Research Article

Overcoming cancer-associated fibroblast-induced immunosuppression by anti-Interleukin-6 receptor antibody

Authors' names:

Noriyuki Nishiwaki[1], Kazuhiro Noma[1], Toshiaki Ohara[1, 2], Tomoyoshi Kunitomo[1], Kento Kawasaki[1], Masaaki Akai[1], Teruki Kobayashi[1], Toru Narusaka[1], Hajime Kashima[1], Hiroaki Sato[1], Satoshi Komoto[1], Takuya Kato[1], Naoaki Maeda[1], Satoru Kikuchi[1], Shunsuke Tanabe[1], Hiroshi Tazawa[1, 3], Yasuhiro Shirakawa[1, 4], and Toshiyoshi Fujiwara[1]

Authors' affiliations:

[1] Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[2] Department of Pathology & Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[3] Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan

[4] Department of Surgery, Hiroshima City Hiroshima Citizens Hospital

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Address correspondence to Kazuhiro Noma, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan Phone: +81-86-235-7255; Fax: +81-86-221-8775; E-mail: knoma@md.okayama-u.ac.jp

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Abbreviations used in this article: BMDM, bone marrow-derived monocytes; CAF, cancer-associated fibroblast; CI, confidence interval; COVID-19, coronavirus Disease 2019; CTL, cytotoxic T-cells; DFS, disease-free survival; EC, esophageal cancer; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; FoxP3, forkhead box protein 3; GLUT, glucose transporter; HIF1α, hypoxia-inducible factor 1α; HR, hazard ratio; Iba1, ionized calcium-binding adaptor protein 1; IF, immunofluorescence; IHC, immunohistochemistry; IL, interleukin; IL-6Rα, interleukin-6 receptor alpha; JCRB, Japanese Collection of Research Bioresources; MDSC, myeloid-derived suppressor cell; M-CSF, mouse macrophage colony-stimulating factor; OS, overall survival; PI3K, phosphatidylinositol-3 kinase; PMA, phorbol-12-myristate-13-acetate; RT, room temperature; SMA, smooth muscle actin; STAT signal transducer and activator of transcription; TAM, tumor-associated macrophage; TCZ, Tocilizumab; TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment; Treg, regulatory T-cell; TTDR, Tumor & Tissue Dissociation Reagent; UICC, union for international cancer control; VEGF, vascular endothelial growth factor; XTT, sodium 2,3,-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium

Abstract

Cancer-associated fibroblasts are a critical component of the tumor microenvironment and play a central role in tumor progression. Previously, we reported that cancer-associated fibroblasts might induce tumor immunosuppression via interleukin-6 and promote tumor progression by blocking local interleukin-6 in the tumor microenvironment with neutralizing antibody. Here, we explore whether an anti-interleukin-6 receptor antibody could be used as systemic therapy to treat cancer, and further investigate the mechanisms by which interleukin-6 induces tumor immunosuppression.

In clinical samples, interleukin-6 expression was significantly correlated with α-smooth muscle actin expression, and high interleukin-6 cases showed tumor immunosuppression. Multivariate analysis showed that interleukin-6 expression was an independent prognostic factor. In vitro, interleukin-6 contributes to cell proliferation and differentiation into cancer-associated fibroblasts. Moreover, interleukin-6 increased hypoxia inducible factor-1α expression and induced tumor immunosuppression by enhancing glucose uptake by cancer cells and competing for glucose with immune cells. MR16-1, a rodent analog of anti-interleukin-6 receptor antibody, overcame cancer-associated fibroblast-induced immunosuppression and suppressed tumor progression in immunocompetent murine cancer models by regulating hypoxia inducible factor-1α activation in vivo. The anti-interleukin-6 receptor antibody could be systemically employed to overcome tumor immunosuppression and improve patient survival with various cancers. Furthermore, the tumor immunosuppression is thought to be induced by interleukin-6 via hypoxia inducible factor-1α activation.

Introduction

Cancer immunotherapy has led to breakthroughs in cancer treatment; however the effects of immunotherapy are limited and have yet to overcome intractable cancers. Esophageal cancer (EC) is the seventh most common cancer and the sixth most common cause of cancer-related deaths globally.(1) Despite recent advances in EC-associated chemotherapy, targeted therapy, and immunotherapy, the prognosis remains poor with a 5-year survival rate of approximately 15–25%.(2, 3) Moreover, preclinical or clinical studies consistently report mixed results, which suggests that the tumor microenvironment (TME), especially the immune microenvironment in EC, may be implicated in the regulation of those therapies.(4, 5)

Numerous studies have demonstrated that the TME composition significantly influences tumor outcomes.(6-8) Cancer-associated fibroblasts (CAFs) are critical components of the TME and play a central role in tumor growth, metastasis, and invasion,(9, 10) and have recently attracted attention as potential therapeutic targets.(10-12) In EC patients specifically, CAFs contribute to tumor development by promoting angiogenesis,(13) chemoresistance,(14) lymph node metastasis,(10) and tumor immunosuppression.(15) Previously, we reported that CAF elimination suppresses tumor growth(16) and neutralizing local IL-6 in the TME secreted by CAFs improves tumor immunosuppression.(15)

Although it is widely known that CAFs are central players in shaping the TME toward immunosuppression by mediating the immune system,(17) we focused on the IL-6-mediated recruitment of tumor-infiltrating immune cells by CAFs and their fate in a hypoxic TME. Most solid tumor regions are permanently, or transiently, hypoxic due to aberrant vascularization and poor blood supply.(18) Hypoxic environments and subsequent activation of hypoxia-inducible factor 1α (HIF1α) are common features of advanced cancers. Under hypoxic conditions, HIF activity contributes to increased tumor glycolysis, causing “metabolic competition” between cancer and T-cells, while suppressing T-cell function and the antitumor

response.(19) Although we reported that CAFs alter T-cell distribution in the TME to an immunosuppressive state via IL-6,(15) the precise mechanism is not yet clear.

Tocilizumab (TCZ) is the first marketed interleukin-6 (IL-6) blocking antibody that targets IL-6 receptors and has been used to treat rheumatoid arthritis.(20) Although the tumor growth effect of IL-6 is well known and the application of anti-IL-6 receptor antibodies to cancer treatment has been attempted, there are few reports showing clear therapeutic effects.(21-23) We hypothesized that IL-6 produced by CAFs promotes tumor growth in the TME and is the target of anti-IL-6 receptor antibody therapy. Specifically, we aimed to determine whether anti-IL-6 receptor antibody overcomes tumor immunosuppression and suppresses tumor progression using systemic administration of MR16-1, which is a rodent analog of TCZ.(24) Further, we explored the mechanism by which CAFs induce immunosuppression via IL-6, especially focused on hypoxic TME.

Material and Methods

Patients and clinical information

A total of 185 EC tumor samples were obtained from patients who underwent esophagectomy with lymph node dissection at Okayama University Hospital between 2008 and 2011. The outline of our study was published on our web page to explain the study, and to provide opportunities for disagreement. Surgeries were performed according to the Japanese EC treatment guidelines.(25, 26) Patients were excluded if they: i) underwent follow up procedures; ii) were diagnosed with melanoma or distant metastases; or iii) were in remission. Resected specimens were fixed with 10% formalin. Tumor classification and stage were determined according to the TNM Classification of Malignant Tumors 7th edition (UICC 7th edition).(27)

Reagents and antibodies

A rat anti-mouse IL-6 receptor antibody, MR16-1, was kindly provided by Chugai Pharmaceutical Co., Ltd. (Kamakura, Japan). Details of the other reagents and antibodies used in this study are listed in Table S1.

Cell lines

Murine colon cancer (Colon26), fibroblast (NIH/3T3), human esophageal squamous cell cancer (TE4), and esophageal adenocarcinoma (OE19) cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) Cell Bank. The murine fibroblast cell line (MEF) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The murine squamous cell carcinoma cell line (SCCVII) was kindly provided by Professor Yuta Shibamoto (Nagoya City University, Nagoya, Japan), and pancreatic ductal adenocarcinoma (Pan02) was obtained from the National Cancer Institute

(Frederick, MD, USA). Primary human esophageal fibroblasts (FEF3), were isolated from the human fetal esophagus as described previously.(13) WI-38 fetal lung human fibroblasts were purchased from the Health Science Research Resource Bank (Osaka, Japan).

Immunohistochemistry

All immunohistochemistry procedures were described previously.(15) Stained slides were evaluated using ImageJ software (http://rsb.info.nih.gov/ij/). Briefly, the number of cells expressing CD8, forkhead box protein 3 (FoxP3), ionized calcium-binding adaptor protein 1 (Iba1), CD163, and HIF1α were counted in four randomly selected high-magnification fields. The scores of alpha smooth muscle actin (αSMA), IL-6, and vascular endothelial growth factor (VEGF) were evaluated using an "area index," calculated in low magnification fields. All evaluations were performed by an independent pathologist who was blinded to clinical information.

Immunofluorescence

Primary antibodies were added to deparaffinized slides for 60 min at room temperature (RT) (20–22 °C) or overnight at 4 °C, followed by secondary antibodies for 60 min at RT. Coverslips were coated with a drop of mounting medium (P36983; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and subsequently photographed using a fluorescence microscope (IX83; Olympus, Tokyo, Japan).

Cell viability assay

Cells were seeded in 96-well plates (10×10^4 cells/well) and treated with recombinant IL-6 and recombinant IL-6 receptor alpha (IL-6Rα). According to the manufacturer's protocol, cell

viability was determined 2 days after treatment using a Cell Proliferation Kit II (XTT; Roche Diagnostics, Rotkreuz, Switzerland).

ELISA

Cell culture supernatants and human serum samples were assessed for the levels of Mouse-IL-6, Mouse-IL-6Rα, and Human-IL-6 using appropriate ELISA kits (R & D Systems), according to the manufacturer's protocol.

Western blot analysis

Proteins were extracted from whole-cell lysates or nuclear proteins, electrophoresed on polyacrylamide gels and transferred onto membranes. The membranes were incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies 60 min at RT, and then visualized using the Amersham ECL chemiluminescence system (GE Healthcare, IL, USA). Equal loading of the samples was confirmed using β-actin.

Animal studies

Animals were maintained under specific pathogen-free conditions at the Department of Animal Laboratory at Okayama University. Mice were purchased from Clea (Tokyo, Japan) and housed under sterile conditions.

Subcutaneous syngeneic cancer mouse model

Colon26 (0.5×10^6) cells with and without NIH/3T3 (0.5×10^6) cells were subcutaneously inoculated into the right flank of six-week-old female BALB/c mice. The perpendicular diameter of each tumor was measured every 3 days. Tumor volume was calculated using the formula:

Tumor volume (mm^3) = $L \times W^2 \times 0.5$

L represents the longest diameter, W represents the shortest diameter, and 0.5 is a constant used to calculate the volume of an ellipsoid. Treatment with intraperitoneal injections of 20 mg/kg of isotype control (BE0088; BioXcell, Lebanon, NH, USA) or MR16-1 every 3 days began when tumors reached 50–100 mm^3. To generate other cancer models, Pan02 and MEF models were established in C57BL/6 mice, while SCCVII and MEF models were established in C3H/He mice, which were then inoculated and treated in the same way as the Colon26 model.

For T-cell depletion studies, anti-CD8α antibodies (BP0061; BioXcell) were injected intraperitoneally at 10 mg/kg per day before the first injection of isotype control or MR16-1, and every 3 days thereafter, for a total of four treatments. The animals were euthanized via cervical dislocation, and serum and tumor tissue were collected for further analyses.

Culture of mouse bone marrow-derived monocytes

Mouse bone marrow-derived monocytes (BMDMs) were isolated from the femur bones of 6- to 10-week-old BALB/c female mice according to previous studies.(28-30) BMDMs were used as a positive control for macrophage differentiation experiments using IL-4 or IL-6 as stimuli.(29)

Isolation of TILs

Tumor tissues were dissected from the mice and TILs were harvested using BD Horizon Dri Tumor & Tissue Dissociation Reagent (TTDR), according to the manufacturer's protocol. All cells, including TILs and tumor cells with indicated fluorescence-labeled antibodies, were subjected to flow-cytometric analysis.

Flow-cytometric analysis

Cells were washed and incubated with monoclonal antibodies for 30 min at RT in PBS containing 2% FBS. Cells were then washed and analyzed on a BD FACSAria III or FACSLyric (BD Biosciences).

Intracellular cytokine staining of tumor infiltrating lymphocytes

Tumor infiltrating lymphocytes (TILs) were harvested as described above and stimulated for 6 h in the presence of phorbol-12-myristate-13-acetate (PMA), ionomycin, and Brefeldin A at 37 °C. Next, cells were harvested and labeled with a cell surface marker followed by intracellular cytokine staining and flow-cytometric analysis on a FACSAria III.

Statistics

All statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA). Overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan–Meier method, with the log-rank test to compare subgroups. Hazard ratios (HRs) and 95% confidence intervals (CIs) for clinical variables were calculated using Cox proportional hazard regression in univariate and multivariate analyses. Spearman's correlation was used to assess relationships between variables. For group comparisons, the Mann-Whitney test or Student's *t*-test was used. For multiple-group comparisons, analysis of variance with Tukey's test was used. Statistical significance was set at $P < 0.05$.

Study approval

This study was conducted in accordance with the Declaration of Helsinki's ethical standards and the ethical guidelines for medical and health research involving human subjects. The use of clinical samples was approved and reviewed by the Ethics Review Board of Okayama University (No. 1801-023; Okayama, Japan). The experimental animal protocol was approved

and reviewed by the Ethics Review Committee for Animal Experiments at Okayama University (OKU-2020166).

Results

IL-6 expression is an independent prognostic factor in EC patients

We conducted IL-6 immunohistochemistry (IHC) analysis of surgically resected specimens, and the mean value was calculated as an "IL-6 area index" (Figure 1A). The expression of IL-6 was significantly correlated with the expression of αSMA (r = 0.67, P < 0.001) (Figure 1B). When patients were divided into high- and low-IL-6 groups based on the median value (7.21) of IL-6 area index, immunofluorescence (IF) imaging revealed that the expression of αSMA (green) and IL-6 (red) overlapped in both high- and low-CAF groups (Figure 1C). We evaluated the relationship between IL-6 expression, clinicopathological features, and clinical outcomes in 185 patients with EC (Supplementary Table 2). Univariate analysis revealed that sex, tumor depth, lymph node status, prior neo-adjuvant therapy administration, αSMA and IL-6 expression, $CD8^+$ (cytotoxic T-cells (CTL)) and $FoxP3^+$ (regulatory T-cells (Treg)) TILs status, and $CD163^+$ tumor-associated macrophages (TAMs; M2 macrophages) status were significant prognostic factors for OS (Table 1). Patients with high IL-6 expression had significantly shorter OS and DFS than those with low expression (Figure 1D). OS stage-related subgroup analysis using intratumoral tissues revealed that lower IL-6 expression tended to reflect a better OS for all stages (Figure S1). Multivariate analysis was performed using all variables via univariate analysis with P < 0.10; a backward selection was performed using the Akaike information criterion. Multivariate analysis identified IL-6 expression as an independent prognostic factor for OS (HR = 1.82, 95% CI = 1.03-3.20, P = 0.039: Table 1). Similar trends were observed for DFS (Table S3).

CAFs induce immunosuppression via IL-6 in the TME

In intratumoral tissues, negative correlations between $CD8^+$ TILs and IL-6 (r = -0.18), and positive correlations between IL-6 and $FoxP3^+$ TILs (r = 0.33), and $CD163^+$ TAMs (r = 0.51; Figure 2A), were observed. In a comparison based on the IL-6 area index, those with a high IL-6 area index showed significantly lower $CD8^+$ with higher $FoxP3^+$ TIL and $CD163^+$ TAM quantities in intratumoral tissues. In contrast, no significant correlation was observed between $Iba1^+$ TAM (pan-macrophage) numbers and IL-6 (Figure S2).

IF imaging revealed that $CD8^+$ TILs were scarce in high IL-6 patients, despite αSMA accumulation. Unlike $CD8^+$ TILs, the abundance of $FoxP3^+$ TILs and $CD163^+$ TAMs increased in the high IL-6 group compared to the low IL-6 group (Figure 2B).

IL-6 directly contributes to cancer and stromal cell proliferation and differentiation into CAFs and TAMs

An XTT assay was conducted to evaluate cell proliferation. IL-6 induced proliferation of both cancers and fibroblasts for murine and human cell lines; no difference was observed in Colon26 and WI38 cells (Figure 3A). Western blot analysis revealed that IL-6 treatment increased the expression of αSMA; thus, IL-6 differentiates normal fibroblasts into CAFs (Figure 3C).

The effect of IL-6 on macrophage polarization was investigated using BMDMs that were primed for differentiation and pretreated with mouse-macrophage colony-stimulating factor (M-CSF). Flow cytometry analysis of F4/80 (pan-macrophage), CD80 (M1 marker), and CD206 (M2 marker) expression showed that, compared with control cells, treatment with IL-6 increased differentiation of $F4/80^+CD80^-CD206^+$ macrophages, indicating an M2-like phenotype (Figure 3D).

MR16-1 overcomes tumor immunosuppression and suppress tumor growth in vivo

Previously, we demonstrated that CAFs contribute to tumor growth by inducing tumor immunosuppression via IL-6 using in vivo experimental models.(15) To evaluate the effect of MR16-1, a TCZ analog for tumor suppression, we performed in vivo experiments using Colon26 cells and BALB/c mice. Tumors that developed through inoculation with cancer cells (Colon26), co-inoculation with fibroblasts (Colon26 + NIH/3T3), or co-inoculation and treatment with MR16-1 (Colon26 + NIH/3T3 + MR16-1) were compared. MR16-1 significantly reduced the accelerated growth (Figure 4A) and tumor weights (Figure 4B and Figure S3A) that were observed in the co-inoculated tumors. IHC demonstrated that the number of $CD8^+$ TILs in the Colon26 + NIH3T3 group was lower than in the Colon26 group. In contrast, an increased proportion of $FoxP3^+$ TILs and $CD163^+$ TAMs were observed in the Colon26 + NIH3T3 group compared to the Colon26 group (Figure 4C). No difference was observed in the number of $Iba1^+$ TAMs (Figure S3B). Notably, MR16-1 influenced the TIL and TAM populations in the TME, with a significant increase in $CD8^+$ TILs and a significant decrease in $FoxP3^+$ TILs and $CD163^+$ TAMs, compared with the Colon26 + NIH3T3 group. IHC revealed that the expression of αSMA was higher in the Colon26 + NIH3T3 group than in the Colon26 group and decreased in the MR16-1 group (Figure 4C).

We performed the same experiment, substituting Colon26 cells for the pancreatic cancer cell line Pan02 in C57BL/6 mice (Figure 4D and Figure S4). The same trends of tumor suppression and overcoming immunosuppression by MR16-1 were observed as for Colon26 tumors. We conducted the same study with a dermal squamous cell carcinoma cell line SCCVII in C3H/He mice to mimic esophageal squamous cell cancer (Figure 4E and Figure S5). The same trends were observed. Importantly, MR16-1 treatment did not induce significant weight loss in any of the animals tested (Figures S3D, S4C, S5C).

MR16-1 suppresses tumor growth by affecting CD8 in the TME in vivo

We hypothesized that MR16-1 suppressed tumor progression by increasing and activating $CD8^+$ TIL and evaluated whether the efficacy of MR16-1 was $CD8^+$ TIL dependent. Colon26 + NIH/3T3 mice were administered the CD8α depleting antibody during treatment. Four fibroblast groups co-inoculated with cancer cells were compared: no treatment (control), treated with MR16-1 (MR16-1), treated with anti-CD8α antibody (anti-CD8α), and treated with MR16-1 and anti-CD8α (MR16-1 + anti-CD8α). Administration of CD8α depleting antibody abrogated the efficacy of MR16-1 in mice bearing Colon26 + NIH/3T3 tumors (Figure 5A and Figure S6). Tumor progression was significantly suppressed in the MR16-1 group compared to the control and combined MR16-1 and CD8α depleting antibody groups.

We investigated the status of $CD8^+$ TILs in the control and MR16-1 groups. IF staining and flow cytometry demonstrated that the number of total $CD8^+$ TILs in the MR16-1 group was higher than the controls (Figure 5B, C). Furthermore, the capacity for triple cytokine production of $CD8^+$ TILs was increased by MR16-1 treatment (IFNγ, $P = 0.170$; TNFα, $P = 0.007$; IL-2 $P = 0.004$).

Serum IL-6 may serve as a biomarker of CAFs in the TME

Serum samples from mice were analyzed for IL-6 and IL-6Rα. Serum IL-6 concentration in the Colon26 + NIH3T3 group was higher than the Colon26 group, while MR16-1 treatment decreased IL-6 (Figure 5D). In contrast, IL-6Rα was highest in the MR16-1 group (Figure S3C). To investigate the relationship between CAFs in the TME and serum IL-6, we analyzed three groups with varying amounts of fibroblasts: cancer cells alone (Colon26), co-inoculated cancer cells and fibroblasts (Colon26 + 1NIH/3T3, 1:1), and co-inoculated cells with Colon26 + 2NIH/3T3 (1:2). The protocol was followed by tumor resection and simultaneous blood sampling once the tumor volume exceeded 500 mm^3 (Figure S7). Tumor growth was accelerated in the co-inoculated groups, although the difference between the three groups was

not significant. Interestingly, serum IL-6 correlated more strongly with the amount of αSMA in the tumor than with tumor weight (Figure 5E).

Two cancer groups were compared to investigate the effect of MR16-1 treatment in the cancer model: no treatment (Colon26) and cancer cells treated with MR16-1 (Colon26 + MR16-1). MR16-1 showed neither tumor suppression nor immune activation in the cancer cells alone model (Figure S8).

IL-6 regulates tumor immunosuppression via HIF1α activation

To evaluate the relationship between IL-6 and HIF1α activity under hypoxic TME, HIF1α, VEGF, and glucose transporter-1 (GLUT-1), a hypoxia marker, were evaluated by IHC in the in vivo and clinical specimens. In vivo expression of HIF1α and VEGF increased in the high IL-6 state of CAFs present and decreased with MR16-1 treatment (Figure 6A, B and Figure S9). Although GLUT-1 expression was heterogeneous within the tumor tissue samples, it was downregulated in the MR16-1 group compared to the control. In both groups, there were significantly fewer $CD8^+$ TILs at the sites of high GLUT-1 expression and more $CD8^+$ TILs at the sites of low expression (Figure 6C, Figure S11A, B). HIF1α and VEGF expression in clinical specimens were elevated in patients with high IL-6 levels (Figure 6D, E). The heterogeneity of GLUT-1 expression within the tumors was similar to the in vivo specimens. Patients with high IL-6 expression also showed high GLUT-1 expression and low $CD8^+$ TILs, while the opposite trend was observed in patients with low IL-6 expression (Figure 6F, Figure S11C, D).

Next, we used Western blotting to evaluate whether IL-6 activated HIF1α signaling by hypoxia-independent mechanisms. This analysis showed that IL-6 administration increased the expression of HIF1α, VEGF, and GLUT-1 in both murine and human cell lines under normoxic conditions (Figure 6G, Figure S12). CAFs are the major regulators of IL-6 in TME and secrete

much higher levels of IL-6 than cancer cells, TAMs, or normal fibroblasts. Furthermore, IL-6 secretion is increased under hypoxia compared to normoxic conditions (Figure S10).

Discussion

We demonstrated that CAFs induced TILs and TAMs within the TME, into an immunosuppressed state via IL-6 to promote tumor growth and explored a mechanism of IL-6-mediated immunosuppression by CAFs. Furthermore, we showed that systemic administration of MR16-1 alleviated CAF-induced immunosuppression and suppressed tumor growth in vivo, suggesting that an anti-IL-6 receptor antibody could be used for cancer treatment. Regarding the induction of IL-6-mediated immunosuppression by CAFs, we found support for the 'metabolic competition' hypothesis between T-cells and tumor cells, which results in T-cell dysfunction and immunosuppression due to the increased glucose metabolism of tumor cells caused by hypoxia-related signals, which were improved by MR16-1 treatment (Figure S13).

Drug repositioning refers to the use of known drugs for the treatment of diseases other than those for which they were initially designed.(31, 32) TCZ was recently repurposed to treat coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2.(33) Therefore, we investigated the application of TCZ in cancer treatment. In this study, we found that the primary source of IL-6 was CAFs. Additionally, we previously reported that cancer stimulus activated normal fibroblasts into CAFs and also triggered IL-6 secretion from CAFs(15). Furthermore, CAFs created an IL-6-mediated positive feedback loop. IL-6 increased the differentiation of CAFs and TAMs, which further increased the secretion of IL-6. Meanwhile, MR16-1 treatment suppressed tumor growth by activating tumor immunity and inhibiting the growth of CAFs. However, no therapeutic effect was observed with CAF-poor models, indicating the limitations of IL-6 blockade therapy. This result may be one of the reasons that TCZ showed no significant benefit for a novel cancer therapy,(22, 34, 35) suggesting that the anti-IL-6 receptor antibody treatment could be specifically effective in

treating tumors with high CAF abundance, and TCZ repositioning is expected to improve the survival of patients who develop refractory cancers.

Although no effective biomarkers for estimating CAF abundance were previously described, we identified blood IL-6 levels as a potential candidate for estimating CAF abundance in the TMEs. The relationship between blood IL-6 levels and survival has been reported in various cancers,(36-38) but few studies have examined this relationship histologically. In vivo, we demonstrated that serum IL-6 concentration in mice showed a stronger correlation with αSMA positive IHC than with tumor weight, indicating that the amount of IL-6-producing CAFs in the TME influences tumor development. Although this trend was observed in other cell types, the difference was not significant, suggesting that the amount of IL-6 produced by CAFs varies from cell to cell. Since our results highlight blood IL-6 concentration as a potential biomarker of CAFs, as well as a predictor of anti-IL-6 receptor antibody efficacy, further analyses using clinical specimens are warranted.

IL-6 suppresses immune functions in TME by increasing competition between tumors and T-cells for glucose in hypoxic TME. $CD8^+$ TILs demonstrate cytotoxicity toward tumor cells, while $FoxP3^+$ TILs and $CD163^+$ TAMs suppress antitumor immunity, contributing to tumor progression.(39, 40) The mechanism by which IL-6 suppresses T-cells remains unclear. Although IL-6 suppresses the function of Tregs,(41) our results showed the opposite. To investigate this contradiction, we focused on hypoxic TME. Tumor hypoxia forms in advanced cancers with actively proliferating cells, and CTL numbers are reduced due to glucose deficiency. Meanwhile, Treg and M2 macrophage numbers increase by using oxidized lipids as a fuel source under hypoxic conditions, leading to an immunosuppressive state.(42-44) Our results demonstrate that CAFs are the major regulators of IL-6 in TME and IL-6 increased cell proliferation, while IL-6 production by CAFs was enhanced under hypoxia, suggesting that IL-6 and hypoxia exert mutually positive feedback.

Although most solid tumors have hypoxic regions, not all regions or tumors are hypoxic and it is difficult to attribute cancer pathogenesis to hypoxia. Evidence has revealed various hypoxia-independent mechanisms for HIF1α signaling activation, which are termed 'pseudohypoxia'.(45) We observed that HIF1α expression was upregulated by the addition of IL-6 in normoxic conditions, while VEGF and GLUT-1 were continuously upregulated. IL-6 is known to increase the transcriptional activity of HIF1α via signal transducer and activator of transcription3 (STAT3) signaling under hypoxia, and furthermore, HIF1a upregulate VEGF expression via STAT3 pathway and activate GLUT-1 via phosphatidylinositol-3 kinase (PI3K) pathway.(46-48) We revealed that IL-6 regulated HIF1α activation through a hypoxia-independent mechanism.(49) On the other hand, HIF1α, VEGF, and GLUT-1 expression correlated with IL-6 expression in clinical samples and decreased following MR16-1 treatment in vivo, which may reflect hypoxia-mediated HIF1α activation. In tumor tissues, HIF1α shifts glucose metabolism from oxidative phosphorylation to anaerobic processes (the Warburg effect).(50-52) GLUT-1 upregulation accompanying accelerated glucose metabolism in the tumor is associated with low infiltration of effector T cells.(53) Meanwhile, HIF1α and VEGF inhibit the development and activation of CTLs while increasing the number and immunosuppressive functions of Tregs and TAMs.(54, 55) IL-6 might induce tumor immunosuppression by decreasing effector T cells by enhancing cancer glucose uptake and by increasing regulatory cells through HIF1α and VEGF function via hypoxia-pseudohypoxia-mediated HIF1α activation. Therefore, CAFs would mediate tumor immunosuppression by regulating hypoxia-pseudohypoxia-mediated HIF1α activation via IL-6. Furthermore, IL-6 secretion was increased under hypoxia (56-58)(supplementary figure S10D), and HIF1α itself is also known to upregulate IL-6 expression. (59, 60) Thus, there would be a positive feedback loop between IL-6 signaling and HIF-1α expression in the TME.

Our study revealed some interesting results, but also has limitations. First, it is known that IL-6 has two signaling pathways, classical signaling and trans-signaling, and we evaluated the effects of trans-signaling of cancers and fibroblasts on tumor immunity by simultaneous administration of IL-6 and IL-6R.(61-63) In this study, the effect of IL-6 via classical signaling on cells originally expressing membrane IL-6R, such as B cells or myeloid cells, was not evaluated. Further additional effects may be observed by assessing the tumor immunity generated by these cells. Second, we demonstrated that HIF1α was elevated in both allograft models and clinical specimens with high IL-6 expression, and anti-IL-6R decreased HIF1α expression in vivo models, suggesting improvement of hypoxia. However, the mechanism of the direct relationship between anti-IL-6 receptor antibody and local hypoxia is still unclear, therefore further investigation is required. Finally, the TCZ analog (MR16-1) was the used to evaluate the effects of the anti-IL-6 receptor antibody. Future trials are needed to evaluate the exact effects of TCZ on cancer treatment.

In conclusion, we demonstrated that CAFs are the major regulators of IL-6 in the TME, and blood IL-6 concentration could be a potential biomarker of CAFs, while systemic administration of an anti-IL-6 receptor antibody overcomes CAF-induced immunosuppression and halts tumor progress. Furthermore, we described the mechanism by which IL-6 mediates tumor immunosuppression by focusing on metabolic competition between T-cells and tumor cells via hypoxia-pseudohypoxia-mediated HIF1α activation. Hence, the anti-IL-6 receptor antibody may be applied for treating tumors with high CAF abundance, overcoming tumor immunosuppression, and improving the survival of patients with various cancers.

Author contributions

Conception and design: N. Nishiwaki, K. Noma, T. Ohara, H. Tazawa, Y. Shirakawa, T. Fujiwara

Development of methodology: N. Nishiwaki, K. Noma

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.Nishiwaki, T. Kunitomo, K. Kawasaki, M. Akai, T. Kobayashi,. T. Narusaka, H. Kashima, H. Sato, S. Komoto, N. Maeda, S. Tanabe

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis): N.Nishiwaki, T.Kato, K. Noma

Writing, review, and/or revision of the manuscript: N.Nishiwaki, T. Kato, K. Noma, T. Fujiwara

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.Nishiwaki, K. Noma, Y. Shirakawa, S. Kikuchi, H. Tazawa, T. Fujiwara

Study supervision: K. Noma, T.Kato, H. Tazawa, Y. Shirakawa, H. Kobayashi, T. Fujiwara

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Footnotes

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Ethics Statement:

The use of clinical samples was approved and reviewed by the Ethics Review Board of Okayama University (No. 1801-023; Okayama, Japan). The experimental animal protocol was

approved and reviewed by the Ethics Review Committee for Animal Experiments at Okayama University (OKU-2020166).

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Figure legends

Figure 1. Correlation of IL-6 expression and CAFs distribution in esophageal cancer tissues.

(A) Tissue staining with H&E and IL-6. ImageJ was used to evaluate the area index at 200 × magnification. The IL-6 area index is plotted as a histogram (red bar, median value). Scale bars: 100 μm. (B) The area index of αSMA at 100 × magnification was recorded using ImageJ. Scale bars: 200 μm. Correlation between IL-6 and CAFs is shown by the scatter plot (Spearman's correlation coefficient). Violin plots show comparisons based on high or low IL-6 area index. $^{**}P < 0.01$, Student's *t*-test. (C) Immunofluorescence images of IL-6 and αSMA. Representative high and low IL-6 cases at 100 × magnification. Scale bars: 200 μm. (D) Survival curve according to the IL-6 expression (low or high group). Cox regression hazard model, 95% confidence intervals, and log-rank test.

Figure 2. Relative distribution of CAFs, IL-6, and immune cells in resected human esophageal cancer samples.

(A) Average number of $CD8^+$ or $FoxP3^+$ TILs, and $Iba1^+$ or $CD163^+$ TAMs at 400 × magnification. Scale bars: 50 μm. Correlation between IL-6 and $CD8^+$ or $FoxP3^+$ TILs, $CD163^+$ TAMs is shown by scatter plot (Spearman's correlation coefficient). Violin plots show comparisons based on high or low IL-6 area index. $^{**}P < 0.01$, Student's *t*-test. (B) Immunofluorescence images of CD8- or FoxP3-expressing lymphocytes, CD163-expressing macrophages, and αSMA. An example of high and low IL-6 cases at 100 × magnification. Scale bars: 200 μm.

Figure 3. Direct contribution of IL-6 to cell proliferation and differentiation of CAFs and TAMs.

(A and B) Percentage of viable cells at various concentrations of recombinant IL-6 (1, 5, 10, 20 ng/mL) relative to untreated cells (0 ng/mL). Recombinant IL-6R alpha was administered at five times the IL-6 concentration; n = 5; mean ± SE. $^{*}P < 0.05$, Student's *t*-test compared to untreated control. (A) Murine cell lines. (B) Human cell lines. (C) Whole-cell lysates of NIH/3T3, MEF, and FEF3 cells collected 2 days after IL-6 treatment (20 ng/mL) subjected to western blot analysis of αSMA and β-actin expression. (C) Flow cytometry analysis of cell surface F4/80 (M1/M2 marker) and CD80 (M1 marker), and intracellular CD206 (M2 marker) expression in BMDMs with or without IL-6 (20 ng/mL) treatment for 2 days. The bar chart shows the quantification of the F4/80^{+}, CD80^{+}, and CD206^{-} (M1) population and F4/80^{+}, CD80^{-}, and CD206^{+} (M2) populations, n = 3.

Figure 4. MR16-1 treatment overcomes tumor immunosuppression and suppresses tumor growth.

(A) Volume and (B) weight of tumors excised from Colon26 + NIH/3T3 mice, n = 7 mice/group; mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$, Tukey's test with ANOVA. (C) Immunohistochemical staining for CD8, FoxP3, CD163, and αSMA in tumor tissues. The average number of $CD8^{+}$ or $FoxP3^{+}$ TILs and $CD163^{+}$ TAMs at 400 × magnification and the area index of αSMA at 200 × magnification. Scale bars: 100 μm (200 ×); 50 μm (400 ×). $^{*}P < 0.05$; $^{**}P < 0.01$, Tukey's test with ANOVA. (D and E) Tumor volume of the transplanted mice in each group. (D) Pan02 + MEF model, n = 5 mice/group, (E) SCCVII + MEF model, n = 5 mice/group; mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$, Tukey's test with ANOVA.

Figure 5. MR16-1 suppresses tumor growth by affecting CD8.

(A) Tumor volume in transplanted mice with or without MR16-1 treatment. Mice depleted of $CD8^+$ T-cells; n = 5 mice/group mean ± SE. *, $P < 0.05$, Tukey's test with ANOVA. (B) Immunofluorescence images of αSMA and CD8-expressing lymphocytes in control and MR16-1 treatment groups at 100 × magnification. Scale bars: 200 μm. (C) Colon26 + NIH/3T3 tumors treated with or without MR16-1 analyzed for TILs via flow cytometry. TILs were stimulated with PMA/ionomycin for 6 hours, stained for surface CD8, and intracellular staining for IFNγ, TNFα, and IL-2. Flow-cytometric analyses of cytokine-producing $CD8^+$ TILs were statistically assessed between control and MR16-1 treatment (n = 5). $^*P < 0.05$; $^{**}P < 0.01$, Student's *t*-test. (D) Serum IL-6 quantification in Colon26 + NIH/3T3 model by ELISA. $^{**}P < 0.01$, Tukey's test with ANOVA. (E) Correlation between serum IL-6 and tumor weight or αSMA (Spearman's correlation coefficient).

Figure 6. IL-6 regulates tumor immunosuppression via hypoxia/pseudohypoxia-mediated HIF1α activation.

(A) Immunohistochemical images for HIF1α and VEGF in tumor tissues. (B) Average number of HIF1α^{+} cells at 400 × magnification and the area index of VEGF at 200 × magnification. Scale bars: 100 μm (200 ×); 50 μm (400 ×). $^{*}P < 0.05$; $^{**}P < 0.01$, Tukey's test with ANOVA. (C) Immunofluorescence images of GLUT-1 and CD8-expressing lymphocytes in control and MR16-1 treatment groups at 100 × and 200 × magnification. Scale bars: 200 μm (100 ×), 100 μm (200 ×). (D) Immunohistochemical staining for HIF1α and VEGF in human esophageal cancer tissues. (E) Average number of HIF1α^{+} cells at 400 × magnification and the area index of VEGF at 200 × magnification. Scale bars: 100 μm (200 ×), 50 μm (400 ×). $^{**}P < 0.01$, Student's *t*-test. (F) Immunofluorescence images for GLUT-1 and CD8-expressing lymphocytes in a high and low IL-6 case at 100 × and 200 × magnification. Scale bars: 200 μm (100 ×); 100 μm (200 ×). (G) Western blot analysis for HIF1α, VEGF, GLUT-1, and β-actin.