Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration

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Abstract  Tumour-associated macrophages (TAMs) of the M2 phenotype are present in the stroma of many tumours and are frequently associated with the progression of several types of cancer. We investigated the role of M2 macrophages in colon cancer progression and found that human colon cancer tissue had elevated numbers of CD68⁺ (macrophage marker) cells and CD206⁺ (M2 macrophage marker) cells and increased CD47 expression. To explore potential interplay between colon cancer cells and M2 macrophages, we differentiated the monocyte cell line THP-1 into M1 and M2 macrophages (CD206high and Th2 cytokine-secreting cells), respectively. M2 macrophages migrated faster than M1 macrophages towards SW480-conditioned medium. Similarly, M2 macrophage-conditioned medium induced SW480 cell migration and CD47 expression. Factors released by macrophages were involved in this induction. In addition, SW480 cells migrated faster when co-cultured with M2 macrophages. Inhibition of CD47 with blocking antibodies or siRNA significantly reduced the migration of SW480 cells in the presence of M2 macrophages. This effect was further decreased via blocking antibodies against the CD47 ligand signal-regulatory protein α (SIRPα). Additionally, cancer cells also secreted significant levels of IL-10, thereby promoting M2 macrophage differentiation. These findings indicate that a TAM-enriched tumour microenvironment promotes colon cancer cell migration and metastasis.

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1. Introduction

Colorectal cancer is the third most commonly diagnosed cancer in the world. Patients suffering from inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis, have an elevated risk of developing colon cancer. In recent years, the tumour microenvironment has been highlighted as an important hallmark of cancer. In tumour microenvironments, macrophages represent a major inflammatory component of the stroma and affect many aspects of the neoplastic tissue. Tumour-associated macrophages (TAMs) play an important role in cancer progression, and accordingly, high levels of macrophage infiltration of the tumour tissues are associated with poor prognosis in cancer patients. Intratumoural macrophages that express the macrophage markers CD68 and CD163 are enriched in colon carcinoma and may promote tumourigenesis; however, the mechanism of such promotion remains unclear. Evidence indicates that intratumoural macrophages are recruited by tumour cells. The tumour microenvironment is thought to induce the tumour-promoting M2 macrophage phenotype. Therefore, specific visualisation of M2 macrophages in colon cancer tissues is crucial.

Macrophages produce a wide array of cytokines and lipid mediators, such as prostaglandins and leukotrienes (LTs). M1 macrophages are pro-inflammatory and potentiate Th1 responses by secreting high levels of interleukin (IL)-1, IL-12, interferon (IFN)-γ and other Th1 cytokines, whereas M2 macrophages secrete high levels of IL-8 and Th2 cytokines, such as IL-4, IL-10 and IL-13, which contribute to the maintenance of an immunosuppressive microenvironment. It has been documented that the prostaglandin E2 increases migration and invasion of colon cancer cells. The pro-inflammatory cysteinyi leukotriene D4 (LTD4) has also been implicated in colon cancer progression, and we have shown previously that LTD4 increases proliferation, migration and survival in colon cancer cells via CysLT1 receptor (CysLT1R) signals. Equally important, we have shown that colon cancer patients with high CysLT1R expression in their tumour tissues have a poor prognosis. Therefore, we hypothesise that LTD4 released from M2 macrophages influences colon cancer cell behaviour.

Apart from soluble factors, cell surface-bound molecules regulate the interaction between intratumoural macrophages and colon cancer cells. One such molecule that was recently shown to be commonly expressed on cancer cells is CD47. CD47 was first described as an anti-phagocytosis molecule based on its interaction with signal regulatory protein alpha (SIRPα), which is expressed on the surface of macrophages. In the context of cancer, such a mechanism could enable cancer cells to escape the immune system. The interaction between CD47 and SIRPα has also been suggested to contribute to cell migration. For example, CD47 has been shown to control the migration of canine kidney epithelial cells and intestinal epithelial cells grown on a collagen I-coated surface. Inhibition of CD47–SIRPα interactions with a CD47 blocking antibody may be a potential therapeutic approach; however, the precise role of CD47 in colon cancer cell migration and metastasis remains to be determined.

In this study, we report that M2-like macrophages secrete immunosuppressive cytokines to create a microenvironment that promotes colon cancer cell growth and migration. In addition, colon cancer cells exhibit elevated CD47 levels, which can be influenced by macrophages, and secrete high levels of IL-10, enhancing the M2 macrophage phenotype. Increased CD47–SIRPα signalling between colon cancer cells and M2-like macrophages promotes cancer cell migration. These findings highlight the importance of TAM activity in the colon cancer microenvironment.

2. Materials and methods

2.1. Cell culture, stimulation and differentiation

The human colon cancer cell line SW480, HCT-116 and human monocyte cell line THP-1 (obtained from ATCC) were cultured following the supplier’s instructions. SW480 cells were stimulated with 20 ng/ml IL-4, 80 nM LTD4, 100 ng/ml IL-8 or M2 macrophage-conditioned medium for 24 h at 37 °C after overnight serum starvation. THP-1 cells were differentiated by treatment with 100 nM phorbol-12-myristate-13-acetate for 7 days. The adhered cells were further differentiated with 100 ng/ml lipopolysaccharide for 72 h and 20 ng/ml IFN-γ during the last 48 h (M1) or 20 ng/ml IL-4 for 48 h (M2; Fig. 2A). For multiplex assays, enzyme-linked immunosorbent assays (ELISAs), and conditioned medium collecting, the differentiated macrophages were washed with serum-free medium to remove the differentiation factors and cultured in 1.5% foetal bovine serum (FBS)-containing medium for an additional 3 days.

2.2. Patient samples

Formalin-fixed and paraffin-embedded colon cancer and control colon specimens from colorectal cancer patients were obtained from the archives of the Department of Pathology at Malmö University Hospital. Tissues from 72 patients with varying grades and stages of disease were included. Staging of the tumours was done using Dukes’ classification. Fresh biopsies were obtained for mRNA analysis. The biopsy samples were placed in RNAlater (Qiagen, Hilden, Germany) and frozen by submersion in liquid nitrogen. The matched control samples from normal colon tissues were surgical specimens from the same patients. Specimens were
Fig. 1. CD68 and CD206 expression in colon cancer tissues. (A) mRNA expression of CD68 and CD206 in normal and tumour tissues from colon cancer patients. (B and E) CD68 and CD206 immunohistochemistry staining. (C, D, F, and G) Number of CD68+ and CD206+ cells in tissues from colon cancer patients. (H) Kaplan–Meier survival curve using log-rank test. Numbers of CD206+ cells in tumour tissues were compared to normal tissues. The results of mRNA expression analysis are shown as the mean ± standard error of the mean (SEM) of four separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test).

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Fig. 2. Differentiation and cytokine profiles of M1 and M2 macrophages derived from the human monocyte cell line THP-1. (A) Schematic diagram showing the in vitro differentiation of THP-1 cells into M1 and M2 macrophages. Images show CD68 expression during differentiation. (B) The mRNA expression levels of CD68 and CD206. (C) Histogram overlay from flow cytometry showing cell surface expression of CD68 and CD206 in M1- and M2-like macrophages. (D) Signal-regulatory protein α (SIRPα) expression in M1- and M2-like macrophages. (E) Cytokine profiles of SW480 cells, M1 or M2 macrophages as well as of co-cultures of SW480 with M1 or M2 macrophages by multiplex assay. The results are shown as the mean ± standard error of the mean (SEM) of 3–9 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (column statistics for B and D, two-tailed Student’s t-test for E).

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obtained with informed consent after ethical approval was granted by the Ethics Committee of Lund University.

2.3. Tumour xenograft study

The Regional Ethics Committee for Animal Research at Lund University, Sweden (M205-10) approved the present animal study. Female 6- to 8-week-old athymic nude mice (BalbCnu/C2) were purchased from Taconic Europe A/S (Ry, Denmark). To generate subcutaneous human colon cancer xenografts, 2.5 × 10⁶ low-passage HCT-116 cells suspended in 100 μl phosphate-buffered saline (PBS) were injected into both flanks of the mice (21 mice in total). Twenty-one days after tumour cell inoculation all mice were sacrificed, and the tumours were removed. Tumour tissues were immediately fixed in 10% buffered formalin and then embedded in paraffin for further immunohistochemical analysis.

2.4. Immunohistochemistry

Formalin-fixed, paraffin-embedded archival colon cancer human specimens were stained with CD68 (1:50; Biolegend, San Diego, CA, United States of America (USA)), CD206 (1:50; BD Biosciences, San Jose, CA, USA), CD47 (1:50; Biolegend, San Diego, CA, United States of America (USA)), CD206 (1:50; BD Biosciences, San Jose, CA, USA), CD47 (1:50; Biolegend, San Diego, CA, United States of America (USA)) antibodies. All stained tissues were manually counterstained with Mayer’s haematoxylin. Slides were scanned with the ScanScope CS (Aperio, Vista, CA, USA) at 10× magnification. NIS-Elements Advanced Research software (Nikon, Tokyo, Japan) and statistically analysed with SPSS software. The total F4/80 positive stained area per tumour was measured with Aeprio ImageScope software (Aperio, Vista, CA, USA).

2.5. RT-PCR

RNA from cells and tissue samples was isolated following the protocol of the Qiagen RNeasy Plus Mini Kit. The following primers were obtained from Applied Biosystems (Cambridge, United Kingdom (UK)): CD68 (Hs00154355_m1), CD206 (Hs00267207_m1), CD47 (Hs00964717_m1), and SIRPα (Hs00388955_m1). Amplification was performed in a Mx3005P system (Agilent Technologies, Inc., CA, USA), and reactions were analysed with MxPro software and normalised against the housekeeping gene HPRT1.

2.6. Immunofluorescent staining

THP-1 cells were attached to coverslips by centrifugation at 1000 rpm for 5 min. Fixation and blocking were performed as previously described. The cells were incubated with SIRPα, CD68, CD206 or mouse immunoglobulin G (IgG) antibodies (1:100) for 1 h, washed with PBS and incubated with Alexa 488 or Alexa 546 goat anti-mouse secondary antibody (1:500) for 1 h. For double staining, an extra blocking step was performed. After washing with PBS, the cells were incubated with DAPI (1:1000) for 3 min and mounted in fluorescent mounting medium (Dako, Glostrup, Denmark). The slides were photographed with a Nikon Eclipse 80i microscope using a PlanApo 60× objective and NIS-Elements Advanced Research software (Fig. 2B), an Olympus Fluoview FV10i Confocal Microscope using a 60× objective (Olympus Corporation, Tokyo, Japan; Fig. 3B), or a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany; Fig. 6A).

2.7. Flow cytometry

The differentiated M1 and M2 macrophages were detached using 0.02% versene and were washed twice in 0.5% bovine serum albumin (BSA)/PBS before blocking the human FcRs with 20 μg/ml heat-aggregated human IgG. Next, 0.5 × 10⁶ cells were suspended in 100 μl 0.5% BSA/PBS and incubated with 2 μg/ml CD68 antibody followed by incubation with Alexa 488 goat anti-mouse secondary antibody (30 min each at 4°C). Cells were incubated with 20 μl allophycocyanin-conjugated CD206 (BD Biosciences) or 5 μl SIRPα antibody (eBioscience). Cells were examined with a FACS-Calibur using the software Cell Quest (Becton–Dickinson, San Jose, CA, USA) and analysed using FCS Express Version 4 (De Novo Software, Los Angeles, CA, USA). Forward and side scatter gates were set to include all viable cells.

2.8. Co-culture of M1 or M2 macrophages with SW480 cells

SW480 cells were counted before adding pre-differentiated M1 or M2 macrophages at a ratio of 1:1 or 2:1 and cultured in 1.5% FBS-containing medium for 72 h. The conditioned medium was collected for a multiplex assay or ELISA. Medium containing 1.5% FBS was used as a negative control.

2.9. Measurement of secreted cytokines by multiplex assay

To remove cell debris, the conditioned medium was collected and centrifuged at 1000 rpm for 5 min.
Electrochemiluminescence assays were performed on macrophage-conditioned medium in duplicate using a 10-plex human TH1/TH2 detection kit (Meso Scale Discovery, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The plates were read using the Meso Scale Discovery SECTOR Imager 6000 and analysed using Discovery Workbench and SoftMax PRO 4.0 software.

Fig. 3. Education of M1-like macrophages. (A) Histogram overlay showing cell surface expression of signal-regulatory protein α (SIRPα) in M1 macrophages, M2 macrophages and M1 macrophages stimulated with IL-10 for 72 h and analysed by flow cytometry. (B) Representative immunofluorescence images showing expression and localisation of CD206 (green) and SIRPα (red) in M2 and M1 macrophages with/without IL-10 stimulation. (C) M1 macrophages co-cultured with SW480 cells. DAPI is shown in blue. Scale bar is 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.10. Transfection with CD47 siRNA oligomers

SW480 cells were cultured for 3 days to 50–60% confluence. The growth medium was aspirated, and the cells were washed with OPTI-MEM I (Invitrogen Corp., Carlsbad, CA, USA). Next, 5 ml of OPTI medium containing 75 nM siRNA against CD47 (ID #: 2811, 145978 and 145979) or a scrambled control siRNA (Ambion, Cambridgeshire, UK) was added to the cells along with lipofectamine 2000. After 4 h, the transfection medium was diluted with RPMI 1640 medium (10% FBS) without antibiotics, and the cells were allowed to grow for an additional 72 h with a medium change after 24 h.

2.11. Western blotting

Whole cell lysates were prepared, and Western blotting was performed as described. The polyvinylidene difluoride (PVDF) membranes were blocked for 1 h at room temperature with either 4% BSA/PBS-T (0.05% Tween 20) or 5% non-fat dried milk. The membranes were incubated overnight at 4 °C with anti-CD47 (MEM122, 1:1000; EXBIO, Prague, Czech Republic), stripped and reprobed with anti-β-actin (1:10,000) antibodies. The membranes were washed and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies diluted at 1:10,000. Proteins were detected after incubation with an Immun-Star Western Chemiluminescence Kit (BioRad, Hercules, CA, USA) using a Bio-Rad ChemiDoc XRS+ System. Bio-Rad Image Lab software was used for densitometric analysis, and the value obtained from the non-stimulated control was set to 100.

2.12. Wound healing assay

SW480 cells were serum starved for 2 h and incubated with or without 2 μg/ml IL-8 receptor CXCR1 antibody (Abcam, Cambridge, UK), 50 μM CysLT1R inhibitor ZM198,615 (AstraZeneca, R&D Lund, Sweden), or 20 μg/ml anti-CD47 antibody B6H12 for 30 min, 15 min or 20 min, respectively. The wound healing assay was performed as described previously.

For the co-cultured cell wound healing assay, M2 macrophages were differentiated in the wells of an ibidi culture-insert (ibidi, Martinsried, Germany). On day 4, 2.5 × 10^6 SW480 cells were added to the dishes outside of the insert. Thereafter, SW480 cells and macrophages were cultured for an additional 5 days. On day 9, the culture medium from the macrophages was aspirated before the insert was carefully removed. In some experiments, transfected SW480 cells were used and M2 macrophages in the ibidi wells were treated with 10 μg/ml anti-SIRPα antibody (Biolegend, San Diego, CA, USA) for 30 min. SW480-conditioned medium was collected, centrifuged to remove cell debris and added back to the dishes. Non-adherent cells were gently washed in PBS. The cells were allowed to migrate for 24 h at 37 °C. Pictures were taken using a Nikon DS-Fi1 microscope using a 10× objective and analysed with NIS-Elements Basic Research software. The area of the wound was measured with Adobe Photoshop CS4 software.

2.13. Boyden chamber assay

M1 and M2 macrophages were detached by the addition of 0.02% versene and collected. Approximately 5 × 10^4 cells were added to the upper well of a Boyden chamber (Neuro Probe, Gaithersburg, MD, USA). The lower well contained supplemented RPMI 1640 (10% FBS) or SW480-conditioned medium. The two wells were separated by an 8.0-µm polycarbonate PVDF membrane. After a 4-h incubation at 37 °C, cells that had not migrated were removed, and the membrane with migrated cells was fixed and stained as described previously. The membrane was washed, the remaining dye solubilised in 10% sodium dodecyl sulphate and the absorbance measured at 590 nm.

2.14. Statistical analyses

SPSS software 16.0 was used for all immunostaining analyses. Univariate survival analysis was performed by Kaplan–Meier analysis with a log-rank test to determine the risk of death. Survival time was measured from the date of surgery to the date of death or 80 months of follow-up. The death information is from the Swedish Cause of Death Registry and the Swedish Cancer Register. The overall survival was calculated as colon cancer-specific death. Deaths due to other causes were censored at the time of death. Prizm software 5.0d (GraphPad Software, San Diego, CA, USA) was used for other statistical analyses. All the data are presented as the mean ± standard error of the mean (SEM), and statistical significance was determined as P < 0.05 by a two-way analysis of variance (ANOVA; labelled #), Column statistics or a two-tailed Student’s t-test (labelled *). All means were calculated from data from at least three independent experiments.

3. Results

3.1. Macrophage content in human colon cancer tissue

Increased macrophage infiltration has been detected in many different solid tumours. To quantify and distinguish the phenotype of these macrophages, expression of CD68, which is expressed at high levels on macrophages, and CD206, which is highly expressed on M2 macrophages, was examined by Q-PCR and compared between normal and tumour tissues from

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colon cancer patients. Expression of CD206 was 1.8-fold higher in tumour tissues than in matched normal tissues, while CD68 expression was similar in both tissue types (Fig. 1A).

To examine macrophage infiltration in colon cancer, tissues from patients were stained for CD68. In normal tissues, the CD68+ cells were primarily located outside the epithelial cell layer, whereas in the tumour tissue, these cells were observed mostly between the epithelial cancer cells (Fig. 1B). The number of CD68+ cells was determined using a colon cancer tissue array, which contains 72 pairs of normal mucosa and tumour tissue patient samples. In this experiment, tissues from 62 of the 72 patients were grouped, according to Dukes’ stage classification. Significantly more macrophages were observed in tumour tissues compared with normal tissues (Fig. 1C), especially in the late stage of colon cancer classified as Dukes C (Fig. 1D).

Similarly, staining of the same colon cancer tissue array with CD206 antibody revealed significantly increased numbers of CD206+ cells in the tumour tissues as compared with normal tissues (Fig. 1F and G), and these cells were located between the epithelial cancer cells (Fig. 1E). This finding indicated that the tumour area contained more M2 macrophages than the normal area. Additionally, we observed that a higher number of CD206+ cells in the tumour than in the normal tissue tends to confer a poorer prognosis (Fig. 1H).

To assess the above results from both the in vitro experiments and human colon cancer tissue we next set up a mouse xenograft-model with human colon cancer cells. We found, in good agreement with our human in vivo results, a higher content of macrophages (F4/80+ cells) in the tumour area as compared with normal tissues (Fig. 1C, especially in the late stage of colon cancer classified as Dukes C (Fig. 1D).

3.2. M1- and M2-like macrophages differentiated from the human monocyte cell line THP-1

To establish an in vitro model, the human monocyte cell line THP-1 was differentiated into M2 or M1 macrophages (Fig. 2A). THP-1 cells that normally grow in suspension and have a rounded morphology became attached and spread more during differentiation. Expression of CD68 increased during differentiation, indicating that the cells became more macrophage-like (Fig. 2A). To confirm the phenotype of these macrophages, the mRNA levels of macrophage markers were investigated. Both M1 and M2 macrophages expressed similar levels of CD68 mRNA, but the mRNA levels of CD206 were significantly higher (23-fold) in the M2 population (Fig. 2B). A similar expression pattern for macrophage marker expression was observed at the protein level using flow cytometry (Fig. 2C).

3.3. Cytokine profiles of THP-1-derived macrophages

Macropages derived from various tissue sites are known to exhibit different cytokine profiles. Thus, we measured the levels of secreted cytokines in conditioned media from cells grown under different conditions, as described in Section 2. The levels of Th2 cytokines, including IL-4 (122 pg/ml), IL-10 (118 pg/ml) and IL-8 (5531 pg/ml), were significantly higher in the M2 macrophage-conditioned medium compared to the M1 macrophage-conditioned media (Fig. 2E). IL-4 secretion was observed in M2 but not M1 macrophage-conditioned media. IL-8 expression was dramatically increased in the conditioned media from M2 macrophages co-cultured with SW480 cells than in media from M2 macrophages alone (from 673 to 4886 pg/ml). Moreover, the Th1 cytokines, including IL-1β, IFN-γ and tumour necrosis factor (TNF)-α, were more highly expressed in the M1 macrophage conditioned medium. Interestingly, the IL-10 concentration in the SW480-conditioned medium (204 pg/ml) was much higher than that in the macrophage-conditioned medium.

The cytokine profiles from the M1 and M2 macrophages confirmed the macrophage differentiation efficiency, and these data highlighted several cytokines known to influence the behaviour of the tumour microenvironment. IL-10 is an immunosuppressive cytokine that is known to be important in the differentiation of M2 macrophages. Therefore, we investigated the role of IL-10 in macrophage polarisation.

3.4. IL-10 secreted from SW480 cells polarise M1 macrophages

SW480 cancer cells secreted significant amounts of IL-10 (Fig. 2E). We therefore hypothesised that M1 macrophages differentiate into M2 macrophages in the cancer cell milieu. We stimulated differentiated M1 macrophages with IL-10 for 72 h and followed changes in expression of CD206 and SIRPα. Fluorescent staining of CD206 was increased at the cell membrane of stimulated M1 macrophages, similar to that observed in differentiated M2 macrophages (Fig. 3B). Expression of SIRPα increased from a mean fluorescent intensity of 178 to 249 in M1 macrophages stimulated with IL-10 for 72 h (Fig. 3A). This increase in SIRPα expression was confirmed by immunofluorescence staining in IL-10-stimulated cells (Fig. 3B). Furthermore,
immunofluorescent staining indicated that CD206 and SIRPa expression were increased in M1 macrophages co-cultured with IL-10-releasing SW480 cancer cells for 72 h (Fig. 3C). These findings support the hypothesis that M1 macrophages are prone to become M2-like when exposed to factors, such as IL-10, that are released by SW480 cells.

3.5. Migration of SW480 colon cancer cells is induced by M2 macrophage-derived factors

Tumour cells are known to recruit macrophages; thus, we performed a Boyden chamber assay to investigate the ability of SW480 colon cancer cells to attract macrophages. Significantly more M2 macrophages than

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

Fig. 4. Migration of SW480 colon cancer cells stimulated by M2 macrophage-derived factors. (A) Boyden chamber assay demonstrates that M1- and M2-like macrophages migrate towards SW480-conditioned medium. (B) Wound healing assay involving SW480 cells with or without M2 macrophage-conditioned medium at indicated time points. (C) Wound healing assay with SW480 cells pre-treated with IL-8 receptor CXCR1 blocking antibody, CysLT1R inhibitor ZM198,615, or CD47 blocking antibody B6H12 and stimulated with M2 macrophage-conditioned medium. (D) CD47 expression in SW480 cells upon IL-4, IL-8, LTD4 or M2 macrophage-conditioned medium stimulation after 6 or 24 h. The results are shown as the mean ± standard error of the mean (SEM) of 3–7 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (column statistics for D, two-tailed Student’s t-test for A and B), #P < 0.05 (two-way ANOVA).
M1 macrophages migrated towards the SW480 cancer cell-conditioned medium (Fig. 4A). The tumour micro-environment is rich with various soluble and non-soluble factors, which are believed to be essential for tumour progression. In this study, M2 macrophages were shown to release cytokines, and inflammatory mediators, and thus, we examined the contribution of these different factors to the interaction between M2 macrophages and colon cancer cells as well as to cell migration. In a wound healing assay, SW480 cells were stimulated with M2 macrophage-conditioned medium, and wound closure was measured at different time points (Fig. 4B). A significant increase in cell migration was observed at 4, 12 and 24 h. IL-8, which was secreted in significant amounts by M2 macrophages, has been shown to induce cancer cell migration. Furthermore, macrophages are a major source of lipid mediators. Our previous data also indicate that CysLT1R signalling is involved in intestinal epithelial cell proliferation, survival and migration. To determine which factors contribute to this increase in cell migration, SW480 cells were pre-treated with a blocking antibody against the IL-8 receptor CXCR1, the CysLT1R antagonist ZM198,615 (the receptor for LTD4), or the CD47 blocking antibody B6H12. All of these treatments significantly blocked the cell migration mediated by the M2 macrophage-conditioned medium (Fig. 4C). After 6 or 24 h of stimulation with IL-8, LTD4, and M2 macrophage-conditioned medium, CD47 expression was significantly increased in SW480 cells (Fig. 4D). These findings suggest that M2 macrophage-secreted IL-8 and LTD4, which is the ligand to CysLT1R, induces colon cancer cell migration. CD47 signalling may be involved in this induction.

3.6. CD47 and SIRPα expression in human colon cancer tissue

The fact that CD47 influences cell migration led us to investigate CD47 expression in colon cancer tissue. CD47 staining revealed that the tumour tissues contained higher levels of CD47 than the normal tissue (Fig. 5A). In addition, the mRNA levels of CD47 were significantly higher (1.9-fold) in the tumour tissues compared to the normal tissues (Fig. 5B). In normal tissues, SIRPα+ cells were found mostly below the epithelial layer, whereas in tumour tissues, a significant number of SIRPα+ cells were observed between the epithelial colon cancer cells (Fig. 5C); however, the mRNA expression in normal and tumour tissues from colon cancer patients. **P < 0.01 (column statistics).
expression of the CD47 ligand SIRPα was similar in normal and colon cancer tissues (Fig. 5D).

3.7. CD47 and SIRPα contribute to cell migration in SW480 and M2 macrophage co-culture

As we have shown (Figs. 5 and 3A), colon cancer tissues expressed high levels of CD47, and M2 macrophages expressed high levels of SIRPα. Direct binding of CD47 and SIRPα may contribute to cell migration. To investigate the role of the CD47–SIRPα interaction in cell migration, we used ibidi inserts to create a co-culture system (Fig. 6A). More pronounced cell migration of SW480 cancer cells was observed under conditions of co-culture with M2 macrophages (Fig. 6A). Under co-culture conditions, SW480 cells and M2 macrophages migrated towards each other, resulting in cell-to-cell contact after 24 h (Fig. 6B). These findings suggest that M2 macrophages and SW480 cells attract each other and promote cellular migration. We next pre-treated SW480 cells with the CD47 blocking antibody B6H12, and this treatment significantly reduced the SW480 and M2 macrophage migration (Fig. 6C). Furthermore, following siRNA-induced downregulation of CD47 in SW480 colon cancer cells (Fig. 6D), these cancer cells exhibited significantly reduced migration in the presence of M2 macrophages compared to SW480 cells transfected with scrambled siRNA. This effect was even further reduced in the presence of M2 macrophages pre-treated with SIRPα blocking antibodies (Fig. 6E). Taken together, these findings suggest that an interaction between CD47 and SIRPα is involved in the regulation of SW480 cancer cells and M2 macrophages migration under co-culture conditions.

4. Discussion

Macrophages constitute a major population of tumour-infiltrating immune cells that reside in the tumour microenvironment. Depending on their phenotype, these cells exhibit either pro- or anti-tumour properties. Many different types of cancer tissues are populated by significant numbers of CD206-positive macrophages, and these cells are commonly referred to as TAMs. In this study, we addressed the interplay between TAMs, the inflammatory microenvironment and tumour cells in the context of colorectal cancer.

We observed a high number of macrophages, which were immunohistochemically defined as CD68+ cells, in the tumour microenvironment of tissue samples obtained from colorectal cancer patients. TAMs have an M2 macrophage-like phenotype, but since CD68 is expressed on both M1 and M2 macrophages, CD68 expression alone does not define TAMs. A number of other and more specific markers, such as CD83, CD80 and CCR7 for M1 macrophages or CD206 and CD163 for M2 macrophages, have been identified. Compared to normal tissues, the number of CD68+ and CD206+ cells was significantly higher in the colon cancer microenvironment, especially in tissues from Dukes C group of patients, compared to normal tissues. The location of these CD68+ and CD206+ cells differed between normal and tumour tissues. In tumour tissues, these cells were mostly observed between tumour cells. In this tumour microenvironment created by colon cancer cells and macrophages, macrophages may be induced to a more tumour-promoting phenotype via interaction of the cancer cells with TAMs, and this binding may be direct. We also observed a tendency of increased CD206+ cells to be associated with a poorer prognosis. Together, these findings suggested that M2 macrophages are tumour-associated cells and that these cells are involved in cancer progression. To further address the importance of macrophages for colon cancer development we also performed experiments with a xenograft mouse model. Interestingly and in agreement with our previous results, we observed higher macrophage content in larger tumours than in smaller tumours in this model. It is quite plausible that our continuous work will take advantage of a colour-coded fluorescence imaging model that can be used to study interactions between human tumour cells and host cells in nude mice. Hoffman and co-workers have recently developed this imaging method further to a tri-colour based imaging method that can be used to visualise interactions between three different types of cells for example that between host macrophages and lymphocytes with human cancer cells in nude mice.

To explore the interaction between M2 macrophages and tumour cells, we used an in vitro model system. Macrophages can be differentiated into M1- or M2-like macrophages in response to specific stimuli, and thereafter, these cells produce Th1 or Th2 cytokines. Using in vitro differentiated macrophages, we observed a significant increase in the secretion of Th1 cytokines, such as IL-1β, IFN-γ and TNF-α, by the differentiated M1 macrophages, although the conditioned medium of M2 macrophages contained low but clearly detectable levels of both IL-1β and TNF-α. Aside from controlling tumour angiogenesis and invasiveness, IL-1β also activates the Wnt signalling pathway in colon cancer cells, thereby promoting proliferation. In addition to activating macrophages, TNF-α has also been shown to promote angiogenesis in the tumour environment. On the other hand, in the conditioned medium of M2-differentiated macrophages, the levels of many cytokines were increased. Importantly, the Th2 cytokines IL-4 and IL-10 were significantly increased, and these cytokines are known to be immunosuppressive and contribute to M2 macrophage differentiation. Interestingly, in this context, we found that SW480 colon cancer cells secrete very high levels of IL-10. Recently, Tanikawa and
Fig. 6. Cell migration of SW480 colon cancer cells and M2 macrophages under co-cultured conditions. (A) Wound healing assay with ibidi inserts shows cell migration between M2 macrophages and SW480 cells at different time points. (B) Representative immunofluorescence images show expression and localisation of CD47 (green) and signal-regulatory protein α (SIRPα) (red) in SW480 cells and M2-macrophages at 0 and 24 h in a wound healing assay with ibidi inserts. DAPI is shown in blue. Scale bar is 20 μm. (C) Cell migration between M2 macrophages and SW480 cells after treatment of SW480 cells with CD47 blocking antibody B6H12. (D) CD47 expression in SW480 cells after transfection with CD47 siRNA (siRNA 1–3) or control scramble siRNA (scr). (E) Wound healing assay with ibidi inserts shows cell migration between SW480 cells transfected with CD47 siRNA (siRNA 2 + 3) or control scramble siRNA (scr) and M2-like macrophages treated with SIRPα blocking antibody. (F) Schematic depicting the crosstalk between SW480 colon cancer cells and M2 macrophages. *P < 0.05, **P < 0.01 (column statistics for D, two-tailed Student’s t-test for A), #P < 0.05 (two-way ANOVA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
coworkers demonstrated the importance of IL-10 in tumour development, growth and metastasis. We continued this line of investigation by studying the effect of IL-10 on CD206 and SIRPα expression on M1-like macrophages. Indeed, IL-10 increased SIRPα expression in M1-like macrophages. Furthermore, we found that the expression pattern of CD206 was similar in M1-like macrophages stimulated with IL-10 or co-cultured with SW480 cells, which secrete IL-10. These findings support the notion that IL-10, which is produced by colon cancer cells and abundant in the tumour microenvironment, contributes to M2 macrophage differentiation.

Another very important cytokine, which is secreted by M2 macrophages at a significant level, is IL-8, which has two receptors, CXCR1 and CXCR2. Functional CXCR1 and CXCR2 mediate IL-8-triggered Ca²⁺ release, contraction and migration. In this study, IL-8 was detected in M2 macrophage-conditioned medium and was found to induce colon cancer cell migration through its receptor CXCR1. IL-8 also influenced the expression of CD47, which is involved in colon cancer cell migration. These results describe a novel role of IL-8 in regulation of colon cancer cell migration.

Macrophages are also a major source of lipid mediators. In our study, a specific CysLT1R antagonist significantly reduced cell migration after M2 macrophage-conditioned medium stimulation of SW480 cells. This finding is consistent with previous studies showing CysLT1R signalling is involved in intestinal epithelial cell proliferation, survival and migration. Taken together, these data highlight the crosstalk between macrophages and colon cancer cells and indicate that it is mediated, at least in part, by inflammatory lipid mediators generated from the tumour microenvironment.

Cancer cells interact with the microenvironment via a number of adhesion molecules. In the present study, a CD47 blocking antibody and CD47 siRNA significantly reduced cell migration in an M2 macrophage/SW480 colon cancer cell co-culture system. We also found that the addition of a SIRPα blocking antibody to the above co-culture system further reduced cell migration. This finding is in accordance with our previous study in which we reported that CD47 signalling participates in the regulation of cyclooxygenase-2 (COX-2) expression and thus, in triggering intestinal epithelial cell migration. We also found that CD47 expression was increased at both the mRNA and protein levels in tissue biopsies from colon cancer patients. Previous reports indicated that CD47 is upregulated on tumour cells, where it interacts with SIRPα expressed on macrophages and allows tumour cells to evade macrophage clearance. Our findings now identify an additional activity of CD47 in promoting cancer progression. The interaction of CD47 and SIRPα is localised to the membrane distal domain of SIRPα. In agreement with such a CD47–SIRPα interaction, we observed that SIRPα cells in tumours were mostly located between tumour cells, and colon cancer cells SW480 interact with M2 macrophages under co-culture conditions, clearly supporting a physical interaction between CD47 and SIRPα. In comparison to M1-differentiated macrophages, SIRPα expression was significantly higher in M2-differentiated macrophages.

Chao and co-workers have previously reported an anti-tumour effect by administration of a blocking anti-CD47 antibody in an animal model of acute lymphoblastic leukaemia (ALL). The authors proposed that the observed effect of the anti-CD47 antibody was due to blockage of the anti-phagocytotic effect of CD47 enabling phagocytosis of ALL cells by macrophages. Furthermore, in a more recent study from the same group, also commented by Spaargaren, it was shown that anti-CD47 antibody treatment also prevents the dissemination of lymphoma cells. Different possible explanations for this effect are presented or proposed. These include an effect on the anti-CD47 antibody on lymphoma cell migration and an increased macrophage phagocytosis of circulating lymphoma cells. Our data suggest that SIRPα expressed on macrophages promotes colon cancer progression by increasing the migratory capacity of colon cancer cells via interaction with CD47 expressed on the tumour cells which is in part in line with the suggested effect of CD47 in lymphoma cell dissemination.

In conclusion, as a major inflammatory component of the tumour microenvironment, TAMs promote colon cancer cell migration via secretion of soluble mediators and a possible cell–cell interaction between CD47 on cancer cells and its ligand SIRPα on macrophages. Our findings reveal important mechanisms whereby the TAM-enriched tumour microenvironment promotes colon cancer cell migration and subsequent metastasis. Targeting these interactions with small antagonists or blocking antibodies may constitute new therapies for patients with colon cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

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References


