Cancer-Associated Fibroblasts Affect Intratumoral CD8$^+$ and FoxP3$^+$ T Cells via Interleukin 6 in the Tumor Microenvironment

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Abbreviations: CAF, cancer-associated fibroblast; CM, conditioned medium; DFS, disease free survival; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; FAP, fibroblast activation protein; FoxP3, forkhead box protein 3; FSP1, fibroblast-specific protein 1; HR, hazard ratio; IF, immunofluorescence; IVIS, in vivo imaging system; MDSCs, myeloid-derived suppressor cells; OS, overall survival; PDA, pancreatic ductal adenocarcinoma; RT, room temperature; SMA, smooth muscle actin; TILs, tumor-infiltrating lymphocytes; TME, tumor microenvironment; UICC, union for international cancer control; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium
Abstract

**Purpose:** Cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) play a central role in tumor progression. We investigated whether CAFs can regulate tumor-infiltrating lymphocytes (TILs) and their role in tumor immunosuppression.

**Experimental design:** 140 cases of esophageal cancer were analyzed for CAFs and CD8+ or forkhead box protein 3 (FoxP3+) TILs by immunohistochemistry. We analyzed cytokines using murine or human fibroblasts and cancer cells. Murine-derived fibroblasts and cancer cells were also inoculated into BALB/c or BALB/c-nu/nu mice, and the tumors treated with recombinant interleukin 6 (IL-6) or anti-IL-6 antibody.

**Results:** CD8+ TILs and CAFs were negatively correlated in intra-tumoral tissues ($P < 0.001$), while FoxP3+ TILs were positively correlated ($P < 0.001$) in esophageal cancers. Co-cultured Colon26 cancer cells and fibroblasts resulted in accelerated tumor growth in BALB/c mice, along with decreased CD8+ and increased FoxP3+ TILs, compared with cancer cells alone. *In vitro*, IL-6 was highly secreted in both murine and human cancer cell/fibroblast co-cultures. IL-6 significantly increased Colon26 tumor growth in immune-competent BALB/c ($P < 0.001$) with fewer CD8+ TILs than untreated tumors ($P < 0.001$), whereas no difference in BALB/c-nu/nu mice. In contrast, FoxP3+ TILs increased in IL-6-treated tumors ($P < 0.001$). IL-6 antibody blockade of tumors co-cultured with fibroblasts resulted not only in regression of tumor growth but also in the accumulation of CD8+ TILs in intra-tumoral tissues.

**Conclusions:** CAFs regulate immunosuppressive TIL populations in the TME via IL-6. IL-6 blockade, or targeting CAFs, may improve pre-existing tumor immunity and enhance the efficacy of conventional immunotherapies.
Translational Relevance

Despite dramatic improvement of clinical outcomes by innovation of T cell-mediated immunotherapies, the clinical response can be strongly associated with the immunosuppressive tumor microenvironment (TME). Here, we show that cancer-associated fibroblasts (CAFs) regulate tumor-infiltrating lymphocytes (TILs) and change the T cell populations. Intra-tumoral CD8\(^+\) and FoxP3\(^+\) TILs are independent prognostic factors in esophageal cancer and CAFs affect the distribution of TILs. \textit{In vitro}, CAFs activated by cancer cells secrete high levels of Interleukin 6 (IL-6). \textit{In vivo}, CAFs accelerate tumor growth in immune-competent mice, along with phenotypic changes in T cell populations, decreased CD8\(^+\) and increased FoxP3\(^+\) TILs. Moreover, the accelerated tumor growth observed in CAF co-cultures is significantly reduced by IL-6 blockade; therefore, the immunosuppressive TIL population is improved. CAFs may be a biomarker of immunosuppressive TME. IL-6 blockade or targeting CAFs may improve pre-existing tumor immunity and enhance the efficacy of conventional immunotherapies.
Introduction

Esophageal cancer is known as one of the most aggressive malignant tumors, frequently showing lymph node metastasis and tumor invasion into adjacent organs, even in the early stages. The five-year survival rate for esophageal cancer remains only 16.9%, even with current advanced modalities(1). The development of novel therapeutic approaches is thus essential in order to improve the prognosis of patients suffering from this disease. Esophageal cancer is a carcinoma with one of the largest numbers of neoantigens and is likely to benefit from immunotherapy approaches in the near future(2-4).

Recently, the technological innovation of targeted immunotherapy has dramatically improved the prognosis of cancer patients(5-8). Immune checkpoint inhibitors have already been applied to the treatment several malignancies(8), with some reports of favorable outcomes. Furthermore, recent new gene engineering approaches for the modification of tumor-specific T cell function, such as the production of chimeric antigen receptor (CAR) T cells, have been introduced, and there is considerable expectation surrounding this innovation(9, 10). However, there are many problems that remain to be solved with regard to T cell-mediated immunotherapies(11, 12). The ratio of responders has so far been limited, and while the precise mechanism is not yet clear(13), the tumor microenvironment (TME) has been strongly implicated in determining outcomes(14-17). It is currently thought that the TME strongly affects tumor immunosuppression(18), as it is composed of immune cells, tumor vasculature, and cancer-associated fibroblasts (CAFs)(19). CAFs are the most abundant cell population in the TME, and are thus thought to play an essential role in tumor immunosuppression(20-22). They are generally defined as activated fibroblasts within the TME that promote tumor progression and therapeutic resistance(23-25). Several recent reports have shown that CAFs regulate tumor-infiltrating lymphocytes (TILs) via CXCL12 signaling and recruitment of myeloid-derived suppressor cells (MDSCs)(15, 26, 27).
Therefore, the importance of the relationship between CAFs and tumor immunosuppression cannot be understated, and it is an area that requires further research.

In contrast, TILs represent the host immune response to tumor proliferation and metastasis(2, 28). CD8$^+$ T cells, which are cytotoxic T lymphocytes (CTL), demonstrate cytotoxic activity toward tumor cells by triggering apoptosis, and are considered to be the frontline defense against cancer(29-32). Several recent clinical studies have evaluated the role of TILs as prognostic and predictive factors (11), and have shown that CD8$^+$ TILs are associated with longer disease free survival (DFS) and overall survival (OS) in colon and ovarian carcinoma. Meanwhile, forkhead box protein 3-positive (FoxP3$^+$) TILs, which are regulatory T cells (Treg), are thought to suppress mainly antitumor immunity and immunotherapy, as they effectively suppress the proliferation and activation of CTL(30-32). It is reported that FoxP3$^+$ TILs are associated with worse prognosis in breast cancer, gastric cancer and melanoma(29-33). Furthermore, in esophageal cancer, it has been reported that a stratification based on PD-L1 expression and CD8$^+$ TIL status is associated with overall survival(34). However, the role of these TIL populations remains controversial in esophageal cancer(35-38). Moreover, T cell infiltration into inflamed tissues is a multi-step process involving tethering/rolling, activation, adhesion, and transmigration, but it is not fully understood how T cell trafficking and infiltration is regulated in the immunosuppressive TME(13, 17, 18, 39). The role of TME stromal cells, especially CAFs, has now become a major focus in this regard(15, 18, 26).

In this study, we hypothesized that CAFs are implicated in the immunosuppression of esophageal tumors, especially with regard to CD8$^+$ and FoxP3$^+$ TILs, and are thus important for patient prognosis. We therefore performed clinicopathological analysis using primary tissue samples, verified whether CAFs affect tumor immunosuppression using an in vivo model, and further explored the mechanisms underpinning these interactions in vitro.
Materials and Methods

Patients and clinical information

A total of 149 patients who received radical surgery for esophageal cancer in the Department of Gastroenterological Surgery at Okayama University Hospital between 2008 and 2010 were registered for this study. Patients who had received endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) followed by surgery, who were diagnosed with melanoma or a distant metastasis, or who had no tumor presence/complete response after neo-adjuvant therapy were excluded. In total, 140 samples were finally analyzed. All patients were reviewed for age, sex, histological type, neoadjuvant therapy, pathological invasion depth (pT) and lymph node status (pN). Tumor classification was applied according to TNM Classification of Malignant Tumors 7th edition (UICC 7th edition).

Mice and cell lines

Athymic female nude mice (BALB/c-\textit{nu/nu}), female BALB/c mice and female C57BL/6 mice were purchased from Clea (Tokyo, Japan). Animals were maintained in specific pathogen-free conditions in the animal laboratory at Okayama University. Protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University, Okayama, Japan. The murine cell lines of colon cancer (Colon26), luciferase-expressing colon cancer (Colon26-luc), and breast cancer (4T1), as well as the murine fibroblast lines (NIH/3T3, BALBc/3T3 and MEF), were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). The murine cell line of squamous cell carcinoma (SCCVII) was kindly provided by Professor Yuta Shibamoto (Department of Quantum Radiology, Nagoya City University, Nagoya, Japan), and
pancreatic ductal adenocarcinoma (Pan02) was obtained from the National Cancer Institute at Frederick (MD, USA). Human cell lines of esophageal squamous cell cancer (TE4), breast cancer (MCF-7), pancreatic ductal adenocarcinoma (Panc-1) and colon cancer (DLD-1) were also purchased from JCRB. The primary human esophageal fibroblasts, designated FEF3, were isolated from human fetal esophagus as described previously(24). The normal lung fibroblast line NHLF was purchased from Cambrex Corporation (NJ, USA) and WI-38 fetal lung human fibroblast was purchased from the Health Science Research Resource Bank (Osaka, Japan).

**Immunohistochemistry for CD8, FoxP3 and αSMA (alpha smooth muscle actin) in clinical specimens**

Tissue blocks of formalin-fixed and paraffin-embedded surgical specimens of esophageal cancer were sectioned into 2-µm slices for immunohistochemistry (IHC). Firstly, the presence of tumor was confirmed using hematoxylin & eosin (HE) stain. Sectioned tissues were then deparaffinized and soaked in 0.3% H$_2$O$_2$ in methanol at room temperature (RT) for 10 min to extinguish endogenous peroxidase activity. Antigen retrieval was performed by heating specimens in a sodium citrate buffer solution using a microwave. After cooling, sections were incubated in Peroxidase Blocking Reagent (Dako) for 10 min at RT. Sectioned tissues were incubated with primary antibody against CD8 (Dako, clone C8/144B, 1:100 dilution) or FoxP3 (Abcam, ab20034, clone 236A/E7, 1:100 dilution) or αSMA (SIGMA, A2547, clone 1A4, 1:1000 dilution) for 30 min at RT. Following three 5-min washes with PBS, sections were incubated with anti-mouse secondary antibody for 30 min at RT. After washing, the enzyme substrate 3,3’-diaminobenzidine (Dako) was used for visualization, and sections were counterstained with Meyer’s hematoxylin.
Immunofluorescence microscopy for CD8, FoxP3 and αSMA in clinical specimens

Slides were deparaffinized and washed, then protein blocking was carried out at RT for 10 min. The primary antibody against αSMA conjugated to FITC (Abcam, ab8211, clone 1A4, 1:100 dilution) was added at 4°C overnight; antigen retrieval was performed in the dark as described above. Primary antibodies against CD8 or FoxP3 were added as described above, at RT for 30 min. The secondary antibody Alexa568 was added at RT for 1 h. Coverslips were covered with a drop of mounting medium (Vector Laboratories, H-1200) and were subsequently photographed using a fluorescence microscope (BZ-X700, KEYENCE, Osaka, Japan).

Immunofluorescence microscopy for αSMA in cultured cells

NIH/3T3 fibroblasts were seeded onto four-chamber glass slides and cultured in DMEM containing 10% FBS or conditioned medium from Colon26 cells for 96 h. NIH/3T3 cells were fixed with 4% paraformaldehyde and permeabilized with methanol. Slides were stained with the primary antibody against αSMA (Abcam, ab5694, 1:250 dilution) overnight at 4°C. The secondary antibody Alexa568 was added at 4°C for 1 h. Slides were covered and photographed using a Confocal Laser Scanning Biological Microscope (FV10i, Olympus, Tokyo, Japan).

Quantitation of TILs (tumor infiltrating lymphocytes) and evaluation of αSMA area index

Firstly, tumor boundaries were confirmed by H&E staining under microscopy at low magnification (10× objective lens). Next, the slides were examined at high magnification (40× objective lens), and four non-overlapping fields with abundant CD8⁺ or FoxP3⁺ TILs were selected from each of the intra-tumoral or the peri-tumoral tissues. The number of
TILs was counted using Image J software (http://rsb.info.nih.gov/ij/) and the intra-tumoral or peri-tumoral tissues were recorded separately. CAFs were defined as fibroblasts expressing αSMA, and αSMA scoring was evaluated using an “Area Index”, calculated by Image J. To ensure that the whole tumor was evenly evaluated, at least four or more fields including stromal cells were carefully selected to evaluate CAFs. The mean value obtained from each sectioned tissue was defined as the αSMA area index. All evaluations were performed by an independent pathologist who was blinded to clinical information.

**Western blotting**

NIH/3T3 fibroblasts, stimulated with Colon26 cell conditioned medium for 96 h, were examined for the expression of αSMA by Western blotting. Cells were homogenized and whole proteins extracted by centrifugation for 10 min at 4°C. Samples containing 30 mg of protein were separated by electrophoresis on a polyacrylamide gel. Proteins were transferred to membranes and probed overnight at 4°C with primary antibody (Abcam, ab5694, 1:500 dilution). Membranes were then washed in buffer and incubated with secondary antibodies for 1 hour at RT. After washing, membranes were visualized using LAS-4000 mini (FUJIFILM).

**Quantification of IL-6 by ELISA**

Colon26 (2.5 \times 10^4, 5.0 \times 10^4, or 10 \times 10^4 cells) or NIH/3T3 (5.0 \times 10^4, 10 \times 10^4, or 20 \times 10^4 cells) were seeded and cultured in a T25 flask with 2% FBS in DMEM. Culture supernatants were collected 96 h later. The level of IL-6 in each supernatant was measured using the Quantikine ELISA mouse IL-6 Immunoassay (R&D Systems, Minneapolis, MN, USA). In co-culture experiments, both cell types were seeded and cultured using the combinations of cell numbers described above. To evaluate the necessity of direct contact
between cancer cells and fibroblasts, Falcon Cell Culture Inserts (Catalog No. 353090) and Falcon Companion TC Plates (Catalog No. 353502, 6 wells) were used. Colon26 (1.0 × 10^4 cells) or NIH/3T3 (2.0 × 10^4 or 4.0 × 10^4 cells) were seeded, respectively. Data are presented as IL-6 levels per 1.0 × 10^5 cells. In addition, other cancer cells (5.0 × 10^4 of 4T1, SCCVII, or Pan02) and fibroblasts (10 × 10^4 of BALBc/3T3 or MEF) were co-cultured and analyzed according to the same protocol. Finally, human cancer cells (5.0 × 10^4 of TE4, DLD-1, Panc-1, or MCF-7) and fibroblasts (10 × 10^4 of FEF3, WI-38, or NHLF cells) were also co-cultured. For these experiments, supernatants were collected as described above and IL-6 levels were measured using the Quantikine ELISA human IL-6 Immunoassay (R&D Systems). Data are presented as IL-6 levels per 1.0 × 10^5 cells.

In vivo experiments

In order to evaluate tumor immunosuppression by CAFs, we used six-week-old female BALB/c-\textit{nu/nu} and BALB/c mice. Mice were anesthetized and 150 μL of a single-cell suspension containing either Colon26-luc (0.25 × 10^6 cells) only, or Colon26-luc (0.25 × 10^6 cells) with NIH/3T3 fibroblasts (0.5 × 10^6 cells), was injected into the skin of the right flank. For analyzing tumor growth, mice were injected intraperitoneally with luciferin (VivoGlo™ Luciferin, In Vivo Grade) and imaged under isoflurane anesthesia 15 min later. Bioluminescence images were obtained with an IVIS Lumina imaging system (Xenogen IVIS Lumina II; Caliper Life Sciences), and image analysis and bioluminescent quantification were performed using Living Image software. This examination was performed every 4 days, from day 4 to day 16.

To investigate the effects of IL-6, BALB/c and BALB/c-\textit{nu/nu} mice were injected with 1.0 × 10^6 Colon26 cells into the right flank. Subsequently, 200 ng/50 μL/body of recombinant mouse IL-6 protein (R&D systems, 406-ML) or 50 μL of PBS was directly
injected into the tumors every 3 days. Tumor volumes and mouse weights were also evaluated every three days. For other cancer models, C57BL/6 mice were inoculated with $1.0 \times 10^6$ Pan02 cells and were treated according to the same protocol. To explore immunosuppression by CAFs and improvement by IL-6 blockade, $0.5 \times 10^6$ Colon26 cells alone (the Colon26 group) or $0.5 \times 10^6$ Colon 26 cells with $1.0 \times 10^6$ NIH/3T3 cells (the Colon26+NIH/3T3 group) were inoculated into the right flank of BALB/c mice. Mice in the Colon26+NIH/3T3 group were treated with 200 µg/body of isotype control (BD Bioscience, Catalog No. 559286) or anti-IL-6 antibodies (BioXcell) by subcutaneous injection every 3 days. Tumor volumes and mouse weights were also evaluated every 3 days. All mice were euthanized after observation; subcutaneous tumors were then harvested and their tumor weights measured.

**IHC of in vivo-derived tumor tissues**

Harvested subcutaneous tumors were formalin-fixed and paraffin-embedded. Subsequent deparaffinization and antigen-retrieval were performed, as described above. Primary antibodies against CD8a (eBioscience, clone 4SM15, 1:100 dilution), FoxP3 (eBioscience, clone FJK-16s, 1:100 dilution), and αSMA (SIGMA, clone 1A4, 1:1000 dilution) were added and incubated for 60 min at RT. Primary antibody against IL-6 (Abcam, Cat, No. ab6672, 1:500 dilution) was added and incubated overnight at 4°C, and after washing, secondary antibody was added and incubated for 30 min at RT. Sections were visualized and counterstained, as described above. Counting of CD8$^+$ and FoxP3$^+$ TILs and evaluation of αSMA and IL-6 area index were performed using Image J software, as described above.

**Statistics**
All statistical analysis was performed with the SPSS advanced statistics 19.0 software (SPSS, Tokyo, Japan). For CD8^+ or FoxP3^+ TIL counts and the area index of alpha smooth muscle actin (αSMA), cutoffs were defined using the median value of the high or low groups. OS and DFS were calculated using the Kaplan–Meier method, with the log-rank test used to compare between subgroups. Hazard ratios (HRs) and 95% confidence intervals (CIs) for clinical variables were calculated using Cox proportional hazards regression in univariate and multivariate analysis. Spearman’s correlation was used to assess the relationship between TIL counts and the area index of αSMA. For two-group comparisons, the Mann-Whitney test or unpaired t test was used. For multiple-group comparisons, ANOVA with Tukey’s test was used. P values < 0.05 were considered statistically significant.

Study approval

This study was performed in accordance with the ethical standards of the Helsinki Declaration and the ethical guidelines for Medical and Health Research Involving Human Subjects (40). All cases were de-identified and details have been removed from their case descriptions to ensure anonymity. The outline of our study was published on our web page to explain the study, and also to provide opportunities for disagreement. Due to its retrospective nature, we requested and were granted waivers of informed consent from the ethics committee. This study was approved and reviewed by the Ethics Review Board of Okayama University (No. 1603-023; Okayama, Japan). Mouse experiments were performed in a specific pathogen-free environment at the Okayama University animal facility according to institutional guidelines, and all animal experimental protocols were approved and reviewed by the Ethics Review Committee for Animal Experimentation of Okayama.
University. All experiments were performed in accordance with the guidelines and regulations indicated by these committees.

Results

**CAFs affect the distribution of TILs in esophageal cancer tissue.** To explore the distribution of CD8+ and FoxP3+ TILs in esophageal cancer, we conducted IHC using surgically resected tissues. There were differences in accumulation of TILs between intra- and peri-tumoral sites, as demonstrated by the fact that CD8+ TILs were found in greater numbers in peri-tumoral than intra-tumoral tissues (Fig. 1A), and a similar trend was observed for FoxP3+ TILs (Fig. 1B). These results raise the possibility that the migration of lymphocytes into intra-tumoral sites is affected by stromal cells in the TME. Next, we evaluated the distribution of CAFs, which were identified as stromal cells expressing αSMA (Fig. 1C and D). In intra-tumoral tissues, the presence of CAFs was highly variable; in contrast, in peri-tumoral tissues, CAFs were generally present at relatively low numbers. To explore the relationship between the distribution of TILs and CAFs, we performed immunofluorescence (IF) imaging using anti-αSMA (green), and anti-CD8 or anti-FoxP3 (red) antibodies. Co-staining of anti-αSMA and anti-CD8 revealed that numerous CD8+ TILs were present in both intra-tumoral and peri-tumoral sites when CAFs were in low numbers. In contrast, in cases where CAFs were present at high numbers (the majority of stromal fibroblasts staining green), CD8+ TILs in the intra-tumoral tissues were scarce, despite an accumulation of CD8+ TILs in peri-tumoral sites (Fig. 1E). Unlike CD8+ TILs, FoxP3+ TILs were found at higher numbers in intra-tumoral sites in high-CAF cases compared with low-CAF cases (Fig. 1F). Thus, CAFs may influence tumor immunosuppression by regulating the migration or invasion of TILs.
Intra-tumoral CD8$^+$ and FoxP3$^+$ TILs are independent prognostic factors for esophageal cancer. To investigate the clinical impact of TILs in esophageal cancer, we evaluated their association with prognosis in 140 consecutively-enrolled cases of esophageal cancer. The number of CD8$^+$ or FoxP3$^+$ lymphocytes was counted in four sites for each intra-tumoral or peri-tumoral tissue (Fig. 2A) using IHC (Fig. 2B, left), and the average calculated (Fig. 2B, right). The number of both CD8$^+$ and FoxP3$^+$ TILs in peri-tumoral sites was thus significantly higher than in intra-tumoral tissues.

Next, we evaluated the relationship between CD8$^+$ and FoxP3$^+$ TIL distribution, clinicopathological features, and clinical outcome. Tumor depth, lymph node status, and neo-adjuvant therapy status were found to be significant prognostic factors for OS by univariate analysis ($P < 0.001$ for all; Supplementary Tables 1 and 2). Furthermore, patients with high CD8$^+$ TIL numbers in intra-tumoral sites had significantly longer OS than those with low numbers. On the other hand, the high FoxP3$^+$ TIL group for intra-tumoral tissues had significantly a shorter OS (Fig. 2C). However, in peri-tumoral tissues, no significant correlations between CD8$^+$ or FoxP3$^+$ TIL numbers and prognosis were identified (Fig. 2D).

In the Stage-related sub-group analysis for OS in intra-tumoral tissues, higher CD8$^+$ or lower FoxP3$^+$ TIL numbers tended to reflect better OS for all Stages (Supplementary Fig. 1 and Supplementary Table 3). Moreover, in multivariate analysis, both CD8$^+$ and FoxP3$^+$ TILs were independent prognostic factors for OS (CD8$^+$, HR = 0.45, 95% CI = 0.27-0.77, $P = 0.004$; FoxP3$^+$, HR = 1.86, 95% CI = 1.05-3.29, $P = 0.034$; Supplementary Table 4). Similar trends were demonstrated for DFS (Supplementary Tables 2-4). Thus, in intra-tumoral but not peri-tumoral sites, both CD8$^+$ and FoxP3$^+$ TILs strongly contribute to esophageal cancer prognosis.
Correlation between CAFs and CD8+/FoxP3+ TIL distribution in intra-tumoral tissues. To confirm the correlation between CAFs and CD8+/FoxP3+ TIL distribution in esophageal cancer, we evaluated the presence of CAFs expressing αSMA in each sample using IHC. The mean value was calculated as an “αSMA area index” (Fig. 3A). In intra-tumoral tissues, a negative correlation between CD8+ TILs and CAFs was demonstrated in scatter plots ($r = -0.416$), and a positive correlation between FoxP3+ TILs and CAFs was also observed ($r = 0.484$; Fig. 3B). Furthermore, in a comparison based on αSMA area index, those with a high αSMA area index (i.e. CAF-rich cases) showed significantly lower CD8+ and higher FoxP3+ TIL numbers in intra-tumoral tissues (CD8+, $P < 0.001$; FoxP3+, $P < 0.001$; Fig. 3C). In contrast, in peri-tumoral tissues, no significant correlation was observed.

Next, we evaluated the ratio of peri-to intra-tumoral CD8+ TILs and identified lower, inter, and upper quartiles using box-and-whisker plots (Fig. 3D, left). We then compared the αSMA area index in each group (Fig. 3D, middle & right), finding that the αSMA area index in the upper quartile of the peri-/intra-tumoral CD8+ TIL ratio was significantly higher than in other groups. It therefore appeared that CAFs strongly affected the distribution of CD8+ TILs in these cases, resulting in lower CD8+ TIL numbers in the intra-tumoral sites. In contrast, in the FoxP3+ TIL analysis, the αSMA area index in the upper quartile group was significantly lower than other groups (Fig. 3E), suggesting that CAFs also accelerate the migration of FoxP3+ lymphocytes into the intra-tumoral tissues. Furthermore, in CD8+ TILs, a positive correlation between peri-/intra-tumoral ratios and CAFs was demonstrated in scatter plots ($r = 0.451$), and a negative correlation was also observed in FoxP3+ TILs ($r = -0.448$; Supplementary Fig. 2). Taken together, these data provide strong evidence that esophageal cancer CAFs restrict the infiltration of CD8+ TILs into intra-tumor tissues, and in contrast, promote the infiltration of FoxP3+ TILs.
CAFs promote tumor immunosuppression in vivo. To further evaluate the relationship between CAFs and immunosuppression, in vivo experiments were performed using two types of mice, namely BALB/c and BALB/c-\textit{nu/nu} mice. We used the luciferase-expressing mouse-derived cell line, Colon26-luc for these experiments. We established two groups, cancer cells alone (Colon26-luc) and cancer cells co-cultured with fibroblasts (Colon26-luc+NIH/3T3). Cancer cells and fibroblasts were subcutaneously inoculated into each mouse, and tumor growth was assessed using a bioluminescent imaging system (IVIS) every 4 days (Fig. 4A). Quantitative analysis of luciferase activity showed that the progression of Colon26-luc+NIH/3T3 tumors was significantly faster than Colon26-luc tumors, in both the BALB/c-\textit{nu/nu} mice (\(P < 0.05\), day 16) and the BALB/c mice (\(P < 0.05\), day 12; and \(P < 0.001\), day 16) (Fig. 4B and C). Moreover, the luciferase activity in the Colon26-luc+NIH/3T3 group was 1.2-1.3 times higher than in the Colon26-luc group by day 16 in BALB/c-\textit{nu/nu} mice, and 2.5-3.0 times higher in BALB/c mice (Fig. 4D). Therefore, the tumor progression ratio of the Colon26-luc+NIH/3T3 group to the Colon26-luc group was demonstrated more strongly in BALB/c, which are immunocompetent mice, than in BALB/c-\textit{nu/nu} mice. In BALB/c mice, IHC showed that CD8\textsuperscript{+} TIL numbers were lower in tumors from the Colon26-luc+NIH/3T3 group (in which numerous fibroblasts overexpressing \(\alpha\text{SMA}\) were also detected), as compared with Colon26-luc group (\(P = 0.0474\)). In contrast, FoxP3\textsuperscript{+} TIL numbers increased (\(P = 0.251\)), thus showing the same trend as in our clinical analysis (Fig. 4E). Thus, these results suggest that CAFs accelerate tumor immunosuppression by regulating TILs in intra-tumoral tissues and promoting tumor progression.
Elevated expression of interleukin 6 (IL-6) in the tumor microenvironment. Next, we performed in vitro experiments to address the physiological basis of the observed TIL regulation by CAFs. First, we evaluated the expression level of TGF-β, which is known as a key tumor immunosuppressive cytokine. However, we found that not only CAFs, but also cancer cells, released TGF-β at high levels (Supplementary Fig. 3A and B). We then evaluated the involvement of other cytokines using a multi-cytokine array. Before the array, we first confirmed that NIH/3T3 normal fibroblasts acquired a CAF phenotype (i.e. overexpressed αSMA), when cultured in conditioned medium from Colon26 cancer cells, using immunofluorescent microscopy and Western blotting (Fig. 5A). Subsequently, we collected the supernatants from cancer cells cultured alone (Colon26 or SCCVII) as a control and the co-culture of those cancer cells and NIH/3T3 fibroblasts, and measured the level of cytokines using the array (Supplementary Fig. 3C). The results demonstrated that the level of IL-6 in co-cultured supernatants was more than five times higher than in supernatants from cancer cells cultured alone. Therefore, we focused on IL-6 and quantified this cytokine by ELISA in the supernatants from Colon26 and NIH/3T3 cells under various conditions (Fig. 5B). Colon26 or NIH/3T3 cells cultured alone released IL-6, but the concentration was quite low. Meanwhile, co-cultures of Colon26 and NIH/3T3 secreted higher concentrations of IL-6. Furthermore, the concentration of IL-6 increased as the number of NIH/3T3 cells seeded increased ($P < 0.001$, among all experimental groups). In contrast, the seeding density of Colon26 cells didn’t significantly affect the levels of IL-6 in the supernatant, suggesting that it is CAFs that release IL-6 at high levels, rather than cancer cells. We also confirmed that IL-6 was still secreted at high levels when cells were cultured without direct contact (Fig. 5C). Various co-culture combinations were tested between other mouse-derived cancer lines (4T1, SCCVII and Pan02) and mouse-derived fibroblasts (MEF.
and BALBc/3T3) (Fig. 5D). The trend to increase IL-6 secretion as the result of co-culture was observed for most of the combinations.

Finally, we repeated these experiments using four human esophageal cancer cell lines (TE1, TE4, TE8 and OE33) and three human fibroblast lines (FEF3, WI-38 and NHLF). As with the murine cells, the concentration of IL-6 released by human CAFs activated by human cancer cells was significantly higher than seen with cancer cells alone or non-activated fibroblasts (Supplementary Fig. 4). Furthermore, we checked the level of IL-6 in cultured supernatants from several types of cancer cells, colon cancer (DLD-1), pancreatic cancer (Panc-1) and breast cancer (MCF-7) under the condition of cancer alone or co-culture with several representative fibroblasts. High concentrations of IL-6 in co-cultured supernatants were detected under almost all conditions (Fig. 5E). These data suggest that under baseline conditions, cancer cells or normal fibroblasts release low levels of IL-6; however, both mouse and human CAFs activated by cancer cells secrete high levels of IL-6.

**IL-6 promotes tumor growth and immunosuppression in vivo.** We next examined whether high levels of IL-6 in intra-tumoral tissues cause immunosuppression in vivo. We used the murine-derived Colon26 cancer cells, and injected them into BALB/c and BALB/c-"nu/nu" mice subcutaneously. To mimic a high level of IL-6 in intra-tumoral tissues, recombinant mouse IL-6 was injected into the subcutaneous tumors directly every 3 days. The results demonstrated that in BALB/c-"nu/nu" mice, IL-6 had a trend towards an additive effect, although this was not significant (Fig. 6A). However, in BALB/c mice, IL-6 had a significantly additive effect and was associated with significantly greater tumor growth (Fig. 6B and C) and tumor weight gain (data not shown) compared to untreated tumors. Furthermore, with respect to tumor immunity, IHC demonstrated that the number of CD8\(^+\) TILs in the Colon26+IL-6 group was lower than in the Colon26 group (Fig. 6D). In
contrast, there were higher numbers of FoxP3+ TILs in the Colon26+IL-6 group compared to the Colon26 group (Fig. 6E). Quantification of both CD8+ and FoxP3+ TILs revealed significant differences between the Colon26 and Colon26+IL-6 groups (*P < 0.001). It was also confirmed by IHC that the local IL-6 concentration of the Colon26+IL-6 group was indeed higher as intended (Fig. 6F). We also performed the same experiment using the pancreatic cancer cell line Pan02 in C57BL/6 mice. The same trends were observed as above, with the exception of quantification of CD8+ TILs by IHC (Fig. 6G). Furthermore, we performed this experiment using the dermal squamous cell carcinoma cell line SCCVII in C3H/HeJ mice as a substitute for esophageal squamous cell carcinoma (Supplementary Fig. 5). Although significance was not demonstrated in tumor growth among both groups, the implantation rate of the xenograft in the IL-6 group was obviously increased as compared to the control group (100% vs. 67%). Furthermore, IHC demonstrated that CD8+ TILs were lower, whereas FoxP3+ TILs were significantly higher in the SCCVII+IL-6 group, which indicates immunosuppressive conditions. These results suggest that IL-6 promotes tumor growth and immunosuppression by regulating CD8+ or FoxP3+ lymphocytes in intra-tumoral tissues.

Finally, to evaluate the reversibility of CAF-induced tumor immunosuppression via IL-6, we performed in vivo experiments using murine Colon26 cells and BALB/c mice. Three tumor groups were compared; cancer cells alone (Colon26), co-cultured cancer cells and fibroblasts (Colon26+NIH/3T3), and co-cultured cells treated with anti-IL-6 antibody (Colon26+NIH/3T3+Anti-IL-6 antibody). The results showed that the accelerated growth observed in co-cultured tumors (*P < 0.05) was significantly reduced by IL-6 blockade (*P < 0.05; Fig. 6H). The same trend was seen in regard to tumor weights (data not shown). Furthermore, in terms of immune status, IL-6 blockade remodeled the TIL population in the TME as expected, with a significant increase in CD8+ TILs (P = 0.031) and a significant
decrease in FoxP3+ TILs \((P = 0.101)\). Thus, we demonstrated that CAFs affect tumor immunosuppression by regulating CD8+ and FoxP3+ TILs in intra-tumoral tissues by a mechanism involving IL-6. Targeting CAFs with anti-IL-6 antibodies may therefore be a therapeutic strategy to improve tumor immunosuppression and contribute to tumor regression.

**Discussion**

In this study, we demonstrated that CAFs have a strong association with a population of TILs, which could lead to immunosuppressive conditions in the TME. These findings were consistent between *in vitro* and *in vivo* experiments, and in our analysis of clinical samples of esophageal cancer. Our data show that CD8+ lymphocytes (cytotoxic T cells) and FoxP3+ lymphocytes (regulatory T cells) are independent prognostic factors for esophageal cancer, but only with regard to their presence in intra-tumoral, not peri-tumoral tissues. Furthermore, CAFs have a significant correlation with CD8+ or FoxP3+ TILs in intra-tumoral tissues, suggesting that CAFs cooperate with cancer cells to control migration of TILs into the tumor. This raises questions as to whether it is the absolute number of TILs that is important (as opposed to their relative balance between the intra- and peri-tumoral tissues), and how the CAFs regulate the TIL population of the TME.

To address these questions, we analyzed the TIL ratio between the peri- and intra-tumoral sites. We found that a high ratio of peri- to intra-tumoral CD8+ or low ratio of FoxP3+ TILs tended to be associated with a high αSMA index, indicating that it is the CAFs that affect the peri- to intra-tumoral migration of those TILs (Fig. 3D and E, Supplementary Fig. 2). Another important finding of our study is the observation that CAFs stimulated by cancer cells release IL-6 at high levels, and that IL-6 may therefore coordinate the migration of TILs. TGF-β in the TME has been known to play an important role in both tumor...
progression (24) and tumor immunity. In our study, although the level of TGF-β in the co-culture system in vitro was found to be elevated, the cytokine is not specifically secreted by fibroblasts. Therefore, in our study, we focused on IL-6, as CAFs are the major source of this cytokine.

IL-6 is a well-known inflammatory cytokine and it has previously been reported that IL-6 plays an important role in tumorigenesis, tumor proliferation, invasion, angiogenesis, and tumor immunosuppression via signaling pathways in the TME involving JAK/STAT, MEK, and AKT (41-43). In our study, we artificially created a high level of IL-6 in intratumoral tissues by injection of the cytokine directly into the tumor site. No significant difference in tumor growth was detected in the immune-deficient BALB/c-nu/nu mice in response to IL-6, whereas significantly higher tumor growth was demonstrated in BALB/c mice, which are immune-competent. Furthermore, IL-6 blockade decreased tumor growth, and remodeled the TIL population in a direction that would be expected to improve anti-tumor immunity. We confirmed using surgically-resected specimens that IL-6 expression in vivo coincides with the location of αSMA+ stromal cells (Supplementary Fig. 6). High concentrations of IL-6 may promote an immunosuppressive TME, as demonstrated by lower numbers of CD8+ and higher numbers of FoxP3+ TILs in CAF-rich tumors.

However, in general, IL-6 inhibits Treg differentiation induced by TGF-β from naive T cells (44), which does not explain our results. We hypothesize that T cell metabolism is strongly related to the distinctive tumor microenvironment, which is under hypoxia conditions caused by CAFs and IL-6 (45). It is known that hypoxia-inducible factor 1α (HIF-1α) is controlled by oxygen level and is stabilized under hypoxic conditions (45-47). It has also been reported that CAFs and IL-6 induce reactive oxygen species (ROS), and ROS affect hypoxic conditions (48, 49). In effector T cells, stabilized HIF-1α results in activation of glycolytic metabolism. In tumor cells, the enhanced conversion of glucose to lactate was
revealed by the Warburg effect (50). As a result of glucose deficiency, effector T cells compete with tumor cells for glucose within the tumor niche (51, 52), and CD8+ T cells tend to decrease under such conditions. On the other hand, Treg cells are highly dependent on mitochondrial metabolism having the flexibility to use oxidized lipid or glucose as a fuel source (51, 52). Thus, Treg cells are tolerant to hypoxic conditions and appear to increase under these conditions. Furthermore, another group reported that ROS themselves induce Treg (46). From the above considerations, it is thought that lower CD8+ T cells and higher FoxP3+ T cells could be present under hypoxic tumor conditions. Conversely to intra-tumoral conditions, the activity of CD8+ T cells is thought to be maintained in an aerobic environment in peri-tumoral tissues. Our hypothesis is supported by the observation that HIF-1α and carbonic anhydrase IX (CAIX), which is a major downstream target of HIF-1α, were overexpressed together with IL-6 expression in cases with high αSMA by IHC (Supplementary Fig. 7). We previously reported that CAFs secreted vascular endothelial growth factor, which also promotes hypoxic conditions (24). Thus, from the viewpoint of tumor metabolism and hypoxia, there may be a different role for IL-6 in tumor immunity in the tumor microenvironment as compared with the systemic non-tumor microenvironment.

Understanding the regulation of TILs by CAFs may improve conventional immune therapies (5-8). Currently, these strategies are focused on activating pre-existing immunity (16). Clinical evidence has demonstrated that immune checkpoint inhibitors are more effective in inflamed tumors, which are characterized as having high CD8+ T cell density (28, 53). Meanwhile, efficacy has been limited in cases where CD8+ TILs in intra-tumoral tissues are low in number. In such cases, combination therapy strategies may be envisaged to both stimulate pre-existing immune cell function and anti-tumor T cell infiltration (17). It is clear that CAFs are a major source of regulation of CD8+ cytotoxic T cells within the TME. Therefore, controlling CAFs presents the possibility of improving the
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...immunosuppressive microenvironment for immune checkpoint inhibitors. To this end, a recent report has confirmed that IL-6 and PD-L1 antibody blockade combination therapy reduces tumor progression in murine pancreatic cancer models(41). In addition, the trafficking of CD8+ T cells into intra-tumoral tissues may prove critical in improving the efficacy of other immune therapies, such as CAR-T cell therapy and cancer vaccine therapies(10, 16).

It should be recognized, however, that targeting CAFs therapeutically has some challenges. Firstly, CAFs may be derived from a number of cell types, such as fibroblasts, myeloid cells, epithelial cells, and adipocytes. Secondly, there are no specific markers to identify CAFs. In this study, however, we demonstrated that IL-6 blockade resulted in improved tumor immunity, and given that IL-6 blocking antibodies are already approved by the FDA, such an approach to restore an immunosuppressive TME may be a viable strategy in the clinic. Despite this, the development of more radical therapies directly targeting CAFs may still be required in the future.

Our study has some limitations. Firstly, we evaluated the location of CD8+ and FoxP3+ lymphocytes by IHC, rather than evaluating their functionality. T cell exhaustion was not examined, and in regard to FoxP3+ lymphocytes, Treg functionality was not assessed. It should be considered whether IHC with the single FoxP3+ would be enough for evaluation of Treg. Furthermore, it is unclear whether the changes in both TILs are caused by trafficking or selection. It is reported that trafficking of Treg cells in the tumor is caused by the chemokine CCL22 produced by tumor-associated macrophages and tumor cells(54); therefore, verification of T cell trafficking by CAFs is necessary. In addition, further experiments are necessary to confirm our hypothesis regarding the correlation between IL-6 and T cell metabolism. Finally, the investigation of effects from T cells to CAFs is also another issue, which is in the opposite direction as demonstrated in this study. Other groups...
have reported that effector T cells control stroma-mediated chemoresistance by altering the metabolism of CAFs in the tumor microenvironment(55). Thus, further investigation is required regarding the crosstalk between T cell activation and suppression, and CAF signaling.

In conclusion, we have described a role for CAFs in modulating tumor immunity in esophageal cancer, in particular in regard to the intra-tumoral migration of TILs. CAFs remodel the immunosuppressive TIL population of the TME, via the secretion of high levels of IL-6. Blockade of IL-6 signaling or therapies directly targeting CAFs could improve T cell trafficking, migration, and tumor immunosuppression, and thus improve the prognosis of patients with many different types of cancer, including esophageal cancer.

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

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

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Kato, H. Kashima, Y. Katsura, R. Katsube, S. Komoto

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

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

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

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Footnotes

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).
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17. Kim JM, Chen DS. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or...


Figure legends

Figure 1. Relative distribution of TILs and CAFs in surgically resected human esophageal cancer samples. (A) IHC for CD8-expressing T cells is shown at low and high magnification (40× and 400×). The distribution of CD8⁺ TILs is different between intra-tumoral and peri-tumoral tissue. (B) IHC for FoxP3 expression is shown. (A and B) Scale bars: 1 mm (HE, 40×), 100 µm (400×). (C, D) IHC for αSMA⁺ CAFs shows a variable distribution in intra-tumoral tissue between different cases, but no differences for peri-tumoral tissues. Scale bars: 2 mm (HE, IHC: 20×), 200 µm (100×). (C) Representative example of a high-αSMA case. (D) Representative example of a low-αSMA case. (E, F) Double staining immunofluorescence (IF) images of CD8- or FoxP3-expressing lymphocytes and αSMA at middle and high magnification (100× and 400×). Scale bars: 1 mm (HE, IHC: 40×), 200 µm (100×), 50 µm (400×). (E) CD8⁺ TILs in intra-tumoral tissues are present at high numbers in a low-CAF case and at low numbers in a high-CAF case. (F) In contrast, FoxP3⁺ TIL numbers in intra-tumoral tissues are high in a high-CAF case and low in a low-CAF case.

Figure 2. Correlation between CD8⁺ and FoxP3⁺ TIL distribution and clinical outcome in 140 cases of esophageal cancer. (A) Protocol for area selection. The intra-/peri-tumoral border was first defined at low magnification (40×). Four areas
with the most abundant lymphocytes under high magnification (400×) were then selected for both intra- and peri-tumoral sites in each specimen. (B) The average number of CD8⁺ or FoxP3⁺ TILs was recorded using ImageJ (n = 140 per group; black bar indicates median). CD8⁺ and FoxP3⁺ TILs in peri-tumoral tissues were more numerous than in intra-tumoral sites (CD8⁺, P < 0.001; FoxP3⁺, P = 0.034; Student’s t test). Scale bars: 50 µm. (C) OS curves according to CD8⁺ and FoxP3⁺ TIL number (high or low groups) in intra-tumoral tissues. The CD8⁺ high group showed significantly better prognosis, while the FoxP3⁺ high group showed significantly worse prognosis (Cox regression hazard model, 95% confidence intervals and log-rank test). (D) Survival curves for TIL distribution in peri-tumoral tissues. No significant difference in outcome was detected for the high/low groups of CD8⁺ and FoxP3⁺.

Figure 3. Correlation of CAFs and CD8⁺/FoxP3⁺ TIL distribution in esophageal cancer tissues. (A) Tissue staining with H&E and anti-αSMA, with the emphasis figure from ImageJ demonstrating the evaluation of area index. The mean of area ratio measurements was calculated as the αSMA area index; the αSMA area index for all cases is plotted as a histogram (black bar, median value). Scale bars: 200 µm. (B) The correlation between CAFs and CD8⁺ or FoxP3⁺ TILs is shown by scatter plot. In intra-tumoral tissues, there was a negative correlation with CD8⁺ TILs (r = -0.416), whereas there was a positive correlation for FoxP3⁺ TILs (r = 0.484). No correlation was observed in peri-tumoral tissues (CD8⁺, r = 0.017; FoxP3⁺, r = 0.230; Spearman’s correlation coefficient). (C) Comparisons based on high or low αSMA area index are shown by the box and whisker plots. In intra-tumoral tissues, significant differences were observed (CD8⁺, P < 0.001; FoxP3⁺, P = 0.034; Student’s t test), however, this was not the case for peri-tumoral tissues (CD8⁺, P = 0.441; FoxP3⁺, P = 0.017; Student’s t test). (D)
Ratios of peri- to intra-tumoral CD8\(^+\) TILs were calculated for all cases and evaluated by histogram and whisker plot (left). Data were then divided into three groups based on quartiles, and plotted by line graph (CD8\(^+\) count, middle), and box and whisker plots (\(\alpha\text{SMA}\) area index, right; \(*P < 0.05\), Tukey’s test with ANOVA). (E) Ratios for peri- to intra-tumoral FoxP3\(^+\) TILs were analyzed as for CD8\(^+\) TILs. The opposite pattern to that seen for CD8\(^+\) TILs for \(\alpha\text{SMA}\) area index was observed (*\(P < 0.05\); Tukey’s test with ANOVA).

**Figure 4. CAFs promote tumor growth by regulating TILs in vivo.** (A) IVIS imaging of subcutaneous tumors of Colon26-luc cells alone, or Colon26-luc cells co-cultured with NIH/3T3 fibroblasts, in BALB/c-\(\text{nu/nu}\) mice and BALB/c mice. (B) Quantitative luciferin activities are shown for BALB/c-\(\text{nu/nu}\) mice (mean \(\pm\) S.E.M; *\(P < 0.05\), day 16; Student’s \(t\) test). (C) Quantitative analysis for BALB/c mice (mean \(\pm\) S.E.M; *\(P < 0.05\), day 12; **\(P < 0.001\), day 16; Student’s \(t\) test). (D) Relative progression of tumor growth in the co-culture and control groups (Colon26-luc/Colon26-luc+NIH/3T3 tumors) (E) IHC of CD8, FoxP3 and \(\alpha\text{SMA}\) in BALB/c mice. Increased \(\alpha\text{SMA}\) staining and FoxP3\(^+\) TILs, and decreased CD8\(^+\) TILs were observed in Colon26-luc+NIH/3T3 tumors (left). The number of CD8\(^+\) and FoxP3\(^+\) TILs in each sample is also plotted (right), showing a significantly lower number of CD8\(^+\) TILs in Colon26-luc+NIH/3T3 tumors (*\(P = 0.0474\), Tukey’s test with ANOVA), and a trend towards greater numbers of FoxP3\(^+\) TILs \(P = 0.251\), Tukey’s test with ANOVA). Scale bars: 200 \(\mu\text{m}\) (HE), 100 \(\mu\text{m}\) (IHC).

**Figure 5. Fibroblasts and cancer cells cooperate to promote the release of IL-6.** (A) Immunofluorescent microscopy showed higher expression of \(\alpha\text{SMA}\) in activated fibroblasts, stimulated by conditioned medium from Colon26 cells. Western blots showed
higher expression of αSMA in CAFs vs. non-activated fibroblasts. Scale bars: 50 µm. (B) Quantification of IL-6 secretion by ELISA. Cancer cells (Colon26) and normal fibroblasts (NIH/3T3) secreted IL-6 in a seeding density-dependent manner (× 10^4 cells). Under co-culture conditions, IL-6 secretion was significantly increased in a CAF-seeding density dependent manner. (C) Quantification of IL-6 secretion from fibroblasts (× 10^4 cells) using conditioned medium from Colon26 cells (1.0 × 10^4 cells) cultured in isolation, to evaluate stimulation under non-contact conditions. (D) The stimulation of IL-6 secretion using other types of cancer cells and fibroblasts was evaluated. Some of these lines had a high baseline level of IL-6 secretion, such as 4T1 or MEF. However, most fibroblasts were stimulated by co-culture with cancer cells to release IL-6 excrete high levels. (E) Quantification of IL-6 secretion by human cells was investigated. The same trend was observed as with mouse-derived cells (panels B-E: data represent mean ± SD; *P < 0.05, Tukey’s test with ANOVA; NS, not significant).

Figure 6. IL-6 accelerates tumor growth and immunosuppression by regulating TILs in vivo. (A, B) Growth of subcutaneous tumors inoculated in BALB/c (n = 6, in each group) or BALB/c-nu/nu mice (n = 5, in each group) with or without IL-6 injection, was observed (mean ± S.E.M; *P < 0.05; Student’s t test). (C) Comparison of tumor progression in IL-6 treated animals relative to untreated animals. (D) IHC and quantification of CD8^+ TILs at high magnification (400×; *P < 0.001, Student’s t test). (E) IHC and quantification of FoxP3^+ TILs at high magnification (400×; *P < 0.001, Student’s t test). (D and E) Scale bars: 200 µm (200×), 100 µm (400×). (F) IHC and quantification of the IL-6 area index at high magnification (400×; *P < 0.001, Student’s t test). Scale bars: 1 mm (40×), 100 µm (400×).
(G) Pan02 subcutaneous tumors in C57BL/6 mice were evaluated and stained for CD8\(^+\) and FoxP3\(^+\) TILs by IHC. Evaluation of tumor weight and the number of TILs is shown for each group (*\(P < 0.05\), Student’s \(t\) test). (H) Tumor growth assay using anti-IL-6 antibody to block IL-6 effects (left). Comparison with or without IL-6 blockade in co-cultured tumors (\(n = 7\) mice in each group; mean ± SD; \(*P < 0.05\), Tukey’s test with ANOVA). Number of CD8\(^+\) and FoxP3\(^+\) TILs in each sample is shown for each of the three groups (middle and right). Significantly decreased CD8\(^+\) TIL numbers in NIH/3T3 co-cultured tumors, and their subsequent rescue by IL-6 blockade is shown (middle) (*\(P < 0.05\), Tukey’s test with ANOVA). The significantly increased FoxP3\(^+\) TIL numbers in co-cultured tumors (*\(P < 0.05\)), also showed a trend to reversal by IL-6 blockade (\(P = 0.101\); Colon26+NIH/3T3 vs. Colon26+NIH/3T3+Anti-IL-6, Tukey’s test with ANOVA).
**Figure 4**

A

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B

BALB/c-nt/nu mice
Colon26-luc subcutaneous tumor

C

BALB/c mice
Colon26-luc subcutaneous tumor

D

Relative value (control=1.0)

E

H&E | αSMA | CD8 | FoxP3

Colon26-luc

Colon26-luc+NIH/3T3

CD8+ TILs (count)

FoxP3+ TILs (count)
Figure 6

A. BALB/c-\textit{nu/nu} mice
Colo26 subcutaneous tumor

B. BALB/c mice
Colo26 subcutaneous tumor

C. Relative value (control=1.0)
BALB/c-\textit{nu/nu} and BALB/c

D. CD8

E. FoxP3

F. IL-6

G. C57BL/6 mice
Pan02 subcutaneous tumor

H. BALB/c mice
Colo26 subcutaneous tumor
Cancer-Associated Fibroblasts Affect Intratumoral CD8+ and FoxP3+ T Cells via Interleukin 6 in the Tumor Microenvironment

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