary human MC can directly support human colon cancer proliferation. Importantly, this effect does not solely depend on mediators secreted by MC without receiving signals from cancer cells. Although Khan *et al* suggested the supernatant of LAD2 is enough to support HT29 colon cancer proliferation,³⁵ in our setting the conditioned supernatant of primary human MC has a much weaker effect on promoting cancer proliferation as compared to the direct coculture, suggesting that the mutual communication between MC and colon cancer cells enhances cancer proliferation. In other words, MC likely receive signals from colon cancer cells and further promote cancer survival and proliferation. Indeed, MC showed upregulated MMP2, VEGFA and PDGFA in gene expression when cocultured with HT29 spheroid. In addition, gene ISG15 (IFN-stimulated factor 15), which is found as a support factor for cancer stem cells in the tumor microenvironment,⁴² was also upregulated in HT29-cocultured MC (Supplemental Figure 6).

A variety of inflammatory factors present in tumor microenvironment can activate MC.⁷ Among those activators, TLR2 ligands, such as laminin-β1, HMGB1 and S100A9, are upregulated in CRC and have been proposed as biomarkers to monitor cancer progression.^{43,44} Furthermore, many TLR2 expressing cells are present in CRC stroma.⁴⁵ In line with the data of Varadaradjalou *et al*,⁴⁶ we show that primary

human MC constitutively express TLR2 (Supplemental Figure 1B). To mimic the inflammatory microenvironment in tumor, we pre-stimulated human MC with TLR2 agonist FSL-1 and co-cultured these MC with HT29 spheroids. It has been reported that TLR2 agonists induce the release of TNF-α, IL-5, IL-10 and IL-13 by human MC.⁴⁶ Nevertheless, we did not detect measurable amount TNF-α, IL-5, IL-10 and IL-13 in the 3D coculture, which might be due to the different source of cultured human MC. Interestingly, TLR2-primed MC significantly enhanced angiogenin and IL-8 production in the co-culture with HT29. We have excluded the possibility of the direct action of FSL-1 on HT29, as illustrated by lack of TLR2 expression in HT29 (Supplemental Figure 1B) and no increase of angiogenin and IL-8 from HT29 stimulated directly with FSL-1 (Supplemental Figure 2). Moreover, secreted IL-8 and angiogenin was not detectable in TLR2-primed MC (Figure 6(b)), and gene expression of CXCL8 (gene coding IL-8) and ANG (gene coding angiogenin) was not upregulated in TLR2-primed MC in coculture with HT29 (data not shown). Therefore, it is reasonable to speculate that the enhanced growth of colon cancer causes increased production of IL-8 and angiogenin. Further RNA sequence analysis revealed that ALOX5 (gene coding 5-lipoxygenase, 5-LOX) was upregulated in TLR2-primed MC in coculture with HT29 (Supplemental Figure 7) and 5-LOX is a key enzyme for leukotriene synthesis. This is of particular interest, as increased expression of 5-LOX promotes colon cancer cell proliferation in a direct manner.^{41,47} However, more work is needed to determine how MC support colon cancer growth via 5-LOX.

Increased cytokines produced in MC-HT29 co-culture correlate with clinical significance. For instance, increased TIMP-1 and TIMP-2 are associated with a poor prognosis in patients, which are produced by colon cancer cells and adjacent stroma cells.^{48–50} Notably, our data reveal for the first time that primary human MC are able to express and secrete both TIMP-1 and TIMP-2. In addition, uPAR expression, especially in tumor-associated stromal cells, is negatively associated with CRC patient survival⁵¹ and MIF can promote intestinal inflammation and colonic carcinogenesis.⁵² Consistent with the finding of Rossi *et al*,⁵³ we show that primary human MC constitutively produce uPAR and are the major source of MIF. Collectively, these findings reflect the capacity of MC to support the tumor microenvironment favoring colon cancer growth and implies a correlation between MC infiltration and colon cancer progression.

The upregulation of NOTCH1 signaling suggests the presence of notch1 ligands in the MC-HT29 coculture. It has been reported that notch1 ligands (e.g. Jagged1) are highly expressed in colorectal cancer tissues.⁵⁴ Since no cell-cell contact was detected in the coculture of MC with HT29 spheroid, we speculate that the upregulated NOTCH1 signaling may be induced by soluble form of ligands, where the shedding extracellular domain of notch1 ligands activate notch1 signaling upon clustering.⁵⁵ Although our RNA sequence data suggest NOTCH1 induces upregulation of COX2 and VEGFA in MC, the contribution of notch1-mediated MC activation to cancer growth is not yet explored. Consistent with the gene expression, VEGF-A is also found

upregulated in the coculture supernatant by cytokine arrays (Supplemental Figure 8). These findings together support MC as a potent source of VEGF-A and point to a direct contribution of MC to tumor angiogenesis.^{56,57} PGE₂ is one of the products from upregulated COX-2 activity. Intriguingly, PGE₂ can strongly induce MC to produce VEGF-A without causing degranulation.^{58,59} This suggests the upregulated VEGF-A production can also be induced by increased PGE₂ activity in MC themselves. Besides, PGE₂ induces activation and expansion of both Tregs and MDSC (myeloid-derived suppressor cells) and thus supports immunosuppression in cancer milieu.⁶⁰ Human MC express multiple PGE₂ receptors EP(2–4) with major expression of EP2 and EP3.⁶¹ EP2 and EP4 have been reported as inhibitory receptors for MC activation.^{59,62} Further research is needed to understand the biological meaning of upregulated PTGER4 (gene coding EP4) in colon cancer-cocultured MC. Collectively, this supports the concept that in the early phase of cancer formation, colon cancer cells may stimulate MC to produce various pro-tumorigenic mediators in order to create a milieu which favors cancer growth and angiogenesis and supports immunosuppression.

In conclusion, a better understanding of MC-colon cancer interaction may provide therapeutic benefit in CRC treatment. The 3D cancer spheroid coculture model with primary human MC potentially serves as a promising tool to study the role of human MC in various cancer. In future studies, the 3D multicomponent coculture should be developed by incorporation of other stromal/immune cells to further validate the interaction of MC in the complexed tumor microenvironment. Our study supports the detrimental role of MC in CRC development and provides a molecular insight into the

bidirectional communication between human MC and colon cancer cells.

Materials and methods

Peripheral autologous hematopoietic stem cells derived from patients were used after written informed consent as approved by the ethics committee (TCBio 16–089) of the Utrecht Medical Center, Utrecht, the Netherlands, in accordance with the Declaration of Helsinki (59th WMA General Assembly, Seoul, October 2008), and in compliance with guidelines from the Ethical Committee and European Union legislation.

Generation of primary human MC

CD34⁺ derived human MC were generated from surplus autologous stem cell concentrates as previously described by Schmetzer et al.³⁷ Briefly, frozen stem cell concentrates were rapidly thawed at 37°C under sterile conditions, and poured in a large cell culture flask (Greiner, 661195). 20% human serum albumin clinical solution (HSA) (Sanquin), 6% hydroxyethyl starch clinical solution (Braun) and RMPI containing 10 U/ml Heparin (LEO pharma) were then added slowly and consecutively to the cell concentrate. Cells were then filtered through a cell dissociation sieve (Sigma, CD1-1KT) and incubated with 200 U.I./ml DNase (Roche, 04536282001) for 15 min. After washing, cells were resuspended in PBS containing 4% HSA and incubated with Fc-Block (Miltenyi, 130–059-901) for 15 min, CD34⁺ positive selection cocktail (StemCell, 18056) for 15 min and nanoparticles for 10 min. Subsequently, CD34⁺ cells were sorted with an EasySep[®] Magnet (StemCell, 18000) according to the manufacturer's protocol. Finally, sorted cells were resuspended in serum-free expansion medium (SFEM) (StemCell, 09650) supplemented with human LDL (50 µg/ml) (StemCell, 02698). On day 1, human recombinant IL-3 (100ng/ml) (Biolegend, 578006) and SCF (100ng/ml) (Miltenyi, 130–096-695) were added. Every three to four days, IL-3 and SCF were added to a final concentration of 20ng/ml. At the end of the second week, MC were maintained under 20ng/ml SCF with the withdrawal of IL-3. From day 17 on, cells were ready to use. They were further purified using a dead cell removal kit (Miltenyi, 130–090-101) followed by CD117 positive selection (Miltenyi, 130–091-332) according to the manufacturer's protocol. The purity of human MC was assessed by flow cytometry based on the surface expression of CD117 (eBioscience, 17–1178-42) and FcεR1α (eBioscience, 25–5899-42) using a BD FACSCanto II (Becton Dickinson, Vianen, the Netherlands).

β-hexosaminidase assays

Human MC were activated with anti-human FcεR1α MAb (eBioscience, 25–5899-42) in varying concentrations of 1.56 – 25 µg/ml at 37°C for 1 hr. Cells were lysed with 5% Triton X-100 in order to quantify the total β-hexosaminidase (β-hex) content. Supernatant was collected and incubated with 200 µM 4-methylumbelliferyl-β-d-glucopyranoside (4-MUG) at 37°C for 1 hr. Enzyme reaction was then terminated by

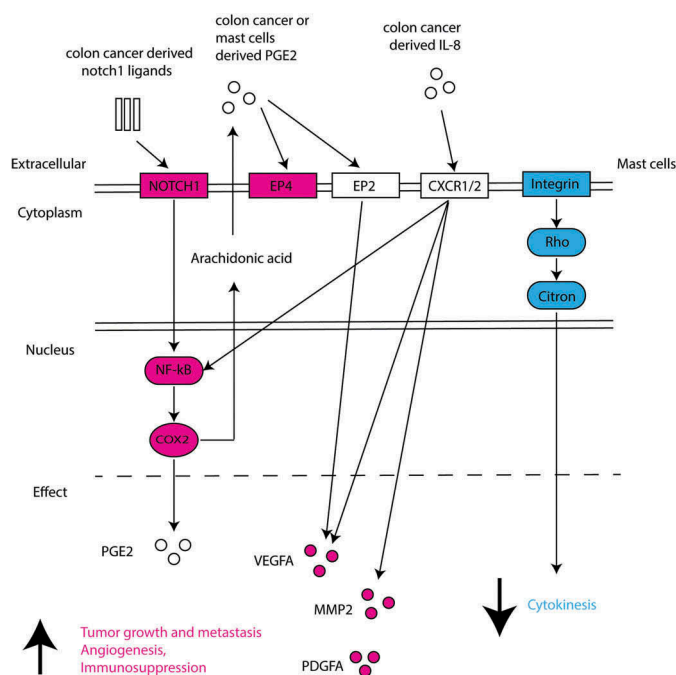


Figure 8. Schematic overview of deregulated genes and associated pathways in colon cancer-cocultured MC.

Differentially upregulated genes are shown in red and downregulated genes in blue. Predicted pathways and effects are presented.

adding 0.1M glycine buffer. The β -hex content was quantified by measuring fluorescence at $\lambda_{\text{exc}}360/\lambda_{\text{em}}460$ nm. The percentage of β -hex release was calculated as: $(A - B)/(T - B) \times 100\%$, where A is the amount of β -hex released from stimulated cells, B is that released from unstimulated cells, and T is total β -hex content released from the positive control.

Colon cancer cell lines

Human colon cancer cell lines, HT29 and Caco2, were obtained from American Type Tissue Culture Collection. HT29 were grown in McCoy medium (Gibco) supplemented with heat-inactivated 10% fetal calf serum (FCS), and 100 $\mu\text{g}/\text{ml}$ pen/strep (penicillin and streptomycin, Gibco, 26600080). Caco2 were cultured in EMEM medium (Lonza, BE12-611F/12) supplemented with 10% FCS, 100 $\mu\text{g}/\text{ml}$ pen/strep, 1% non-essential amino acids, 1% L-glutamine and 1% sodium pyruvate (Gibco, 11360-039). All cells were cultured in a humidified 37°C/5% CO₂ incubator. Green fluorescent protein (GFP) expressing HT29 were generated by transfection with pMONO-neo-GFP plasmid containing GFP and neomycin resistance genes (Invivogen, pmonon-gfp) by electroporation (Nucleofector, Lonza, Belgium). GFP-transfected HT29 were then selected under Geneticin (Gibco, 10131035).

Preparation of conditioned medium

For the production of colon cancer (HT29- and Caco2-) conditioned medium, cells were grown to 100% confluency in standard medium. This medium was removed and replaced with serum-free medium for 48 hr. The conditioned medium was then collected, centrifuged and used for migration assays. For the production of human MC supernatant, $2 \times 10^5/\text{ml}$ cells were suspended in the serum-free medium of colon cancer cells (McCoy or EMEM, respectively) supplemented with 1% BSA (Sigma, A4161) and 20ng/ml SCF for 24 hr. Subsequently, MC-conditioned supernatant was collected, centrifuged and used for proliferation assays.

Chemotaxis assay

The upper chamber of 5- μm transwell 96-well plate was loaded with CD34⁺ derived human MC in serum-free medium. The lower chamber was filled with HT29- or Caco2-conditioned medium with or without SCF blocking Ab (PeproTech, 500-P48) or CCL15 blocking Ab (R&D, AF628) or isotype Abs at concentrations of 1.25- 5 $\mu\text{g}/\text{ml}$. After 4 hr, the filter inserts were removed and the number of MC migrating into the lower chamber was quantified by flow cytometry.

Cancer proliferation assay

2×10^3 cells/well colon cancer cells were seeded and grown in 96-well plate for 24 hr. The standard medium was removed and replaced with either serum-free medium containing human MC at various ratio or MC-conditioned supernatant. The cells were then cocultured for 72 hr. Followed by the removal of MC suspension, DNA synthesis of colon cancer

cells was measured by BrdU ELISA kit (GE Healthcare, RPN250) according to the manufacturer's protocol.

Colon cancer spheroid

Multicellular cancer spheroids were formed using the hanging drop method⁶³. Briefly, drops of 250 GFP-transfected HT29 cells in 20 μl were made in standard medium and incubated upside-down for 4 days. Cancer spheroids were then embedded in extracellular matrix (ECM) as previously described⁶⁴. Shortly, ECM mixture was prepared on ice containing Matrigel (Corning, 356231) and non-pepsinized rat-tail collagen type I (2.3mg/ml) (Corning, 354249) at a ratio of 1:1 or 1:100. Spheroids suspended in 40 μl of pre-heated culture medium were mixed with 200 μl of 1:1 ECM (Matrigel: Collagen type I). 50 μl drops of spheroid-ECM mixtures were then placed in a 24-well plate previously coated with 1:100 ECM (Matrigel: Collagen type I) base layer. For full polymerization of the gel, plates were incubated at 37°C for 1hr. Subsequently, 1ml coculture medium with or without human MC ($2 \times 10^5/\text{ml}$) was added. TLR2-stimulated MC was pre-treated with FSL-1 (1 $\mu\text{g}/\text{ml}$) for 4 hr. MC were stained with cell tracker CMPTX dye (ThermoFisher, C34552) prior to the coculture. After 6-day coculture, GFP-HT29 spheroids were stained with Hoechst to visualize cell nuclei (Life technologies, H1399) and imaged using confocal microscopy (Yokogawa CV7000, Wako, San Diego, USA). Spheroid area and invasion were analyzed using ImageJ software.

Cytokine array

Medium was collected from cocultures of HT29 spheroids and human MC or from cultures of HT29 spheroids alone at the end of incubation. Samples were centrifuged (10,000 rpm, 2 min) to remove particulates and the medium was used for measuring cytokines by multiplexed protein detection using a human cytokine antibody array C1000 (RayBiotech, AAH-CYT-1000) according to the manufacturer's instructions (the map of cytokine arrays is provided as supplemental table 1).

Elisa

Medium was collected from the coculture or the culture of HT29 spheroids or human MC alone at the end of incubation. Samples were centrifuged (10,000 rpm, 2 min) to remove particulates and the medium was used for quantification of Angiogenin, IL-8, MIF, TIMP-1, TIMP-2 and uPAR by ELISA (Thermo Scientific) according to the manufacturer's instruction.

Transcriptome analysis of spheroid cultured mast cells

To obtain RNA samples from MC in the spheroid coculture, 1mg/ml collagenase/dispase (Sigma, 10269638001) was added to the ECM mixture and incubated at 37°C for 1hr. Cells were then washed with PBS supplemented with 1% BSA and subsequently stained with YO-PRO1 and antibodies for CD117 and Fc ϵ RI α . CD117 and Fc ϵ RI α double positive viable cells

were sorted using BD Influx™ cell sorter (BD Biosciences, San Jose CA, USA). Control MC cultured in the EMC in absence of HT29 spheroid were stained and sorted in an identical way as HT29-cocultured MC. RNA of purified MC was isolated using PureLink RNA mini kit (ThermoFisher, 12183025), according to the manufacture's protocol.

RNA samples of human MC were sequenced on an Illumina Nextseq500 platform according to the manufacture's procedure by the Utrecht Sequencing Facility of the Utrecht University (<http://www.useq.nl/>). Sequencing libraries were generated using TruSeq Stranded mRNA poly A kit. Sequence reads were checked for quality by FastQC (v0.11.4) after which reads were aligned to GRCh37 using STAR (v2.4.2a) and read groups were added using Picard (v1.141). All samples passed QC and were subsequently processed using HTSeq-count (v0.6.1) on ENSEMBL gene definitions (GRCh37, release 74). R statistical software was used to identify deregulated genes. The P values were computed by Wald test and corrected by the Benjamini-Hochberg procedure. Transcript with a log₂-transformed gene expression fold change (log₂FC) > 0.2 and adjusted P value < 0.05 were deemed differentially expressed. Canonical pathways and MC marker genes were predicted by IPA software (Ingenuity Pathway Analysis, QIAGEN).

Statistical analysis

Statistical analyses were performed using Graphpad Prism 7 (version 7.03) statistical program, SPSS or R statistical software. Comparison of groups were assessed using the Student's t test or ANOVA, where appropriate. P values < 0.05 were considered statistically significant.

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Disclosure of interest

The authors report no conflict of interest.

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