

Tnf α Expression by Myeloid Cells in Ascites Regulate Colorectal Cancer

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Conflict-of-interest statement: The authors declare that there is no conflict of interest regarding the publication of this paper.

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Received: November 29, 2021

Revised: November 2, 2021

Accepted: December 4, 2021

Published online: December 21, 2021

ABSTRACT

OBJECTIVES: CD14⁺ macrophages within a tumor or in peripheral blood (PB) are cytotoxic during the early stages but support cancer cell proliferation in the late stages. We investigated the role of inflammatory cells in ascites in patients with colorectal cancer (CRC).

METHODS: We prospectively enrolled 18 consecutive patients with CRC and 5 patients with inguinal hernia (IH) requiring laparoscopic approach. Inflammatory cells in the peritoneal fluid were enumerated. To investigate the function of the major fraction, we collected CD45⁺ and CD14⁺ cells (approximately 5×10^5 cells) by fluorescence-activated cell sorting and analyzed CD14⁺ cells in ascites and PB using M1 (TNF α , iNOS, and CCR2) and M2 markers (ARG1, IL-10, and TGF- β). HCT116 cells (colon cancer) were co-cultured with CD14⁺ macrophages from ascites and PB from patients

to investigate cancer cell proliferation.

RESULTS: There were no significant differences in CD14⁺ cell numbers (mean, 5.5 vs. 10.1%) in the peritoneal fluid of the two groups, but TNF α levels in CRC ascites macrophages were significantly higher than those from PB of IH ($p < 0.01$). CD14⁺ cells from ascites of patients with CRC better suppressed cancer cell proliferation ($p < 0.01$), but cancer cell proliferation persisted in the presence of TNF α antibodies ($p < 0.01$).

CONCLUSIONS: Myeloid-derived CD14⁺ cells in the environment of ascites can infiltrate in tumors of patients with CRC; they appear to be M1 macrophages, and expression of TNF α can suppress the growth of CRC cells.

Key words: Colorectal cancer; Ascites; Surgery; Tumor necrosis factor- α ; Tumor-associated macrophages

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Tsuchiya Y, Munakata S, Ishihara H, Honjo K, Sugimoto K, Sakamoto K. Tnf α Expression by Myeloid Cells in Ascites Regulate Colorectal Cancer. *Journal of Gastroenterology and Hepatology Research* 2021; **10(6)**: 3635-3640 Available from: URL: <http://www.ghrnet.org/index.php/joghr/article/view/3235>

INTRODUCTION

Inflammatory cells are a key component of the ecological niche of colorectal cancer (CRC), and an inflammatory microenvironment is now recognized to be integral to cancer progression. Thus, paradigms have shifted from a cancer-cell-centric view to one that encompasses the tumor microenvironment, including inflammatory cells^[1]. Monocytes and macrophages are a major component of the leukocyte infiltrate in all tumors and they work to effectively suppress anti-cancer immunity and assist cancer promotion by interacting with lymphocyte subsets. Recently, tumor-associated macrophages (TAM) have reported to both support and inhibit cancer. Thus, while bisphosphonate, zoledronic acid, and CCL2 inhibitor have been shown to deplete macrophages or their migration to breast or prostate cancer^[2,3]. Zhao et al. have reported that, in CRC, myeloid CD11b⁺/Gr1mid cells were recruited to liver metastases to promote tumor cell proliferation^[4]. TAM have been primarily described as having an

M2-like phenotype but switching to a predominantly M1 phenotype has been proposed as a key anti-cancer immunotherapeutic treatment strategy^[5,6]. TAMs are known to promote tumor progression and are associated with poor prognosis^[7].

Recent data show that myeloid-derived suppressor cells (MDSC; Lin-/lowHLA-DR-CD11b+ CD33+) are present in the circulation in CRC, that they inhibit T-cell proliferation, and that, compared to those from healthy individuals, are closely correlated with clinical cancer stage and tumor metastasis^[8].

On the other hand, Foxp3+ CD25+ CD4+ regulatory T-cells, which suppress aberrant immune response against self-antigens, also suppress anti-tumor immune response. Additionally, it is now well substantiated that a large number of these regulatory T-cells infiltrate the tumor tissues of various cancers and that their abundant presence is often associated with poor clinical prognosis^[9]. CD8+ T lymphocytes are a known crucial component of cell-mediated immunity and Mei et al have demonstrated that greater CD8+ T-cell numbers in tumor stroma infiltrates indicates good survival^[10].

While previous studies have investigated the roles of MDSC and regulatory T-cells in cancer tissue and peripheral blood (PB)^[8,11], little is known about inflammatory cells in ascites and how they display immunotolerance toward malignant cells in the ascites. Therefore, we compared inflammatory myeloid cell fraction in CRC ascites with normal ascites using markers for macrophages (CD14), in addition to CD4 T-cells, CD8 T-cells, and CD19 B-cells.

MATERIALS AND METHODS

Patients with clinically confirmed CRC or inguinal hernia (IH), diagnosed at our hospital between January 2017 and July 2019, were eligible before inclusion in this study was performed in accordance with the ethical standards of the Committee on Human Experimentation of our institution (Institutional Review Board No.18-257), and we excluded all cases of emergency surgery or multiple cancers. The following factors were compared between the CRC group and the non-CRC group (IH group), namely, age, gender, white blood cell counts, cancer stage, and inflammatory cell population in ascites. Ascites was aspirated during laparoscopic surgery using the Opti4 system (Medtronic Inc., Minneapolis, MN). In the absence of ascites in the peritoneal cavity, ascites was diluted with saline.

Flow cytometry

Ascites were filtered through a cell strainer (Mesh 35µm) with 5mL tube, centrifuged at 300 ×g for 10 minutes at 4°C, and the supernatant removed. Next, 0.01 mol/L of PBS/FBS was added, mixed thoroughly, the cells counted, the concentration adjusted to approximately 1 × 10⁶ cells, and 100 µL of the prepared cell suspension distributed to test tubes. FC Block (BD/Pharmingen Franklin Lakes, NJ) was aliquoted into 2.5 µL tubes, allowed to react for 10 minutes in a cool dark place, the FC Receptor blocked, and antibody finally added. For cell sorting, leukocyte fractions were identified by forward scatter (FSC) and side scatter (SSC) analyses, and dead cells were removed by 4',6-diamidino-2-phenylindole (DAPI) staining (Vector Laboratories, Burlingame, CA). For staining of surface antigens, inflammatory cells were stained with CD4-PE, CD8-FITC, CD14-PE, CD19-APC, and CD45-PerCP (all from TONBO). Viability was determined via DAPI staining. Flow cytometry analyses were performed on Fluorescence-activated cell sorting (FACS) Verse (BD, Immunocytometry Systems, San Jose, CA). For data analysis, we used CellQuestPro (BD/Pharmingen, Franklin Lakes, NJ), and FlowJo (Tree Star, Ashland, OR). CD45

positive and CD14 positive cells were sorted by FACS Aria III (BD Biosciences Franklin Lakes, NJ) and collected approximately 5 × 10⁵ cells (Figure 1A).

After surgical resection of the CRC, all specimens were histopathologically reviewed, and the pathological classification and stage determined according to the TNM staging system (American Joint Committee on Cancer, AJCC).

Quantification of Cytokine mRNA Expression in CD14 cells by Reverse- Transcription Polymerase Chain Reaction

For qPCR, using NucleoSpin RNA Plus (TAKARA Bio, Otsu), we isolated RNA from macrophages sorted by ARIA III (BD Biosciences Franklin Lakes, NJ). For quantitative real-time PCR, we used RNA-direct™ SYBR Green™ Real-time PCR Master Mix (TOYOBO, Osaka) wherein RNA can be directly amplified without generating cDNA before reaction on 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Relative mRNA expression was calculated using the 2-ΔΔCT method. Each sample was analyzed thrice.

PCR was performed using the following specific forward and reverse primer pairs, respectively: ACTB: 5'-AGAGCTACGAGCTGCCTGAC-3' and 5'-AGCACTGTGTTGGCGTACAG-3'; TGFB1: 5'-GGCCTTTCCTGCTTCTCATGG-3' and 5'-CCTTGCTGACTGCGTGTC-3'; IL-10: 5'-GCCTAACATGCTTCGAGATC-3' and 5'-TGATGTCTGGGTCTTGGTTC-3'; ARG1: 5'-GGCAAGGTGATGGAAGAAAC-3' and 5'-AGTCCGAAACAAGCCAAGGT-3'; TNFα: 5'-CAGAGGGAAGAGTCCCCAG-3' and 5'-CCTTG-TCTGGTAGGAGACG-3'; CCR2: 5'-GACCAGGAAAGAATGTGAAAGTGA-3' and 5'-GCTCTGCCAATTGACTTTCCTT-3'; iNOS: 5'-GTTCTCAAGGCACAGGTCTC-3' and 5'-GCAGGTCACTTATGCACTTATC-3'.

Transwell co-cultures

Co-cultures using 0.4µ cell culture inserts (Nunc, Thermo Fisher Scientific, Waltham, MA) were performed in RPMI-1640 (Wako Pure Chemicals, Tokyo) supplemented with 10% Fetal Bovine Serum (Biosera, Chile, Osaka) and 1% Penicillin–Streptomycin Solution (Wako Pure Chemicals, Tokyo). CD14+ macrophages and HCT 116 cells were co-cultured as follows. HCT 116 (ATCC® CCL-247TM) cells were seeded at a density of 104 cells in the bottom of the plate while CD 14+ cells from the PB of patients with IH or CRC with or without TNFα antibody treatment (1.5 ng/ml AF-410; R&D Systems, Minneapolis, MN) were added to the culture inserts. The cells were cultured for 48 hours, after which cell numbers were enumerated on average of 5 fields of view on BZ-X700 microscope and BZ-X analyzer (KEYENCE, Osaka). Each sample was analyzed thrice.

Statistical analyses

Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Continuous variables are presented as median values and were compared using the Mann–Whitney U-test. Data were analyzed using JMP 10 software (SAS, Cary, NC).

RESULTS

Fraction of the inflammatory cells in ascites

Table 1 summarizes the clinicopathologic characteristics of the 18 CRC patients and the 5 IH patients who underwent surgical treatment. This study group comprised 16 males and 7 females aged 53–84 years (median 66 years). In the CRC patients, the most frequently encountered tumor location was the Sigmoid colon (S; n =

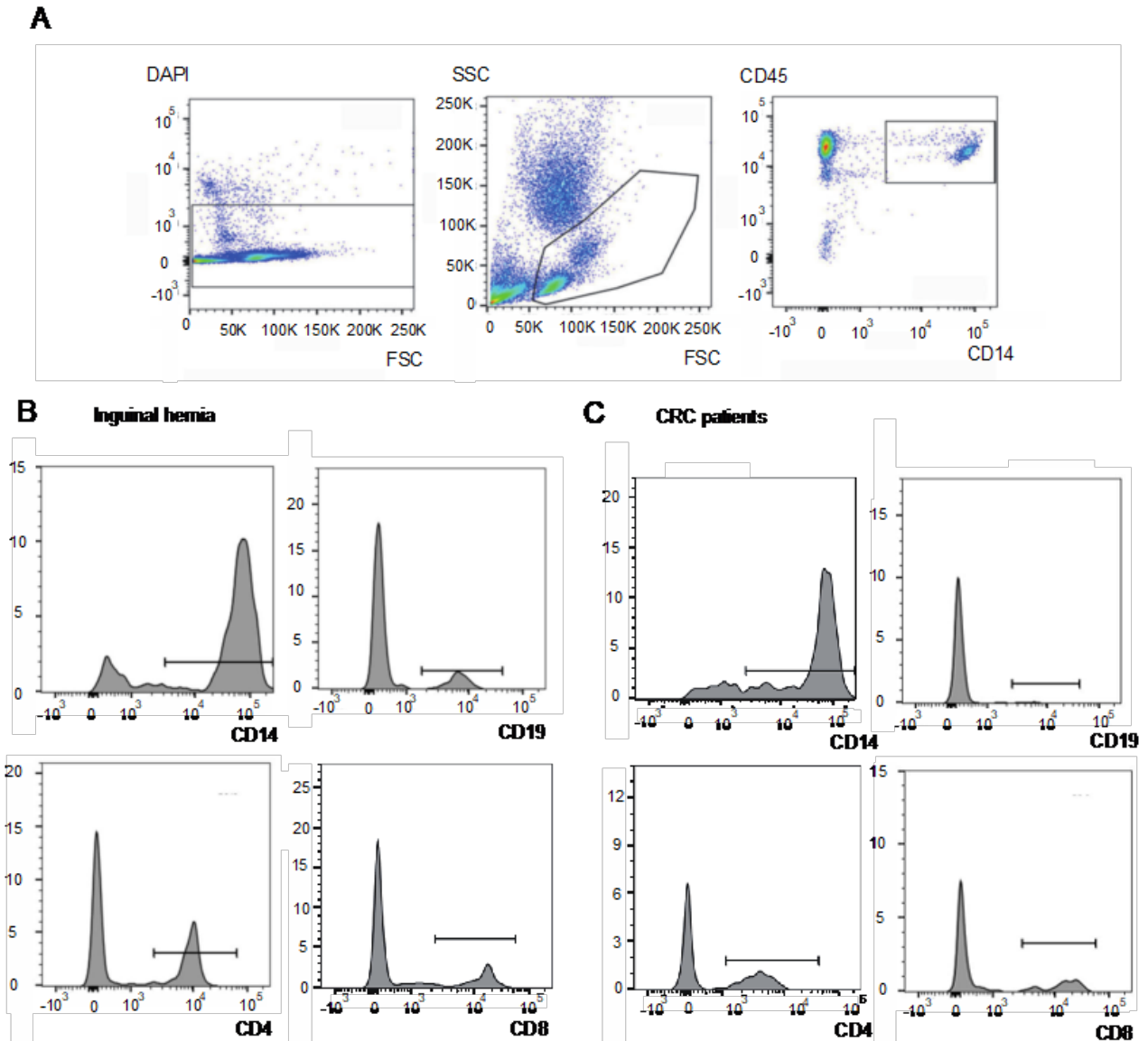


Figure 1 CD14⁺ myeloid cells are a major component of ascites from colorectal cancer (CRC) patients. (A) As shown in the representative data for sorting, leukocyte fractions were identified by forward scatter (FSC) and side scatter (SSC) analyses, and dead cells were removed by DAPI staining. Finally, CD14⁺ and CD45⁺ cells were collected. Representative inflammatory cell analyses of cancer ascites and non-cancer ascites by Fluorescence-activated cell sorting (FACS). Dot-plots show fluorescence pattern of stained myeloid cells from ascites (CD14-PE and CD45-PerCP) and lymphocytes (CD4-PE, CD8-FITC, CD19-APC, and CD45-PerCP) from (B) inguinal hernia (IH), (C) colorectal cancer (CRC) patients.

10, 55.6%), followed by the rectum in 8 patients (44.4%). The TNM stages (7th pathological T category) were pT1 in 2 patients (11.1%), pT2 in 3 patients (16.7%), pT3 in 10 patients (55.6%), and pT4 in 3 patients (16.7%), while TNM pathological staging revealed 6 patients (33.4%) as stage I, 3 patients (16.7%) as stage II, 5 patients (27.8%) as stage III, and 4 patients (22.2%) as stage IV.

No significant differences in age, gender, or white blood cell counts were found between patients undergoing laparoscopic IH repair and CRC resection. We found 8.7×10^6 cells in the ascites of CRC patients, of which 10.1% were CD14⁺ myeloid cells. Representative histogram of ascites is shown in Figure 1B and C.

There were no differences in the number of peritoneal inflammatory cells in the ascites between the CRC and the IH group after dilution and FACS did not reveal an increase in the percentage of inflammatory cells, viz., CD14⁺ (mean, 5.5 vs. 10.1%; $p = 0.73$), CD4⁺ (mean, 1.0 vs. 2.9%; $p = 0.37$), CD8⁺ (mean, 1.0 vs. 2.9%; $p = 0.7$), and CD19⁺ (mean, 0.04 vs. 0.41%; $p = 0.11$). Further, the ratio of CD14⁺ /CD45⁺ cells (mean, 0.39 vs. 0.29; $p = 0.23$) was also not

significantly different between the two groups. (Table 2). These data indicate that CD14⁺ cells comprise the major fraction of inflammatory cells in ascites from IH and CRC patients.

TNF α was released from peritoneal CD14⁺ macrophages of colorectal cancer

We next examined the function of CD14⁺ cells from CRC patients and compared it to CD14⁺ cells derived from the PB of IH; PB was used as the source for CD14⁺ macrophages as it was difficult to obtain peritoneal macrophages from IH because of the smaller number of inflammatory cells in the ascites. We selected the following M1 makers, tumor necrosis factor α (TNF α , inducible nitric oxide synthase (iNOS), C-C chemokine receptor type 2 (CCR2), and the following M2 makers, Arginase-1 (ARG1), Interleukin-10 (IL-10), and Transforming Growth Factor- β (TGF- β).

We found that while the relative expression of TNF α in macrophages from ascites of CRC patients was significantly higher compared to those from PB of IH, expression of CCR2 and iNOS was not

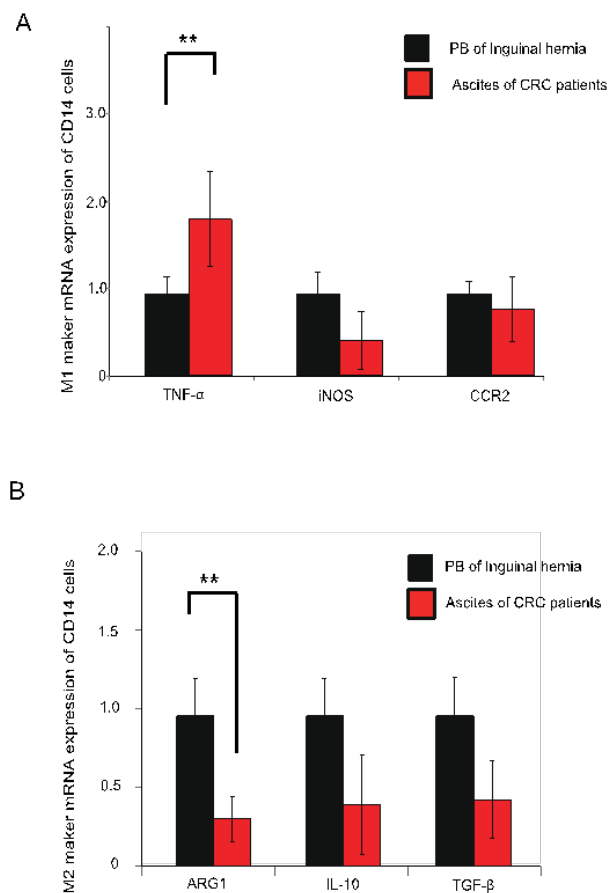


Figure 2 Tumor necrosis factor α (TNF α) inhibits colorectal cancer (CRC) progression. (A) M1 markers in CD14 $^{+}$ myeloid cells, such as TNF α , inducible nitric oxide synthase (iNOS), and C-C chemokine receptor type 2 (CCR2) were compared between ascites from CRC patients and peripheral blood (PB) of inguinal hernia (IH). Relative gene expression of TNF α in sorted CD14 $^{+}$ myeloid cells in CRC ascites was significantly higher than that from PB of IH ($n = 3$ /group). (B) M2 markers in CD14 $^{+}$ myeloid cells, such as Arginase-1 (ARG1), Interleukin-10 (IL-10), and Transforming Growth Factor- β (TGF β), were compared between ascites of CRC patients and PB of IH. $n = 5$ /group. Values represent the means \pm SEM. ** $P < 0.01$ by two-tailed Student's t -test.

different ($p < 0.01$; Figure 2A). On the other hand, ARG1 expression in the macrophages from ascites of CRC patients was significantly lower than that seen in macrophages derived from PB of IH; However, TGF- β , and IL-10 levels were similar ($p = 0.04$; Figure 2B). These observations imply that the CD14 $^{+}$ cells from ascites of CRC patients were M1 type macrophages.

CD14 $^{+}$ macrophages inhibit the proliferation of cancer cells

To investigate the cytokine effects of TNF α , we used a co-culture system with equal numbers of HCT 116 and CD 14 $^{+}$ cells that were sorted from either the PB of IH or from ascites of CRC patients (Figure 3A). We found that CD14 $^{+}$ macrophages from the PB/ascites of patients with CRC and the PB of patients with IH suppressed the proliferation of cancer cells ($p < 0.01$) (Figure 3B). In contrast, there was no significant difference when CD14 $^{+}$ from PB of CRC patients was used with or without anti-TNF α antibody at 48 hours (Figure 4A). Next, we compared HCT 116 cell growth when CD14 $^{+}$ cells from ascites from CRC patients were co-cultured with and without anti-TNF α antibody treatment (at 48 hours) and found that antibody treatment promoted proliferation (Figure 4B). Together, these data show that CD14 $^{+}$ macrophages in ascites of CRC patients inhibit the cancer cell proliferation through TNF α .

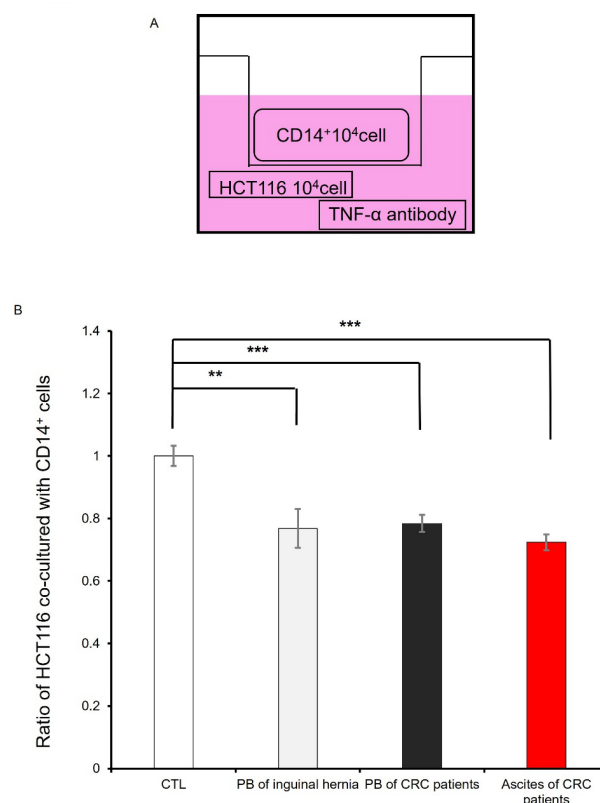


Figure 3 The role of CD14 $^{+}$ myeloid cells in ascites of colorectal cancer (CRC) for cancer cells. (A) Experimental setting: human cancer cell line (HCT116) and CD14 $^{+}$ cell sorted from ascites and peripheral blood (PB) of patients with CRC or inguinal hernia (IH) were co-cultured with or without anti-TNF α antibody for 48 hours and co-cultured in a transwell chamber system. (B) CD14 $^{+}$ macrophages from the ascites of patients with CRC suppressed HCT 116 cancer cell proliferation. Values represent the means \pm SEM. ** $P < 0.01$, *** $P < 0.001$ by two-tailed Student's t -test. Control: HCT 116 with 104 cells were seeded into the lower chamber and none into the upper chamber.

DISCUSSION

The data reported herein demonstrate that CD14 $^{+}$ cells are the predominant fraction of inflammatory cells in ascites of patients with CRC and IH, and that the expression of TNF α by these peritoneal CD14 $^{+}$ macrophages can inhibit cancer cell proliferation in vitro compared with peritoneal CD14 $^{+}$ macrophages. Previous reports have described the use of TNF α for the regional treatment of locally advanced soft tissue sarcomas, metastatic melanomas, and other non-resectable tumors^[12], and exogenous TNF α therapy has been found to be effective for metastatic lesions in CRC^[13,14]. Unfortunately, systemic TNF α administration is associated with severe toxicity and the induction of a 'cytokine storm' with symptoms such as fever and chills, fatigue, headache, decreased performance status, hypotension, leukopenia, and thrombocytopenia, which resemble many signs and symptoms of endotoxic shock^[15]. Concurrently, recent studies have also indicated that TNF α therapy can enhance many processes of carcinogenesis in ways that are associated with its central role in inflammation. Thus, TNF α antagonist treatment has been reported to result in a period of disease stabilization or better in 20% of patients with advanced cancer^[16-18]. To gain a greater understanding of the roles of malignant and organ specific stromal cell-derived TNF α , data on its effects on a case-by-case basis and on its relative importance in early and late cancers are needed^[19]. Our data represent a first look at CD14 $^{+}$ macrophages in ascites of CRC patients and it is notable

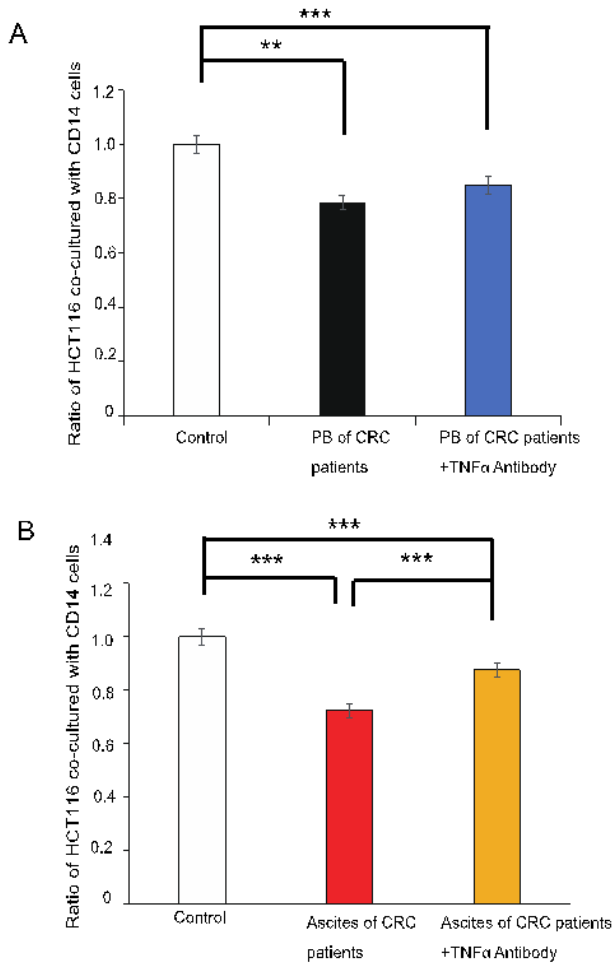


Figure 4 Ratio of colorectal cancer (CRC) cell proliferation co-cultured with CD14+ cells. (A) Peripheral blood (PB) of CRC patients were co-cultured with or without anti-TNF α antibody for 48 hours. n = 5/group. (B) Ascites of CRC patients were co-cultured with or without anti-TNF α antibody for 48 hours. n = 5/group. Values represent the means \pm SEM. **P < 0.01, ***P < 0.001 by two-tailed Student's t-test. Control: HCT 116 with 10 4 cells were seeded into the lower chamber and none into the upper chamber.

that studies using ascites are very rare. Nonetheless, previous studies have shown that ascites from patients with epithelial ovarian cancer contained CD14+ macrophages, which supports a scenario of a pervasive immune suppressive environment^[20]. Myelomonocytic cells originally act as resistance against pathogens (the unsung heroes of immunity) and activate adaptive responses; however, they undergo reprogramming of their functional properties in response to signals derived from microbes, damaged tissues, and resting or activated lymphocytes^[6]. During the development of peritoneal CRC metastasis, which represents a late stage of CRC, these alterations in the environment become necessary. Initially, we expected such changes in peritoneal fluids to occur in late stage of CRC, but interestingly, we could show that CD14+ myeloid cells inhibited CRC proliferation even in the late stage, including at stage \square , using TNF α . For peritoneal metastasis, immunotolerance may be needed to escape the effect of TNF α . We should have selected patients who had peritoneal metastasis to investigate the function of CD14+ myeloid cells.

However, patients with ovarian malignancies show elevated serum concentrations of IL-10 and low serum levels of TNF α ^[21]. Further, survival in patients with tumors expressing high levels of TNF α in CRC was significantly poorer compared to those with low TNF α expression^[22]. It cannot be classified easily by measuring the M1/

Table 1 Clinical characteristics of 18 CRC patients who underwent surgery and 5 IH patients.

	Inguinal hernia (n=5)	CRC patients (n=18)	p-value
Age (Mean)	66.4 \pm 1.5	63.4 \pm 2.5	0.65
Sex(M/F)	4/1	12/6	0.54
WBC(/ μ l) (Mean)	5160 \pm 464	6367 \pm 444	0.41
Cells in ascites (10 ⁶) (Mean)	10.8 \pm 7.39	8.7 \pm 2.61	0.47
Tumor location(S/R)		10/8	
T1/2/3/4		2/3/10/3	
N0/1/2/3		10/4/3/1	
M0/1		15/3	
pStage (\square / \square / \square / \square)		6/3/6/3	

S, sigmoid colon; R, rectum. CRC, colorectal cancer; IH, inguinal hernia. Values represent the means \pm SEM.

Table 2 Ratio of inflammatory cells in the ascites of IH and CRC groups divided by CD45 cells.

Inflammatory cells in peritoneal fluid	Inguinal hernia (n=5)	CRC patients (n=18)	P-value
CD4 (%)	1.04 \pm 0.39	2.88 \pm 0.91	0.37
CD8 (%)	1.02 \pm 0.35	2.98 \pm 1.03	0.7
CD14 (%)	5.5 \pm 1.6	10.1 \pm 2.9	0.73
CD19 (%)	0.04 \pm 0.13	0.41 \pm 0.04	0.11
CD4/CD45	0.089 \pm 0.011	0.21 \pm 0.12	0.41
CD8/CD45	0.12 \pm 0.03	0.23 \pm 0.12	0.41
CD14/CD45	0.39 \pm 0.07	0.29 \pm 0.03	0.23
CD19/CD45	0.0043 \pm 0.0016	0.055 \pm 0.029	0.46

CRC, colorectal cancer; IH, inguinal hernia. Values represent the means \pm SEM.

M2 cytokine level. Thus, it appears that the effects of TNF α can vary based on patient background, inflammatory state, metastatic location, and cancer stage.

Our study has several limitations, including the small number of patients, and further studies are warranted to acquire more data on the mechanism(s) causing the presence of myeloid cells. We hope that this study would significantly contribute to our understanding of the tumor microenvironment and immunomodulation in the peritoneal fluid.

In conclusion, we show that myeloid-derived CD14+ cells that express TNF α in the environment of ascites could infiltrate in CRC patients. It appears that CD14+ macrophages in ascites of CRC patients are of the M1 type and that TNF α expression by these cells can suppress the growth of cancer cells.

Abbreviations

Colorectal cancer; CRC, Fluorescence-activated cell sorting; FACS, Tumor necrosis factor α ; TNF α , Inguinal hernia; IH, Peripheral blood; PB, Tumor-associated macrophage; TAM

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science Wakate Grant 19K18104 (to SM).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

This study was performed in accordance with the ethical standards of

the Committee on Human Experimentation of our institution (Institutional Review Board No.18-257).

Author contributions

Study concept and design (SM); data acquisition (YT, SM, TU, HI, SI, KS, and HK); analysis and interpretation of data (SM); drafting of the manuscript (SM); critical revision of the manuscript (SM and KS).

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