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ORIGINAL ARTICLE

Interleukin-38 promotes tumor growth through regulation of CD8$^+$ tumor-infiltrating lymphocytes in lung cancer tumor microenvironment

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Abstract

Background Interleukin (IL)-38 was discovered in 2001 and is a member of the IL-1 family of cytokines. IL-38 shows anti-inflammatory activity in several inflammatory diseases. In lung adenocarcinoma, we previously demonstrated that high IL-38 expression in tumor cells was associated with poor prognosis. However, the role of IL-38 in the tumor microenvironment has not been clarified.

Methods IL-38-plasmid-transfected Lewis lung carcinoma cells (LLC-IL38) and empty vector-transfected LLC cells (LLC-vector) were established. Cell proliferation in vitro and tumor growth in vivo were examined, and immunohistochemical staining was used to assess tumor-infiltrating lymphocytes (TILs). A CD8$^+$ lymphocyte depletion model was established to show the association between IL-38 and CD8$^+$ lymphocytes. Moreover, we examined the association between IL-38 expression and CD8$^+$ TILs in human samples, analyzing immunohistochemical staining in 226 patients with radically resected lung adenocarcinoma.

Results Tumor growth of LLC-IL38 in vivo was significantly increased compared with that of LLC-vector, although cell proliferation of LLC-IL38 in vitro was lower than that of LLC-vector. CD8$^+$ TILs were significantly decreased in LLC-IL38 tumor compared with LLC-vector tumor. The difference in tumor growth between LLC-IL38 and LLC-vector became insignificant after depletion of CD8$^+$ lymphocytes. In immunohistochemical staining in tissues from patients with lung adenocarcinoma, multivariate analysis showed high IL-38 expression was an independent negative predicter of high density of CD8$^+$ TILs.

Conclusion We demonstrated that high IL-38 expression in tumor cells was significantly associated with reduction of CD8$^+$ TILs and tumor progression. These results suggest that IL-38 could be a therapeutic target for lung cancer.

Keywords Interleukin-38 · Lung cancer · Tumor microenvironment · Tumor-infiltrating lymphocytes · CD8 + lymphocytes

Abbreviations

LLC-vector	Empty-vector-transfected LLC cells
IL	Interleukin
IFN-γ	Interferon-γ
IL-36R	IL-36 receptor

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LLC-IL38	IL-38-plasmid-transfected LLC cells
LLC	Lewis lung carcinoma
MDSCs	Myeloid-derived suppressor cells
NK	Natural killer
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
RT	Reversed transcription
Tregs	Regulatory T cells
TILs	Tumor-infiltrating lymphocytes
TNF-α	Tumor necrosis factor-α

Introduction

Lung cancer is a major cause of cancer-related death worldwide [1]. Recently, immunotherapy, such as anti-programmed cell death-1 (PD-1) inhibitor and anti-programmed cell death-ligand 1 (PD-L1) inhibitor, has shown superiority to conventional chemotherapy and has become standard in non-small cell lung cancer (NSCLC) [2–4]. However, in past clinical trials, the objective response rate of immunotherapy was low [5], and elucidation of the immune mechanism in the tumor microenvironment is necessary for improving treatment outcome in patients with lung cancer.

Interleukin (IL)-38, also known as IL-1F10 or IL-1HY2, was discovered in 2001 and is a member of the IL-1 family of cytokines [6, 7]. In healthy tissue, IL-38 is expressed in skin, fetal liver, placenta, brain, thymus and tonsils, but barely expressed in lungs [7, 8]. IL-38, like IL-36 receptor (IL-36R) antagonist, blocks signaling from IL-36R [9]. IL-36R is mainly expressed in naïve $CD4^+$ T lymphocytes or dendritic cells [10], and the IL-36R signaling pathway produces inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-17A [11]. IL-38 is reported to have strong anti-inflammatory activity in several diseases, such as idiopathic pulmonary fibrosis [8], childhood asthma [12], and rheumatoid arthritis [13]. However, there are few reports describing the significance of IL-38 in malignant tumors, including lung cancer.

In the field of lung cancer, we previously showed that lung adenocarcinoma tissues expressed IL-38 protein, and high IL-38 expression in tumor cells was associated with poor prognosis and PD-L1 expression in lung adenocarcinoma [14]. In contrast, another study showed that IL-38 suppresses migration, invasion, proliferation and colony formation of NSCLC cells through suppression of β-catenin; therefore, IL-38 plays an inhibitory role in NSCLC [15]. Thus, the significance of IL-38 in lung cancer is controversial. Furthermore, the role of IL-38 in the tumor microenvironment has been little studied.

In this study, we proposed a hypothesis that the anti-inflammatory activity of IL-38 is associated with inhibition of antitumor immune response in the tumor microenvironment. In order to prove our hypothesis, we demonstrated the association between IL-38 and tumor progression in an immunocompetent syngeneic mouse model of lung cancer. We further explored the effect of IL-38 on tumor-infiltrating lymphocytes (TILs) and intratumoral inflammatory cytokines. Moreover, we analyzed the association between IL-38 expression of tumor cells and TILs in patients with lung adenocarcinoma.

Materials and methods

Cell culture and generation of IL-38-expressing cell lines

Lewis lung carcinoma (LLC; LL/2, RRCID: CVCL_4358) cells were obtained from the American Type Culture Collection. LLC cells were maintained in RPMI-1640 containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The IL-38 plasmid (NM_153077; Origene) was transfected into LLC cells using the jetPRIME kit (Polyplus Transfection). An empty vector (pCMV6-Entry Vector; Origene) was also transfected into LLC cells as a control. Forty-eight hours after transfection, LLC cells were selected with 500 µg/ml G418 (Sigma). The limiting dilution method was used for the isolation of single cell clones from the transfected LLC cells. Selected single cells were placed in each well of the culture plates, and the clonal populations growing from each single cell were isolated. We established two clones of IL-38-plasmid-transfected LLC (LLC-IL38) cells. LLC cells were checked for mycoplasma infection before and after transfection, and all were negative.

Cell proliferation assay

Five thousand cells in 1 mL RPMI-1640 containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin were plated per well in 12-well plates. The number of cells was counted after incubation for 1–4 days at 37 °C. Assays were performed in triplicate and repeated three times.

Reverse transcription (RT)-PCR

Total RNA was isolated from cells and tissue samples with the Maxwell RSC Simply RNA Tissue kit (Promega). One microgram of total RNA was converted into cDNA using SuperScript III First-Standard Synthesis SuperMix (Invitrogen). The cDNA was diluted fivefold and used for real-time PCR analysis with the StepOnePlus Real-Time PCR System (Applied Biosystems) using a TaqMan Gene Expression Assay probe and primer mix (Applied Biosystems). PCRs were run for 40 cycles with denaturation at 95 °C for 5 s

and annealing at 60 °C for 30 s. RT-PCRs were performed in duplicate for each sample and repeated three times, and the average value was used for calculation of mRNA expression level. Relative mRNA expression was determined by the ΔΔCt method that is based on the relative expression of the target gene versus a reference gene (GAPDH) and normalized to the control samples. ΔCt was defined as mRNA (target gene) − mRNA (reference gene) and ΔΔCt as ΔCt (target sample) − ΔCt (control sample). Primer sets for all genes were purchased from Applied Biosystems. The following primers were used: *Il1f10*: Mm00462022_g1, *Ifng*: Mm01168134_m1, *Tnf*: Mm00443258_m1, *Il17a*: Mm00439618_m1, *Il6*: Mm00446190_m1, *Il1b*: Mm00434228_m1 and *Gapdh*: Mm99999915_g1.

Western blotting

Samples were lysed in lysis buffer containing 50 mmol/l Tris HCl (pH 6.8) and 10% SDS, and the protein concentration for each sample was determined using a Bio-Rad Protein Assay kit. Samples were heated at 95 °C for 5 min and subjected to electrophoresis using SuperSep Ace 15% gels (Fujifilm) at 40 mA for 80 min. The Trans-Blot Turbo Transfer System (Bio-Rad) was used to transfer proteins onto a PVDF membrane (Bio-Rad). Primary and secondary antibodies were diluted in iBind solution (Invitrogen). Primary antibodies were anti-mouse IL-38/IL-1F10 antibody (rat monoclonal; 1:250; clone #797036R; R&D systems) and anti-β-actin (rabbit monoclonal; 1:2000; clone #13E5; Cell Signaling Technology). Secondary antibodies were goat anti-rat IgG H&L (1:10000; Abcam) and goat anti-rabbit IgG H&L (1:5000; Abcam). The membrane was incubated in iBind solution (Invitrogen) with primary and secondary antibodies. Each blot was incubated with Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific) and imaged using Amersham Imager 600 (GE Healthcare).

Animal studies

Animal experiments were reviewed and approved by the Institutional Committee for Animal Care and Use of Kyushu University (A30-256-0, A19-323-0) and by the Biosafety Committee for Recombinant DNA Experiments of Kyushu University (30–84). The experiments were also performed in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

We used an immunocompetent syngeneic mouse model. A six-week old female C57BL/6J mice (CLEA Japan) were kept under specific pathogen-free and humane conditions in the animal care facility. After adequate anesthesia, 5×10^6 LLC-IL38 or empty-vector-transfected LLC (LLC-vector) cells in 100 µl PBS were injected subcutaneously into the backs of the mice. The tumor volume was assessed using microcalipers at 6, 9, 12, 15, 18 and 21 days after subcutaneous injection according to the formula: tumor volume (mm^3) $= S^2 \times L/2$, where L and S indicate the longest and shortest dimensions of the tumor, respectively. The tumor was harvested and weighed at 21 days after subcutaneous injection. The experiments were repeated twice (total $n = 6$ for each group).

Immunohistochemical staining

Immunohistochemical staining was performed on 4-µm formalin-fixed and paraffin-embedded sections. Sections were first deparaffinized and then blocked with 10% normal goat serum. The tissue sections were incubated with the following primary antibodies at 4 °C overnight: anti-mouse CD3 (rabbit monoclonal; 1:1000; clone #EPR20752; Abcam); anti-mouse CD8 (rabbit monoclonal; 1:2000; clone #EPR20305; Abcam); anti-mouse CD4 (rabbit monoclonal; 1:1000; clone #EPR19514; Abcam); anti-mouse forkhead box protein (Fox)P3 (rabbit monoclonal; 1:200; clone #D608R; Cell Signaling Technology); anti-human CD8 (mouse monoclonal; 1:100; clone #C8/144B; Dako); anti-human CD4 (rabbit monoclonal; 1:100; clone #SP35; Abcam); and anti-human FoxP3 (mouse monoclonal; 1:100; clone #236A/E7; eBioscience). The immune complexes were detected with a Dako EnVision Detection System. The sections were reacted in 3,3′-diaminobenzidine, and counterstained with hematoxylin. Stained slides were scanned using the NanoZoomer (Hamamatsu Photonics KK).

The number of $CD3^+$, $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs in mouse specimens were evaluated by counting the number of cells per 0.1 mm^2 over three fields, then averaging the cell counts. The density of $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs in human specimens was also evaluated per 0.04 mm^2 over five fields. In this study, all hematoxylin–eosin images and immunohistochemical images were evaluated by at least two observers, including a pathologist, and TILs were distinguished from other cells by their morphology. The cutoff values of the number of $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs in human specimens were 1455, 1230 and 160 /mm^2, respectively, defined by receiver operating characteristic curve analysis.

Immunohistochemical staining of IL-38 was performed as described previously (14). We used mouse monoclonal anti-human IL-38 antibody (0.5 µg/ml, clone #H127C; kindly provided by T. Hoshino, Kurume University, Fukuoka, Japan) [13]. The evaluation of IL-38 was also conducted as described previously [14]. Cases with weak staining in the cytoplasm and membrane of tumor cells were judged as having low expression, and moderate and strong staining as high expression.

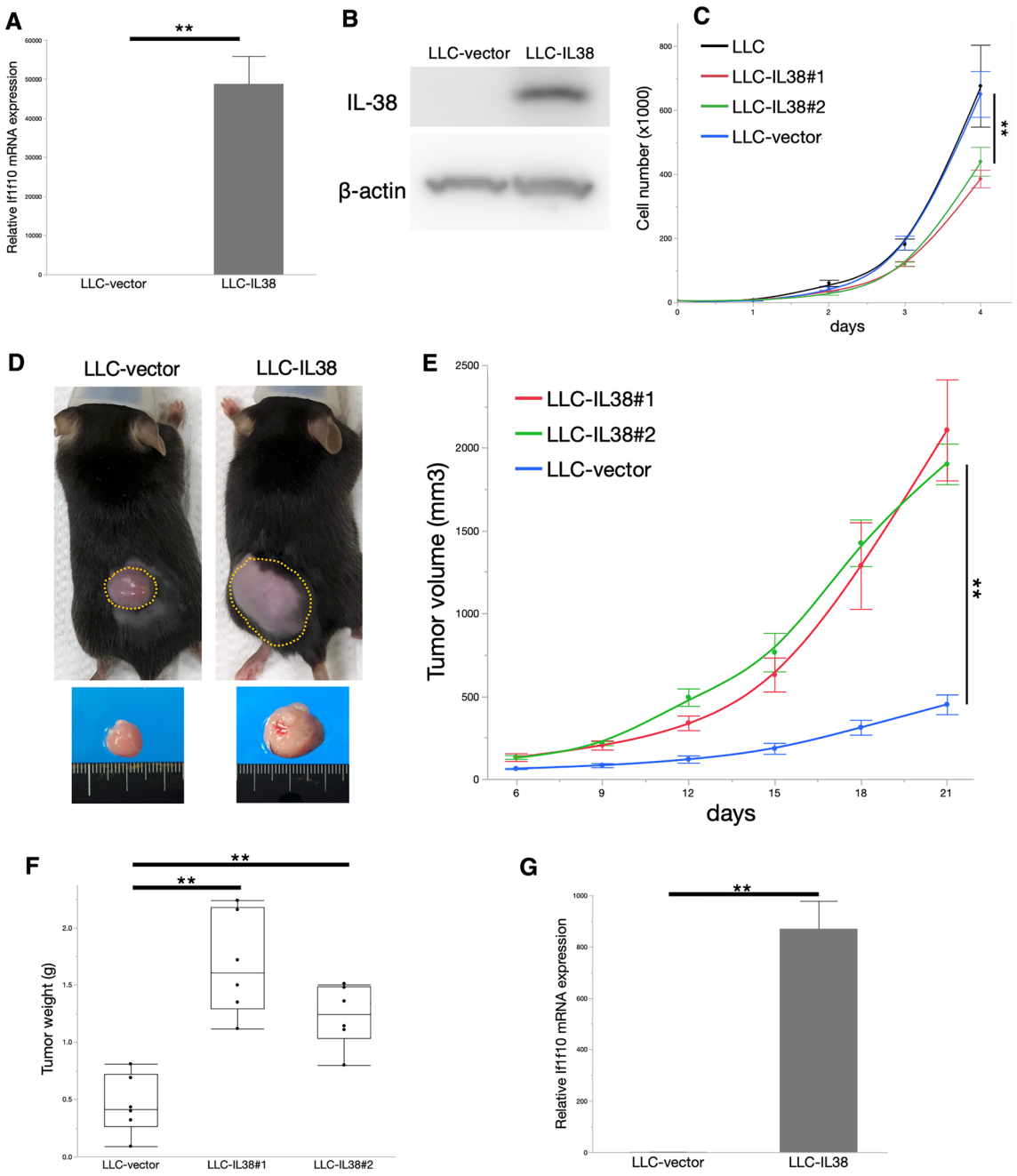


Fig. 1 IL-38 overexpression of tumor cells significantly suppressed cell proliferation in vitro, but strongly promoted tumor growth in vivo. IL-38 plasmid was transfected in LLC cells, and IL-38-plasmid-transfected LLC (LLC-IL38) cells were established. Empty-vector-transfected LLC (LLC-vector) cells were also established as a control. **a** IL-38 mRNA expression of LLC-IL38 and LLC-vector cells were evaluated by RT-PCR. The analysis of RT-PCR was performed by the ΔΔCt method. **b** IL-38 protein expression of LLC-IL38 and LLC-vector cells were evaluated by western blotting. **c** Cell proliferation curve of LLC-IL38, LLC-vector and LLC. Compared with LLC-vector and LLC, the cell proliferation of LLC-IL38 was significantly decreased. LLC-IL38 cells (5×10^6 cells) and LLC-vector cells (5×10^6 cells) were subcutaneously injected into the backs of C57BL/6J mice, and an immunocompetent syngeneic mouse model was established. **d** Macroscopic findings of mice and tumors with LLC-IL38 and LLC-vector at 21 days after subcutaneous injection. **e** Growth curve of LLC-IL38 and LLC-vector tumors. The tumor volume was measured at 6, 9, 12, 15, 18 and 21 days after subcutaneous injection. Tumor volume (mm^3) was defined as $S^2\times L/2$, where L and S indicate the longest and shortest dimensions of the tumor, respectively. **f** Tumor weight of LLC-IL38 and LLC-vector tumors at 21 days after subcutaneous injection. **g** IL-38 mRNA expressions in tumor tissues of LLC-IL38 and LLC-vector were evaluated by RT-PCR. The analysis of RT-PCR was performed by the ΔΔCt method. The experiments were repeated at least twice (total $n=6$ for each group; $^{**}p<0.01$; $^{*}p<0.05$; ns, not significant)

Depletion of CD8$^+$ lymphocytes in vivo

Mice were given 150 µg rat monoclonal anti-mouse CD8α antibody (clone #YTS169.4; Bio X Cell) or isotype control antibody (clone #LTF-2; Bio X Cell) in 200 µl PBS by intraperitoneal administration at days—3, 0, 7 and 14 post-injection with tumor cells. The tumor volume was assessed using microcalipers at 6, 9, 12, 15, 18 and 21 days after inoculation of cells according to the formula: tumor volume (mm^3) = $S^2 \times L/2$, where L and S indicate the longest and shortest dimensions of the tumor, respectively. The tumor was harvested and weighed at 21 days after subcutaneous injection. The experiments were repeated twice (total $n = 5$ for each group). The depletion efficiency was determined by immunohistochemical staining of spleen tissue.

Patients

We retrospectively examined 226 patients with lung adenocarcinoma radically resected between January 2003 and December 2012 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Japan. The clinicopathological characteristics including age, sex, smoking history, pathological T status, pathological N status, pathological stage, pleural invasion, vascular invasion, lymphatic invasion, histological subtype and *EGFR* status were reviewed. IL-38 expression had been previously determined in our laboratory [14]. The clinical information and follow-up data were obtained from the patients' medical records. This study was approved by our Institutional Review Board (Kyushu University, IRB No. 2019-232).

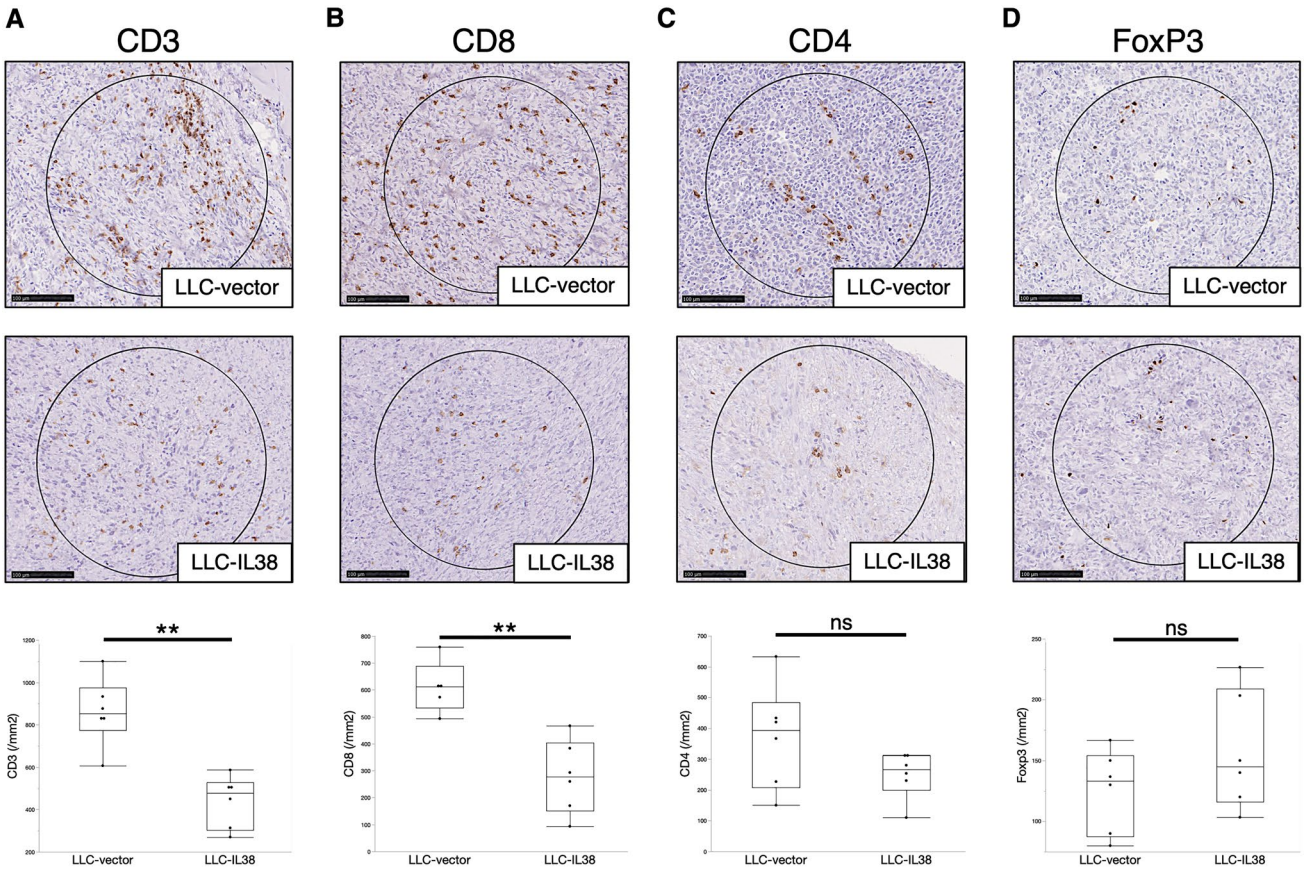
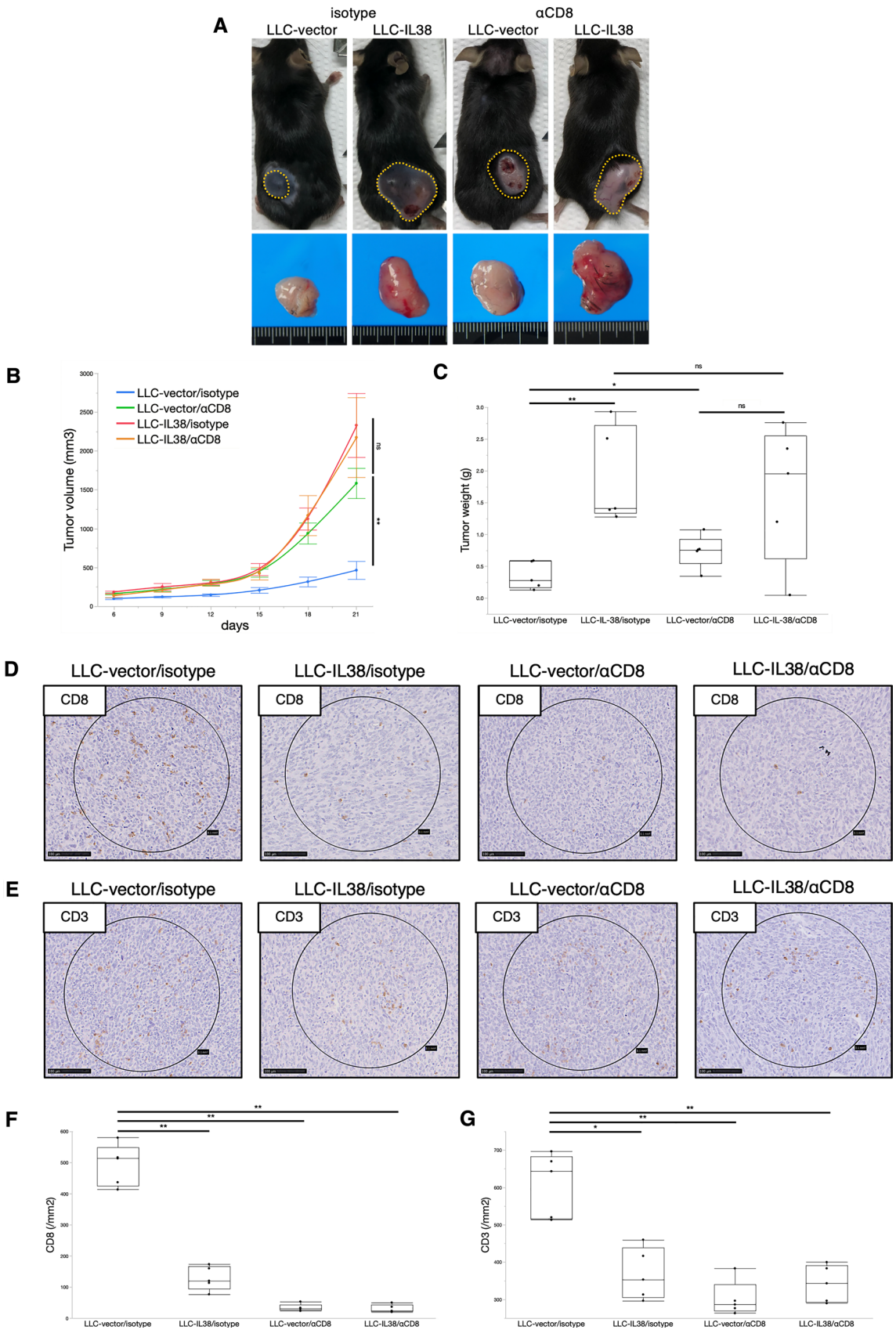


Fig. 2 Overexpression of IL-38 significantly decreased the number of CD3$^+$ and CD8$^+$ TILs. TILs in LLC-IL38 and LLC-vector tumor tissues were evaluated by immunohistochemical staining. The number of CD3$^+$ (**a**) and CD8$^+$ (**b**) TILs was significantly reduced in LLC-IL38 tumor compared with LLC-vector tumor. There was no significant difference in the number of CD4$^+$ (**c**) and FoxP3$^+$ (**d**) TILs between LLC-IL38 and LLC-vector tumors (total $n = 6$ for each group; $^{**}p < 0.01$; *ns*, not significant; scale bars, 100 µm)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◂**Fig. 3** Effect of IL-38 on tumor growth became insignificant after depletion of $CD8^+$ lymphocytes. Mice were given 150 μg of rat monoclonal anti-mouse CD8α antibody or isotype control antibody by intraperitoneal administration at days − 3, 0, 7 and 14 post-injection with LLC-IL38 or LLC-vector cells, and we developed a $CD8^+$ lymphocyte depletion model. **a** Macroscopic findings of LLC-IL38 tumor with CD8α antibody (LLC-IL38/αCD8) or isotype control antibody (LLC-IL38/isotype), and LLC-vector tumor with CD8α antibody (LLC-vector/αCD8) or isotype control antibody (LLC-vector/isotype) at 21 days after subcutaneous injection. **b** Growth curve of LLC-IL38/αCD8, LLC-IL38/isotype, LLC-vector/αCD8 and LLC-vector/isotype tumors. The tumor size was measured at 6, 9, 12, 15, 18 and 21 days after subcutaneous injection. Tumor volume (mm^3) was defined as $S^2 \times L/2$, where L and S indicate the longest and shortest dimensions of the tumor, respectively. **c** Tumor weight of LLC-IL38/αCD8, LLC-IL38/isotype, LLC-vector/αCD8 and LLC-vector/isotype tumors at 21 days after subcutaneous injection. Immunohistochemical staining showed that the number of $CD8^+$ (**d**, **f**) and $CD3^+$ (**e**, **g**) TILs in LLC-vector/isotype tumor was significantly higher than in LLC-IL38/isotype, LLC-vector/αCD8 and LLC-IL38/αCD8 tumors. The experiments were repeated twice (total $n = 5$ for each group; $^{**}p < 0.01$; $^{*}p < 0.05$; *ns* not significant)

Statistical analyses

All data were expressed as mean ± standard deviation. Data were compared using Student's *t* test. Associations between clinicopathological characteristics and the density of $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs were analyzed by Fisher's exact test. Univariate and multivariate analyses of high density of $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs for clinicopathological characteristics were performed by logistic regression analysis. A probability value of $p < 0.05$ was considered statistically significant. All analyses were performed using JMP 13.0.0 software (SAS Institute).

Results

Overexpression of IL-38 significantly decreased tumor cell proliferation in vitro, but strongly promoted tumor growth in vivo

To elucidate the significance of IL-38 in lung cancer, LLC-IL38 cells were established. LLC-vector cells were also established as a control. IL-38 mRNA and protein expression of LLC-IL38 and LLC-vector were examined by RT-PCR (Fig. 1a) and western blotting (Fig. 1b), and confirmed that, compared with LLC-vector, LLC-IL38 stably expressed IL-38. We examined cell proliferation of LLC-IL38 in vitro. The cell proliferation of LLC-IL38 was significantly lower than that of LLC and LLC-vector (Fig. 1c). These results demonstrated that IL-38 overexpression significantly suppressed the proliferation of LLC cells in vitro. To investigate the effect of IL-38 expression on tumor growth in vivo, we established an immunocompetent syngeneic mouse model, and examined tumor growth of LLC-IL38. The tumor volume of LLC-IL38 tumors were significantly increased compared with that of LLC-vector tumor (LLC-IL38#1: 2109.0 ± 746.8 mm^3 and LLC-IL38#2: 1902.2 ± 300.4 mm^3 vs. LLC-vector: 502.5 ± 190.2 mm^3; $p = 0.0003$ and $p < 0.0001$, respectively; Fig. 1d, e). The tumor weight of LLC-IL38 tumors were also significantly heavier than that of LLC-vector tumor (LLC-IL38#1: 1.68 ± 0.45 g and LLC-IL38#2: 1.23 ± 0.27 g vs. LLC-vector: 0.46 ± 0.26 g; $p = 0.0002$ and $p = 0.0005$, respectively; Fig. 1f). These results demonstrated that overexpression of IL-38 strongly promoted tumor growth in vivo, in spite of the suppressive function of IL-38 in tumor cell proliferation in vitro. The relative IL-38 mRNA expression in tumor tissue was also significantly high in LLC-IL38 tumor, comparing to LLC-vector tumor (869.7 ± 265.4; $p < 0.0001$; Fig. 1g).

Overexpression of IL-38 significantly reduced the number of $CD3^+$ and $CD8^+$ TILs in the tumor microenvironment

There was a discrepancy in IL-38 function between cell proliferation in vitro and tumor growth in vivo. We hypothesized that anti-inflammatory activity of IL-38 suppresses immune cells in the tumor microenvironment and, as a result, IL-38 inhibits the antitumor immune response and allows tumor progression. To prove this hypothesis, we investigated the effect of IL-38 on $CD3^+$, $CD8^+$, $CD4^+$ and $FoxP3^+$ TILs by immunohistochemical staining of tumors from mice injected with LLC-IL38 or LLC-vector. The number of $CD3^+$ and $CD8^+$ TILs in LLC-IL38 tumor at 21 days after subcutaneous injection was significantly lower than in LLC-vector tumor ($CD3^+$ TILs: $438 \pm 122/mm^2$ vs. $862 \pm 161/mm^2$; $p = 0.0004$; $CD8^+$ TILs: $278 \pm 136/mm^2$ vs. $611 \pm 97/mm^2$; $p = 0.0013$; Fig. 2a, b). However, the number of $CD4^+$ and $FoxP3^+$ TILs showed little difference between LLC-IL38 and LLC-vector tumors at 21 days after subcutaneous injection ($CD4^+$ TILs: $249 \pm 76/mm^2$ vs. $372 \pm 170/mm^2$; $p = 0.1389$, $FoxP3^+$ TILs: $157 \pm 48/mm^2$ vs. $126 \pm 34/mm^2$; $p = 0.2174$; Fig. 2c, d). This change of TILs caused by IL-38 overexpression also could be observed at 7 and 14 days after subcutaneous injection (data not shown). These results demonstrated that overexpression of IL-38 in tumor cells reduced the number of $CD3^+$ and $CD8^+$ TILs in the tumor microenvironment.

Effect of IL-38 overexpression on tumor growth became insignificant by depletion of $CD8^+$ lymphocytes

To clarify whether the increased tumor progression in LLC-IL38-injected mice was attributed to decreased $CD8^+$ TILs, we examined the effect of $CD8^+$ lymphocyte depletion on tumor growth of LLC-IL38-injected mice. Mice were

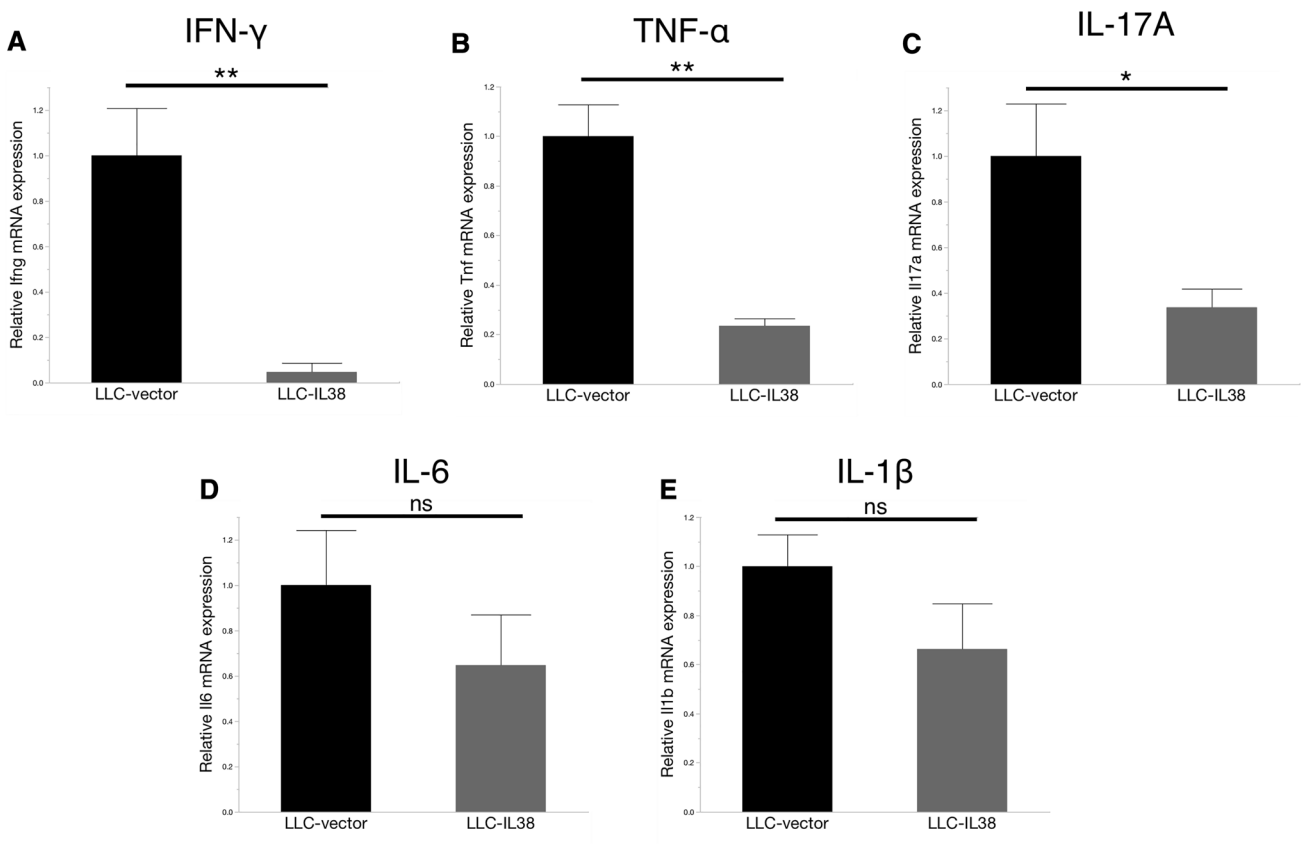

Fig. 4 Overexpression of IL-38 suppressed intratumoral inflammatory cytokines, such as IFN-γ, TNF-α and IL-17A. The mRNA expression of intratumoral inflammatory cytokines in LLC-IL38 and LLC-vector tumors was analyzed by RT-PCR. The analysis of RT-PCR was performed by the ΔΔCt method. IL-38 suppressed mRNA expression of IFN-γ (**a**), TNF-α (**b**) and IL-17A (**c**). There was no significant difference in mRNA expression of IL-6 (**d**) or IL-1β (**e**). The experiments were repeated twice (total $n=6$ for each group; $^{**}p<0.01$; $^{*}p<0.05$; *ns* not significant)

injected with anti-mouse CD8α antibody or isotype control antibody at days—3, 0, 7 and 14 post-injection with LLC-IL38 or LLC-vector cells. In terms of tumor volume and weight, while there was a significant difference between LLC-IL38 with isotype control antibody (LLC-IL38/isotype) and LLC-vector with isotype control antibody (LLC-vector/isotype) (tumor volume: 2331.3 ± 920.1 mm^3 vs. 464.9 ± 259.1 mm^3 $p=0.0024$; tumor weight: 1.90 ± 0.76 g vs. 0.36 ± 0.22 g; $p=0.0024$; Fig. 3a–c), there was no significant difference between LLC-IL38 with anti-mouse CD8α antibody (LLC-IL38/αCD8) and LLC-vector with anti-mouse CD8α antibody (LLC-vector/αCD8) (tumor volume: 2174.7 ± 1149.4 mm^3 vs. 1584.9 ± 434.7 mm^3; $p=0.3145$; tumor weight: 1.66 ± 1.07 g vs. 0.74 ± 0.26 g; $p=0.0974$; Fig. 3a–c). Immunohistochemical staining showed that LLC-vector/isotype tumor had significantly higher number of CD8$^+$ TILs compared to LLC-IL38/isotype, LLC-vector/αCD8 and LLC-IL38/αCD8 tumors (492 ± 67/mm^2 vs. 129 ± 39/mm^2; $p<0.0001$, vs. 34 ± 11/mm^2; $p<0.0001$, vs. 31 ± 13/mm^2; $p<0.0001$, respectively; Fig. 3d, f). The number of CD3$^+$ TILs in LLC-vector/isotype tumor was also significantly higher than in LLC-IL38/isotype, LLC-vector/αCD8 and LLC-IL38/αCD8 tumors (609 ± 86/mm^2 vs. 368 ± 69/mm^2; $p=0.0012$, vs. 301 ± 47/mm^2; $p=0.0001$, vs. 343 ± 50/mm^2; $p=0.0003$, respectively; Fig. 3e, g). These results demonstrated that the difference in tumor growth between LLC-IL38 and LLC-vector became insignificant by depletion of CD8$^+$ lymphocytes, suggesting that the reduced number of CD8$^+$ TILs induced by IL-38 overexpression in tumor cells promoted tumor growth.

Overexpression of IL-38 decreased expression of inflammatory cytokines in tumor tissues

To elucidate further the effect of IL-38 in the tumor microenvironment, we examined expression of inflammatory cytokines in tumor tissue. The IL-36R signaling pathway, which is a target of IL-38, produces inflammatory cytokines such as IFN-γ, TNF-α, IL-17A, IL-6 and IL-1β [11]. IL-38 was reported to block the IL-36R signaling pathway, and

we hypothesized that IL-38 decreased the release of these inflammatory cytokines. Thus, we investigated by RT-PCR mRNA expression of IFN-γ, TNF-α, IL-17A, IL-6 and IL-1β in tumor tissues. In LLC-IL38 compared with LLC-vector tumor, relative mRNA expression of IFN-γ, TNF-α and IL-17A was significantly decreased (IFN-γ: 0.05 ± 0.10; $p = 0.0012$; TNF-α: 0.24 ± 0.07; $p = 0.0002$; IL-17A: 0.34 ± 0.20; $p = 0.0214$; Fig. 4a–c), but not that of IL-6 and IL-1β (IL-6: 0.65 ± 0.55; $p = 0.3093$; IL-1β: 0.66 ± 0.45; $p = 0.1665$; Fig. 4d, e). These results implied that IL-38 inhibited production of IFN-γ, TNF-α and IL-17A in the tumor microenvironment. Suppression of inflammatory cytokines by IL-38 overexpression might be associated with the decrease in $CD8^+$ TILs and inhibit antitumor immune response in the tumor microenvironment.

IL-38 expression in tumor tissue was an independent predictive factor for the density of $CD8^+$ TILs in patients with lung adenocarcinoma

We showed that IL-38 overexpression in tumor cells reduced $CD8^+$ TILs and allowed tumor progression in an immunocompetent syngeneic mouse lung cancer model. We examined the association between IL-38 expression and $CD8^+$ TILs in human samples, analyzing immunohistochemical staining of 226 patients with radically resected lung adenocarcinoma. Immunohistochemical staining of IL-38 was previously performed in our laboratory, and we used the data from the previous study here [14]. The samples from 99 (43.8%) and 127 (56.2%) patients were judged to have low and high expression of IL-38, respectively. In terms of $CD8^+$ TILs, 135 (59.7%) and 91 (40.3%) samples were evaluated as having low and high density of $CD8^+$ TILs, respectively. Fisher's exact test showed that high density of $CD8^+$ TILs was significantly associated with smoking ($p = 0.0301$), advanced T status (0.0014), advanced stage ($p = 0.0250$), pleural invasion ($p < 0.0001$) and vascular invasion ($p = 0.0002$). Although not significant, there was a trend between high IL-38 expression and low density of $CD8^+$ TILs ($p = 0.0566$; Table 1). Multivariate analysis showed that pleural invasion (odds ratio [OR]: 3.73, 95% confidence interval [CI] 1.674–8.312, $p = 0.0013$), vascular invasion (OR: 2.56, 95% CI 1.260–5.191, $p = 0.0094$), and IL-38 expression (OR: 0.44, 95% CI 0.243–0.802, $p = 0.0073$) were independent predictive factors for the density of $CD8^+$ TILs (Table 2). These results demonstrated that, in patients with lung adenocarcinoma, high IL-38 expression in tumor cells was significantly associated with low density of $CD8^+$ TILs.

We also evaluated $CD4^+$ and $FoxP3^+$ TILs in patients with lung adenocarcinoma. The relationship between IL-38 expression and $CD4^+$ TILs was not significant ($p = 0.1049$; Supplementary Table 1), and there was no independent predictive factor for high density of $CD4^+$ TILs (Supplementary Table 2). However, high density of $FoxP3^+$ TILs was significantly associated with high IL-38 expression ($p < 0.0001$; Supplementary Table 3), and high IL-38 expression was an independent predictive factor for high density of $FoxP3^+$ TILs in multivariate analysis (OR: 3.63, 95% CI 1.836–7.186, $p = 0.0002$; Supplementary Table 4).

Table 1 Association between $CD8^+$ TILs and clinicopathological characteristics of patients with lung adenocarcinoma

Characteristics	$CD8^+$ TILs				*p* value
	Low ($n = 135$)		High ($n = 91$)		
Age, years					
< 70	74	(54.8%)	53	(58.2%)	0.6821
≥ 70	61	(45.2%)	38	(41.8%)	
Sex					
Female	77	(57.0%)	45	(49.5%)	0.2788
Male	58	(43.0%)	46	(50.5%)	
Smoking					
Never smoker	81	(60.0%)	41	(45.1%)	0.0301
Smoker	54	(40.0%)	50	(54.9%)	
pT					
T1	94	(69.6%)	44	(48.4%)	0.0014
≥ T2	41	(30.4%)	47	(51.6%)	
pN					
N0	127	(94.1%)	80	(87.9%)	0.1414
≥ N1	8	(5.9%)	11	(12.1%)	
p Stage					
I	116	(85.9%)	67	(73.6%)	0.0250
≥ II	19	(14.1%)	24	(26.4%)	
Pleural invasion					
Negative	122	(91.0%)	61	(67.0%)	< 0.0001
Positive	12	(9.0%)	30	(33.0%)	
Vascular invasion					
Negative	112	(83.0%)	55	(60.4%)	0.0002
Positive	23	(17.0%)	36	(39.6%)	
Lymphatic invasion					
Negative	123	(91.1%)	76	(83.5%)	0.0967
Positive	12	(8.9%)	15	(16.5%)	
Histological subtype					
Micropapillary/solid	5	(3.7%)	8	(8.8%)	0.1453
Others	130	(96.3%)	83	(91.2%)	
EGFR mutation[a]					
Wild type	40	(49.4%)	26	(51.0%)	1.0000
Mutant	41	(50.6%)	25	(49.0%)	
IL-38 expression					
Low	52	(38.5%)	47	(51.6%)	0.0566
High	83	(61.5%)	44	(48.4%)	

[a]Available data were counted, excluding unknown data

Table 2 Univariate and multivariate analyses of high density of $CD8^+$ TILs and clinicopathological characteristics in patients with lung adenocarcinoma

Characteristics	High density of $CD8^+$ TILs					
	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Age, years						
≥ 70	0.87	0.508–1.488	0.6107			
Sex						
Male	1.36	0.796–2.314	0.2623			
Smoking						
Smoker	1.83	1.068–3.131	0.0277			
pT						
≥ T2	2.45	1.412–4.249	0.0014			
pN						
≥ N1	2.18	0.842–5.659	0.1083			
P Stage						
≥ II	2.19	1.116–4.286	0.0226			
Pleural invasion						
Positive	5.00	2.394–10.44	< 0.0001	3.73	1.674–8.312	0.0013
Vascular invasion						
Positive	3.19	1.724–5.894	0.0002	2.56	1.260–5.191	0.0094
Lymphatic invasion						
Positive	2.02	0.899–4.553	0.0887			
Histological subtype						
Micropapillary/Solid	2.51	0.793–7.921	0.1177			
EGFR mutation[a]						
Mutant	0.94	0.465–1.891	0.8581			
IL-38 expression						
High	0.59	0.343–1.004	0.0518	0.44	0.243–0.802	0.0073

[a]Available data were counted, excluding unknown data

We demonstrated serial images of the representative cases with lung adenocarcinoma in Fig. 5. A case with high IL-38 expression showed low density of $CD8^+$ and $CD4^+$ TILs, and high density of $FoxP3^+$ TILs (Fig. 5a): on the other hand, in another case with low IL-38 expression, the density of $CD8^+$ and $CD4^+$ TILs were high and that of $FoxP3^+$ TILs was low (Fig. 5b).

Discussion

In this study, we demonstrated that high IL-38 expression in tumor cells contributed to tumor progression by reducing the number of $CD8^+$ TILs and expression of intratumoral inflammatory cytokines.

Little is known about the significance of IL-38 in malignant tumors, including lung cancer. We previously examined the expression of IL-38 in resected human lung specimens by immunohistochemical staining and found that patients with lung adenocarcinoma with high expression of IL-38 had worse prognosis than those with low expression of IL-38 [14]. In contrast, Wang et al. showed using immunohistochemical staining of human specimens, in vitro experiments and in vivo animal studies that reduction of IL-38 expression increased malignancy of NSCLC [15]. Our in vitro examination and the animal work of Wang et al. [15] suggested that the direct function of IL-38 on tumor cells was inhibitory to cell proliferation. However, there was a clear difference in the presence or absence of cell-mediated immunity between our study and that of Wang et al. [15]. Our immunocompetent mouse model showed that IL-38 reduced the number of $CD8^+$ TILs and intratumoral inflammatory cytokines. Therefore, we speculate that IL-38 indirectly promotes tumor growth by suppressing the antitumor immune response. We think that the reason for the discrepancy in results was because of the difference between direct and indirect effects of IL-38.

Our study demonstrated the association between high IL-38 expression and low density of $CD8^+$ TILs in mice and humans. $CD8^+$ lymphocytes, known as cytotoxic T

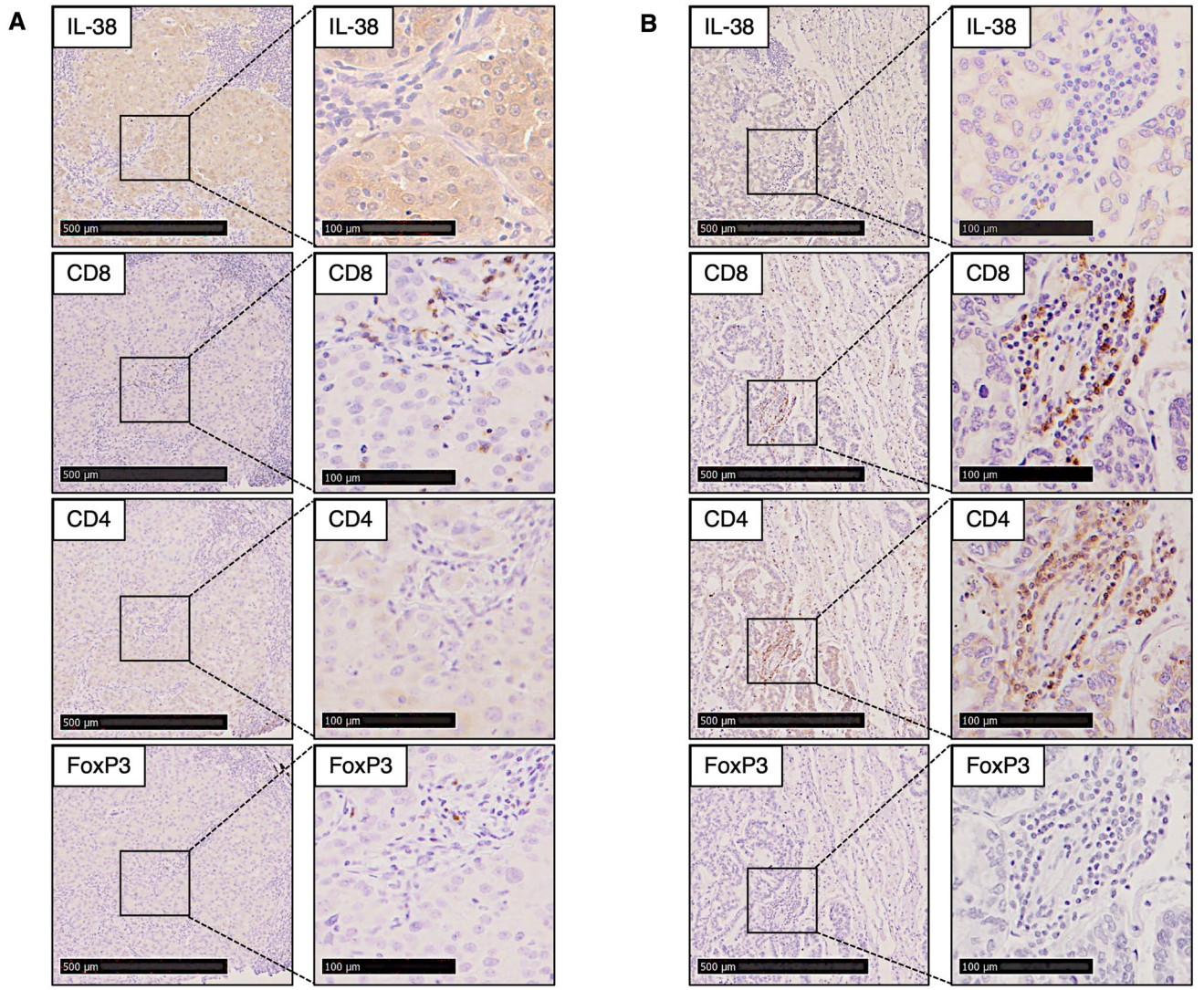

Fig. 5 We demonstrated serial images of the representative cases with lung adenocarcinoma. A case with high IL-38 expression showed low density of $CD8^+$ and $CD4^+$ TILs, and high density of $FoxP3^+$ TILs (**a**): on the other hand, in another case with low IL-38 expression, the density of $CD8^+$ and $CD4^+$ TILs were high and that of $FoxP3^+$ TILs was low (**b**) (scale bars, 500 μm or 100 μm)

lymphocytes, play a critical role in antitumor immune response in the tumor microenvironment [16]. In lung cancer, meta-analyses have shown that high density of $CD8^+$ TILs is significantly associated with good prognosis [17, 18]. In our $CD8^+$ lymphocyte depletion model, tumor growth in the LLC-vector/αCD8 group was more progressive than in the LLC-vector/isotype group. Therefore, $CD8^+$ lymphocytes contributed to suppression of tumor progression in our mouse model, and the reduction of $CD8^+$ TILs seemed to be a crucial mechanism of IL-38 for promoting tumor growth.

However, while not significant, tumor growth in the LLC-IL38/αCD8 group increased compared with that in the LLC-vector/αCD8 group. Therefore, there was a possibility that IL-38 affected other factors besides $CD8^+$ TILs. In the present study, while our mouse model did not show significant results, there was a significant relationship between high IL-38 expression and high density of $FoxP3^+$ TILs in patients with lung adenocarcinoma. $FoxP3^+$ regulatory T cells (Tregs) play an important role in tumor progression; therefore, an increase of $FoxP3^+$ TILs by IL-38 might be an important factor for inhibition of the antitumor immune response. Furthermore, the association between IL-38 and other immune cells, such as natural killer (NK) cells, B cells, macrophages and myeloid-derived suppressor cells (MDSCs), is still unclear. IL-38 may affect the immune status, including these cells, in the tumor microenvironment.

We demonstrated that IL-38 overexpression in tumor cells decreased expression of intratumoral inflammatory cytokines such as IFN-γ, TNF-α and IL-17A. These results suggested that IL-38 could decrease production of IFN-γ, TNF-α and IL-17A through inhibiting IL-36R signaling. Particularly in IL-17A, past research showed that IL-38 suppressed IL-17A production by γδT cells, and was consistent with our study [19]. IFN-γ plays an important role in promoting differentiation and activation of $CD8^+$ lymphocytes [20–22]. In terms of TNF-α, some studies have described TNF-α as both promoter and inhibitor of tumor growth [23, 24]. As an inhibitor of tumor growth, TNF-α causes tumor necrosis by disrupting tumor vessels and generating tumor-specific T lymphocytes [24]. The role of IL-17A is also controversial. IL-17A is reported to promote tumor growth by recruiting myeloid cells [25], but other studies have shown that IL-17A enhances antitumor response of $CD8^+$ lymphocytes and NK cells [26, 27]. Therefore, these cytokines seem to influence $CD8^+$ lymphocytes, and suppression of these cytokines by IL-38 might be associated with the reduction of $CD8^+$ TILs in the tumor microenvironment.

The expression of IL-38 by immune or stromal cells as well as tumor cells in tumor tissues is an important point. Immunohistochemical staining of patients with lung adenocarcinoma showed that immune or stromal cells were weakly stained; however, the staining intensity of immune or stromal cells was much lower than that of tumor cells, and we could not evaluate the IL-38 expression of immune or stromal cells in this study.

There were several limitations to our study. We used cells with overexpression of IL-38 and our animal study was not truly physiological. Additionally, we mainly evaluated the number but not the activity of $CD8^+$ TILs. There was also a possibility that IL-38 affected other immune cells besides $CD8^+$ TILs, such as $FoxP3^+$ Tregs, NK cells, B cells, macrophages, and MDSCs. Moreover, there are many unclear points about the IL-38 expression of immune or stromal cells. Further studies are therefore needed to clearly elucidate the role of IL-38 in the tumor microenvironment. In terms of immunohistochemical staining in patients with lung adenocarcinoma, the study cohort was small and did not include other histological types of lung cancer; therefore, a larger study including all histological types of lung cancer is required.

Conclusion

We demonstrated in a mouse model that IL-38 overexpression in tumor cells strongly promoted tumor growth through regulation of $CD8^+$ TILs and expression of intratumoral inflammatory cytokines. We also demonstrated an association between high IL-38 expression and low density of $CD8^+$ TILs in patients with lung adenocarcinoma. These results suggest that IL-38 could be a therapeutic target for lung cancer.

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Data availability The data used to support the findings of our study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest Not applicable.

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