

TNF- α secreted by myeloid cells in ascites regulate colorectal cancer

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Abstract

Background

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

Methods

We prospectively enrolled 18 consecutive CRC patients who required surgery and 5 patients (healthy donors) requiring inguinal hernia surgery. Inflammatory cells in the peritoneal fluid were enumerated using FACS. Demographic and clinical characteristic such as age, gender, peritoneal inflammatory CD14+, CD4+, CD8+, and CD19 + cells in ascites were compared between the two groups. The M1 (TNF- α iNOS CCR2) and M2 (ARG1 IL-10 TGF- β) characteristics of macrophages in ascites in cancer patients and those from the peripheral blood (PB) of healthy donors were investigated. HCT116 cells (colon cancer) were co-cultured with CD14 + from ascites and PB of CRC patients and with CD14 + macrophages from healthy donors.

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 healthy donors ($p < 0.01$). CD14 + cells from ascites of CRC patients 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 it in CRC patients, they appear to be M1 macrophages, and secreted TNF- α can suppress the growth of CRC cells.

Introduction

Inflammatory cells are a key component of the ecological niche of colorectal cancer, 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 colorectal cancer (CRC), myeloid CD11b⁺ /Gr1^{mid} 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 [8, 11], little is known about inflammatory cells in ascites and how they display immunotolerance towards 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.

Methods

Patients with clinically confirmed CRC or inguinal hernia, 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 (healthy donors), 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, USA). In the absence of ascites in the peritoneal cavity, ascites was diluted with saline.

Flow cytometry

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 (Vector Laboratories). Flow cytometric analyses were performed on a Fluorescence activated cell sorting (FACS)

Verse (BD, Immunocytometry Systems, San Jose, CA). For data analysis, we used CellQuestPro (BD/Pharmingen), and FlowJo (Tree Star, Ashland, OR). CD45 positive and CD14 positive cells were sorted by a FACS Aria III (BD Biosciences).

After surgical resection of the CRC, all specimens were histopathologically reviewed, and the pathological classification and stage determined according to the TNM staging system.

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

Quantitative Real-Time PCR was performed using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primers used were as follows: ACTB: 5'-AGAGCTACGAGCTGCCTGAC-3' and 5'-AGCACTGTGTTGGCGTACAG-3'; TGFB1: 5'-GGCCTTTCCTGCTTCTCATGG-3' and 5'-CCTTGCTGTACTGCGTGTCC-3'; IL-10: 5'-GCCTAACATGCTTCGAGATC-3' and 5'-TGATGTCTGGGTCTTGGTTC-3'; ARG1: 5'-GGCAAGGTGATGGAAGAAAC-3' and 5'-AGTCCGAAACAAGCCAAGGT-3'; TNF- α : 5'-CAGAGGGAAGAGTTCCCCAG-3' and 5'-CCTTGGTCTGGTAGGAGACG-3';

CCR2: 5'-GACCAGGAAAGAATGTGAAAGTGA-3' and 5'-GCTCTGCCAATTGACTTTCCTT-3'; iNOS: 5'-GTTCTCAAGGCACAGGTCTC-3' and 5'-GCAGGTCACTTATGTCACTTATC-3'

Transwell co-cultures

Co-cultures using 0.4 μ cell culture inserts (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were performed in RPMI-1640 (Wako Pure Chemicals, Tokyo, Japan) supplemented with 10% Fatal Bovine Serum (Biosera, Chile) and 1% Penicillin-Streptomycin Solution (Wako Pure Chemicals, Tokyo, Japan). CD14⁺ macrophages and HCT 116 cells were co-cultured as follows. HCT 116 cells were seeded at a density of 10^4 cells in the bottom of the plate while CD 14⁺ cells from PB of healthy donors or CRC patients with or without TNF- α antibody treatment (1.5ng/ml; R&D Systems, Minneapolis, MN) were added to the culture inserts. The cells were cultured for 48 hours, after which cell numbers were enumerated by imaging on a BZ-X700 microscope (KEYENCE, Osaka, Japan).

Immunohistochemistry

Colon cancer tissues were snap-frozen in liquid nitrogen, cut into 5- μ m sections, and labeled with anti-CD14-PE primary antibody (TONBO). Nuclei were stained using DAPI (Vector Laboratories).

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, USA).

Results

Table 1 summarizes the clinicopathologic characteristics of the 18 CRC patients and the 5 hernia (healthy donors) patients who underwent surgical treatment. No significant differences in age, gender, or white blood cell counts were found between patients undergoing laparoscopic inguinal hernia repair (healthy donors) and CRC resection. However, a small population of CD14⁺ myeloid cells had infiltrated the cancer tissue (Figure 1a) and we found 8.7×10^6 cells in the ascites of CRC patients, of which 10.1% were CD14⁺ myeloid cells. Representative FACS dot plot of ascites is shown in Fig. 1b.

There were no differences in the number of peritoneal inflammatory cells in the ascites between the CRC and the healthy donors 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 healthy and CRC patients.

We next examined the function of CD14⁺ cells from CRC patients and compared it to CD14⁺ cells derived from the peripheral blood of healthy donors; PB was used as the source for CD14⁺ macrophages as it was difficult to obtain peritoneal macrophages from healthy group 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 healthy donors, expression of CCR2 and iNOS was not different ($p < 0.01$; Fig. 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 healthy donors; However, TGF- β , and IL-10 levels were similar ($p = 0.04$; Fig. 2b). These observations imply that the CD14⁺ cells from ascites of CRC patients were M1 type macrophages.

To investigate the cytokine effects of TNF- α , we used a CD14⁺ cells and HCT116 colon cancer co-culture system with equal numbers of HCT 116 and CD 14⁺ cells that that were sorted from either the PB of healthy donors or from ascites of CRC patients (Fig. 3a). We found that CD14⁺ macrophages from CRC ascites suppressed cancer cell proliferation compared those from PB of healthy donors (representative data in Fig. 3b). To support this observation, we treated the CD14⁺ cells with anti-TNF- α antibody at 48 h and found that suppression of cell growth was reversed ($p < 0.01$) (Fig. 3c). In contrast, there was not significant difference when CD14⁺ from peripheral blood of CRC patients were used with or without anti-TNF- α antibody at 48 hours (Fig. 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 (Fig. 4b). Together, these data show that CD14⁺ macrophages in ascites of CRC patients inhibit the cancer cell proliferation through TNF- α secretion.

Discussion

The data reported herein demonstrate that CD14⁺ cells are the predominant fraction of inflammatory cells in ascites of CRC patients compared with healthy donors, and that the secretion of TNF- α by these peritoneal CD14⁺ macrophages can inhibit cancer cell proliferation in vitro. Previous reports have described the use of TNF- α for the regional treatment of locally advanced soft tissue sarcomas, metastatic melanomas, and other irresectable 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 colorectal cancer patients and it is notable 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 4, using TNF- α . Thus, it is possible that peritoneal metastasis is another outcome wherein inflammatory cells in the ascites lose their aggressiveness to cancer 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]. 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.

Conclusions

We show that myeloid-derived CD14⁺ cells in the environment of ascites could infiltrate it in CRC patients. It appears that CD14⁺ macrophages in ascites of CRC patients are of the M1 type and that TNF- α secreted by these cells can suppress the growth of cancer cells.

Abbreviations

CRC: Colorectal cancer FACS: Fluorescence activated cell sorting

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by the Juntendo University Faculty of Medicine and a written informed consent was obtained from all individual participants before surgery for collection and analysis of the data. This article does not contain any studies with animals performed by any of the authors. 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)

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed in this study are not publicly available (to maintain privacy) but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

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

Acknowledgements

The authors declare no conflict of interest.

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Tables

Please see the supplementary files section to view the tables.

Table 1: The clinical characteristics of the 18 colorectal cancer (CRC) patients who underwent surgery and the 5 inguinal hernia patients (healthy donors). Univariate analyses of the ascites in CRC patients and healthy donors used by the Mann-Whitney U-test. S; sigmoid colon R; rectum.

Table 2: The ratio of inflammatory cells in ascites of healthy donors and CRC group divided by CD45 cells and analyzed by the Mann-Whitney U-test.

Figures

Figure.1

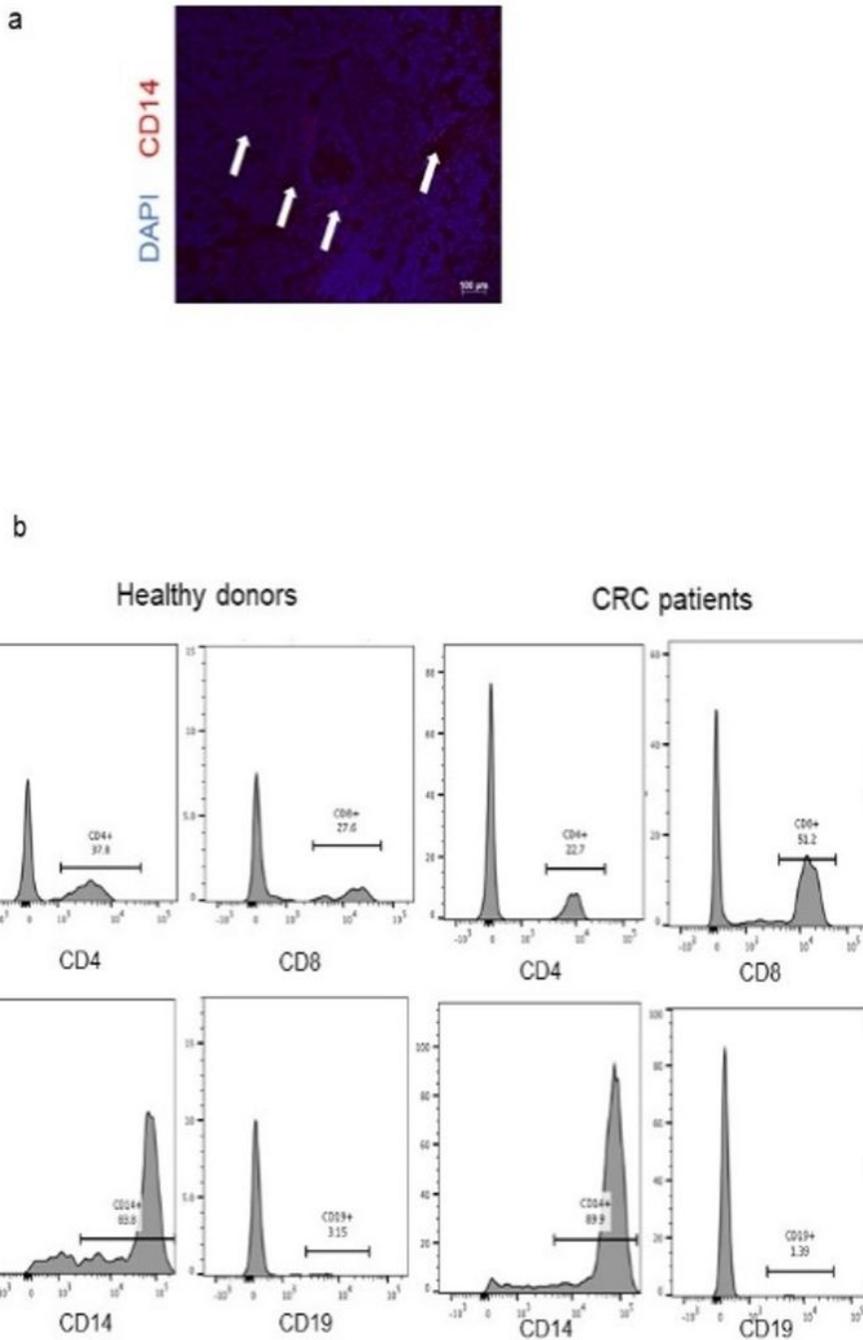


Figure 1

CD14+ myeloid cells are a major component of ascites from colorectal cancer (CRC) patients. (a) Representative immunohistochemistry images of colorectal cancer tissues showing CD14 staining after surgical resection (n = 3). (b) 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 (a) inguinal hernia (healthy donors), (b) colorectal patients.

Figure.2

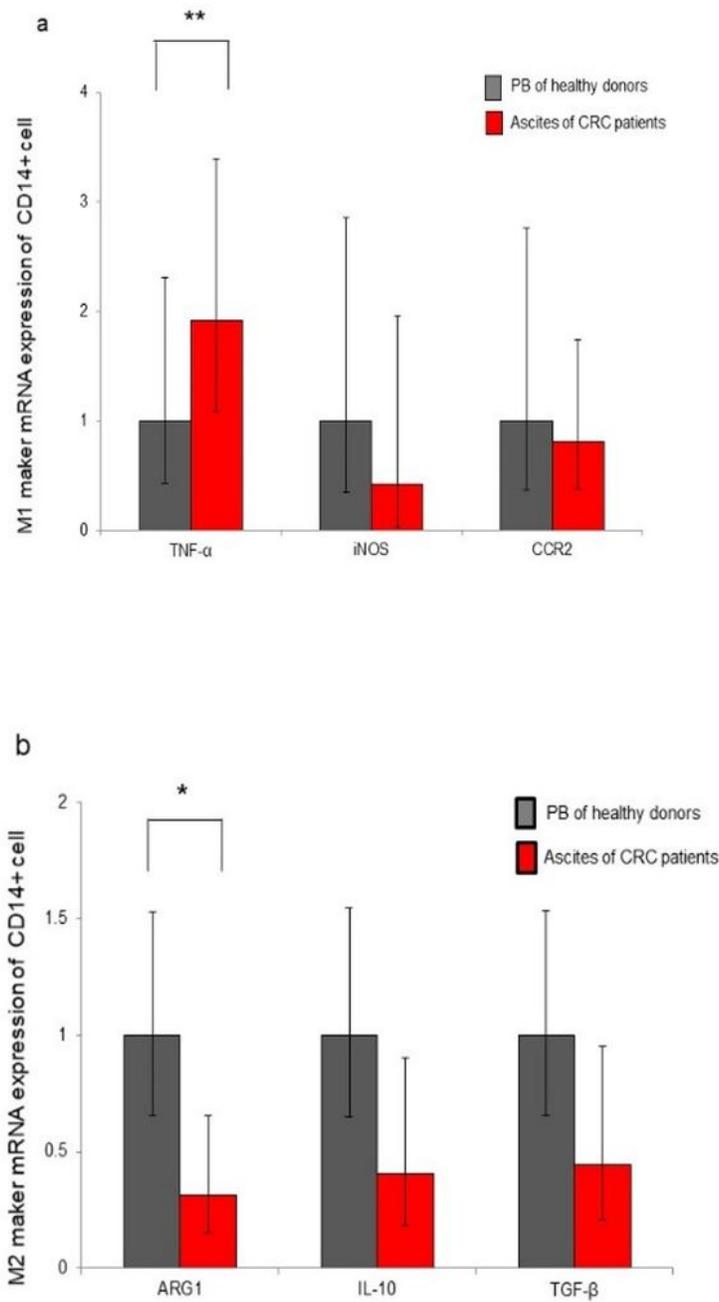


Figure 2

Tumor necrosis factor- α (TNF- α) inhibits colorectal cancer 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 healthy donors. Relative

gene expression of TNF- α in sorted CD14⁺ myeloid cells in CRC ascites was significantly higher than that from PB of healthy donors (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 healthy donors. n = 5/group. Values represent the means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by two-tailed Student's t-test.

Figure.3

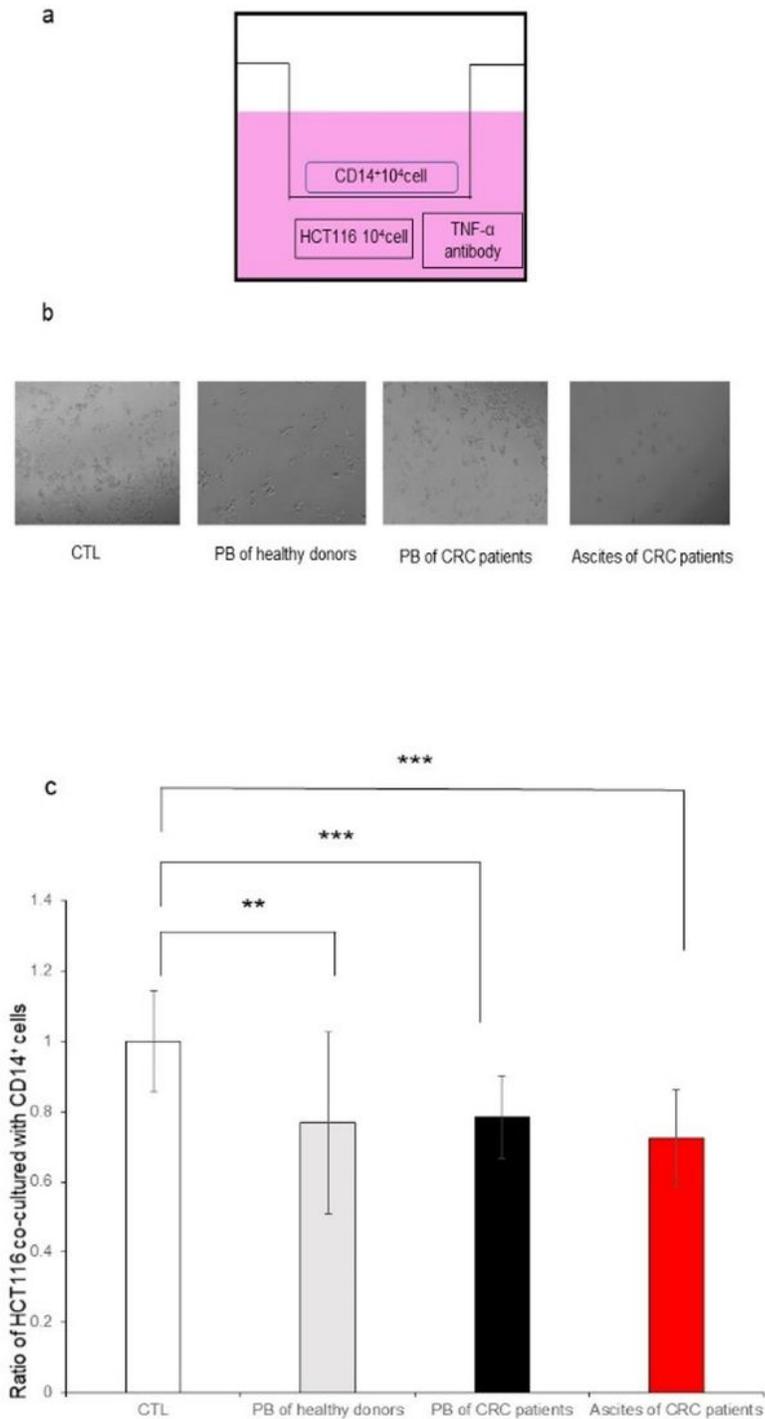


Figure 3

The role of CD14⁺ myeloid cells in ascites of colorectal cancer for cancer cells. (a) Experimental setting: human cancer cell line (HCT116) and CD14⁺ cell sorted from ascites and peripheral blood (PB) of CRC patients or healthy donors were co-cultured with or without anti-TNF- α for 48 hours and co-cultures in a transwell chamber system. (b) Representative images show that CD14⁺ macrophages in ascites of CRC patients suppressed cancer cell proliferation. (c) CD14⁺ macrophages from ascites of CRC patients suppressed HCT 116 cancer cell proliferation. Values represent the means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by the Mann-Whitney U-test. CTL; control.

Figure. 4

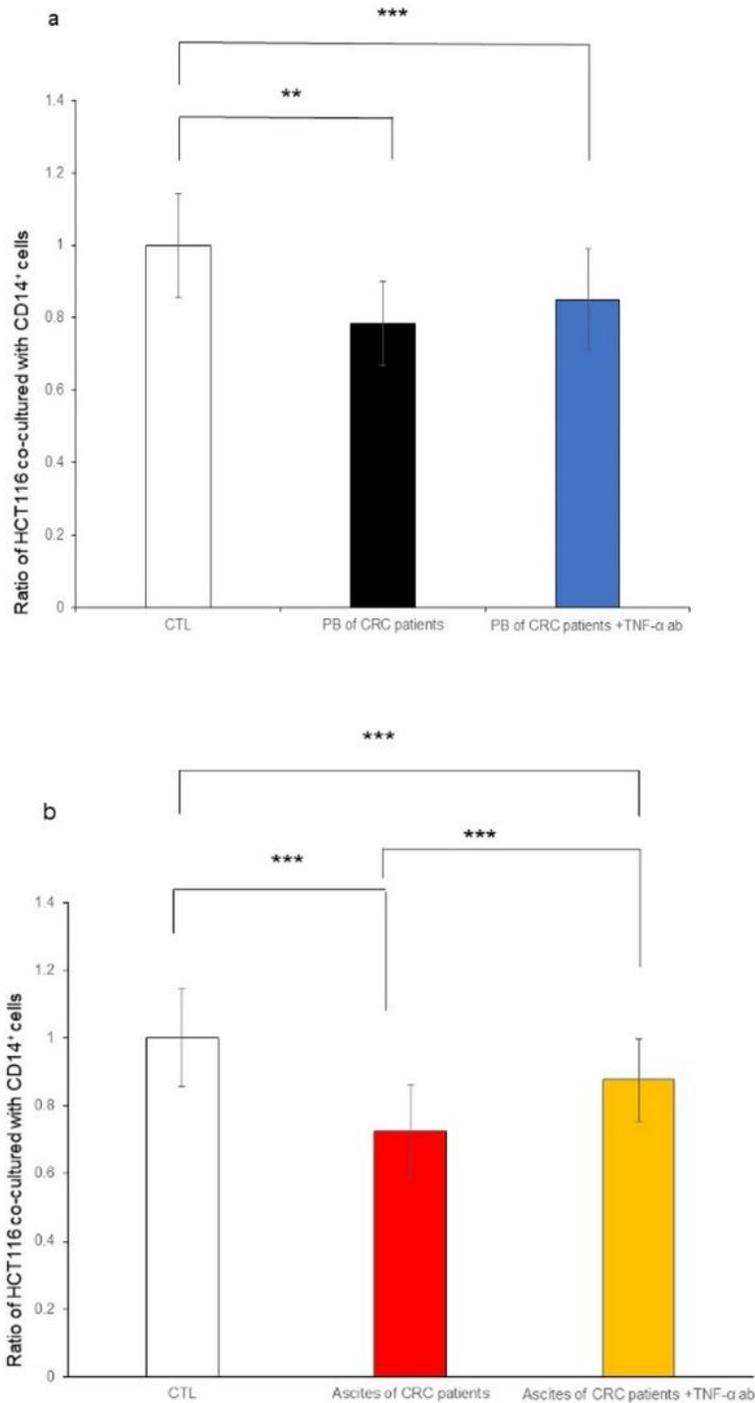


Figure 4

Ratio of colorectal cancer cell proliferation co-cultured with CD14+ cells (a) Peripheral blood (PB) of CRC patients were co-cultured with or without anti-TNF- α for 48 hours. n = 5/group. (b) Ascites of CRC patients were co-cultured with or without anti-TNF- α for 48 hours. n = 5/group. Values represent the means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by the Mann-Whitney U-test.

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