Cancer Letters 452 (2019) 244–253

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Macrophage-derived CCL22 promotes an immunosuppressive tumor microenvironment via IL-8 in malignant pleural effusion

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ARTICLE INFO

Keywords:
CCL22
IL-8
Macrophage
Malignant pleural effusion
Immunosuppressive tumor microenvironment

ABSTRACT

Immune dysfunction often occurs in malignant pleural effusion (MPE). In our previous study, TGF-β derived predominantly from macrophages plays an important role in impairing T cell cytotoxicity in MPE. Therefore, we aimed to investigate whether other immunoregulatory cells and factors mediated TGF-β secretion from macrophages, involved in the immunosuppressive microenvironment of MPE, and to provide clues for potential immune therapy for MPE as well. We found that CCL22 level in MPE was significantly higher than that in non-malignant pleural effusion. The high level of CCL22 was closely associated with poor survival in MPE patients with lung cancer. CCL22 was dominantly produced by tumor-associated macrophages (TAMs) in MPE. Meanwhile, TAM-derived TGF-β mediated CCL22 expression in TAMs via c-Fos. CCL22 promoted the recruitment of regulatory T cells (Tregs) in MPE. Lastly, Treg-secreted high level of IL-8 further induced TGF-β production from TAMs, and promoted the immunosuppressive tumor microenvironment in MPE. Our results indicate that macrophage-derived CCL22 plays an important role in the immunosuppressive tumor microenvironment via IL-8 in MPE.

1. Introduction

Malignant pleural effusion (MPE) is closely associated with poor prognosis of lung cancer patients [1–3]. Moreover, an immunosuppressive tumor microenvironment often occurs in MPE, which affected therapeutic effect of MPE and promoted tumor growth. Increasing evidence indicates that immunoregulatory cells, such as tumor-associated macrophages (TAMs), regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), play an important role in promoting an immunosuppressive tumor microenvironment and tumor progression. TAMs are a potential diagnostic and prognostic biomarker for MPE, and is associated with therapeutic effect on MPE of lung cancer patients [4,5]. Tregs are considered a key factor in immune escape [6–8]. MDSCs, playing a crucial role as cellular regulators of immune responses, have been extensively shown to restrain tumor immunity through a vast array of molecular mechanisms and to promote tumor progression [9–11].

Our previous study has shown that TAM-derived TGF-β was involved in the immune dysfunction in MPE [12]. We found that levels of Tim-3, PD-1, and CTLA-4 in T cells from MPE were upregulated compared to those from peripheral blood, but levels of IFN-γ and Granzyme B were downregulated. The amount of TGF-β mainly produced by macrophages was significantly higher in MPE than in peripheral blood. When T cells were co-cultured with TAMs, levels of Tim-3, PD-1, and CTLA-4 were significantly higher than controls, whereas levels of IFN-γ and Granzyme B were significantly decreased, in a dose-dependent manner. Treatment with anti-TGF-β antibody restored the impaired T cell cytotoxic activity in MPE *in vitro*. All of the results indicate that TGF-β derived predominantly from macrophages plays an important role in impairing T cell cytotoxicity in MPE.

However, the underlying mechanism that how TGF-β produced by macrophages in MPE, whether other immunoregulatory cells and factors mediated TGF-β secretion from macrophages is unknown. Therefore, in this study we aimed to investigate how immunoregulatory cells and mediators involved in the immunosuppressive microenvironment of MPE, and to provide clues for potential immune therapy for

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https://doi.org/10.1016/j.canlet.2019.03.040
Received 22 January 2019; Received in revised form 16 March 2019; Accepted 22 March 2019

MPE as well.

2. Materials and methods

2.1. Patients

From March 2016 to June 2017, samples of MPE from 50 patients with lung cancer and samples of non-malignant pleural effusion (NMPE) from 50 patients were obtained at The First Affiliated Hospital of Zhengzhou University. These patients were subjected to routine laboratory diagnosis, and the samples were analyzed using conventional cytology. Slides were evaluated as being positive for malignant cells. Inclusion criteria of MPE were lung cancer, proved by histopathological examination of lung biopsy material, an age greater than 18 years, and no underlying disease of the immune system. Inclusion criteria of NMPE were pneumonia and tuberculosis. Exclusion criteria of NMPE were a history of malignant disease within the last five years and solid organ or bone marrow transplantation. Clinical pathological characteristics of patients with NSCLC are showed in Supplementary Table S1. All patients gave written informed consent. The whole consent procedure was in accordance with standards defined by Institutional Review Boards of The First Affiliated Hospital of Zhengzhou University.

2.2. Multiplex assay

In order to compare the differences of the microenvironment in MPE and NMPE, a multiplex assay was used to further analyze the levels of immune cell-related chemokines and cytokines in MPE and NMPE. Supernatants derived from MPE and NMPE were analyzed using a multi-analyte flow assay kit (BioLegend, USA) including 13 human cytokines and 13 human chemokines, according to the manufacturer's instructions.

2.3. RNA extraction and qPCR

Total RNA was extracted from cells using TRIzol (Invitrogen Corporation, USA). cDNA was obtained using a PrimeScript™ RT reagent kit (Takara, Japan) according to the manufacturer's instructions. Briefly, the cDNA was used as a template for qPCR using SYBR Premix ExTaq II (Takara, Japan) on an ABI PRISM 7300 (Applied Biosystems, USA) to detect the expression levels of immune cell-related chemokines. The abundance of mRNA for each gene of interest was normalized to GAPDH.

2.4. ELISA

Concentrations of CCL22, IL-6, IL-8, IL-10 and TGF-β in several supernatants were measured using ELISA (R&D Systems Inc., Minneapolis, MN). Anti-human CCL22 antibody was used in 96-well plates overnight at 4 °C. Wells were washed (0.05% Tween 20 in PBS) and blocked for 1 h at room temperature. Samples and standards were then added to the plates and incubated for 2 h at room temperature. The detection antibody and Streptavidin–HRP solution were applied and incubated for 2 h and 20 min, respectively. Protein concentration was determined by nanospectroscopy using a standard curve at 450 nm. All samples were assayed in duplicate. The sensitivity of ELISA tests was 15 pg/mL, and both the coefficients of variation for intra-assay and inter-assay variability were less than 10%.

2.5. Flow cytometry analysis

Isolated fresh human mononuclear cells from MPE, NMPE or peripheral blood (PB) were isolated using Ficoll-Hypaque (Huajing Biology Co., Shanghai) density gradient centrifugation. For cell surface phenotype assessment, cells were incubated with fluorochrome-conjugated primary antibodies. Cells (1×10^5) were stained with anti-human CD4, CD11b, CD14, CD163, CCR4 antibodies. Intracellular detection of Foxp3 was also performed. An isotypic control was performed for each. Cells were analyzed using flow cytometry (BD CantoII, USA) and Diva analysis software (BD, USA). During analysis, the percentage of positive cells was recorded.

2.6. Immunofluorescence staining

Immunofluorescence staining was used to determine whether CCL22 is mainly derived from TAMs, and IL-8 is dominantly produced by Tregs. Briefly, mononuclear cells from MPE were stained with CD163 (Abcam, 1:400), CCL22 (Proteintech Group Inc., 1:300), Foxp3 (Abcam, 1:400) and IL-8 (Abcam, 1:400) antibodies, respectively. Cy3- and FITC-conjugated secondary antibodies (BioLegend, 1:500) were used to detect the primary antibodies. Stained cells were counterstained with DAPI (Roche, 1:1000), and analyzed using an inverted fluorescence microscope (100 × , Olympus, IX71, Japan).

2.7. Induced M1-like or M2-like macrophages

Human monocytic THP-1 cells were maintained in culture in 1640 medium containing 10% of heat inactivated fetal bovine serum. THP-1 cells were differentiated into macrophages by 24 h incubation with 150 nM phorbol 12-myristate 13-acetate (PMA; Sigma, USA). Macrophages were polarized in M1 macrophages by incubation with 20 ng/ml of IFN-γ (R&D, USA) and 10 pg/ml of LPS (Sigma, USA) for 24 h.

THP-1 cells were cultured in 1640 medium with PMA (100 nM, Sigma, USA) at 37 °C *in vitro*. PMA and rhIL-4 (20 ng/mL, PeproTech, USA) were added to these cells at 36 h of cell culture [13]. 6-well plate was used to differentiate M2-like macrophages. After 72 h of cell culture, these cells were evaluated by morphological, phenotypic, and functional analyses.

2.8. RT^2 profiler PCR array

Total RNA was isolated from $CD163^+$ and $CD163^-$ macrophages according to the manufacturer's instructions (QIAGEN, Germany). The extracted RNA was reversed transcribed using a similar method as described above for qRT-PCR analysis. Expression of genes involved in transcription was analyzed by PCR array using a 96-well Human Transcription Factors RT^2 Profiler PCR Array Kit (QIAGEN, Germany) and a MX3005P PCR system (Agilent, USA) according to the kit manufacturer's instructions. The data were analyzed using web-based programs at web of QIAGEN. Gene expression was normalized to the mean of all house-keeping genes in the array.

2.9. ChIP and qChIP assay

A ChIP assay was performed with 2.5 μg anti-CCL22 (Cell Signaling Technology, USA) and goat anti-rabbit IgG (Cell Signaling Technology, USA) using a ChIP assay kit (Cell Signaling Technology, USA) according to the manufacturer's protocol. The antibody-bound DNA was detected in 2% agarose gel. Subsequently, antibody-bound DNA was used to perform qChIP. SYBR green Mix (Roche Diagnostics GmbH, Germany) was used to perform qPCR. The thermocycling conditions were as follows: 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s followed by extension at 60 °C for 30 s. The following primers were used: c-Fos, forward, 5′-TCCATCATCTCTTCTGACTCTGACCCACAG-3′; reverse, 5′-CTGTGGGTCAGAGTCAGAAGAGATGATGGA-3′. The fold of enrichment was normalized to that of IgG and quantified using the $2-\Delta\Delta Cq$ method. Each experiment was repeated three times.

2.10. Migration assay

A 8-μm diameter chamber (Corning, USA) was used in a transwell

assay, whereby 1×10^5 Treg cells sorted from MPE were seeded in the upper chamber and 600 μL of MPE and NMPE supernatants were co-cultured in the bottom chamber. rhCCL22 (PeproTech, USA) and anti-CCL22 antibody (Abcam, UK) was treated in these cells. After incubation for 8 h, the migrated cells stained by counting boardwere counted. All experiments were repeated independently three times.

2.11. Statistical analysis

All statistical analyses were performed using the Statistical Program for Social Sciences 17.0 software. Data were expressed as mean ± SD. Data between different groups were compared using a *t*-test. Overall survival curves were plotted according to the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MPE exhibits a high level of CCL22

Our previous study has shown that macrophage-derived TGF-β was involved in the T cell dysfunction in MPE [12]. Therefore, to investigate the underlying mechanism and or factors mediating TGF-β secretion from macrophages, in the study, we firstly measured the levels of inflammatory cytokines and chemokines in MPE and NMPE using multiplex assay. The results showed that the level of chemokine CCL22 in MPE was significantly higher than that in NMPE (Fig. 1A). To confirm these results, mRNA expression of these chemokines (similar with multiplex assay) in mononuclear cells from MPE and NMPE was analyzed using qPCR. We found that CCL22 mRNA expression in mononuclear cells from MPE was significantly higher than that in mononuclear cells from NMPE ($P < 0.05$, Fig. 1B). Moreover, CCL22 protein levels in MPE and NMPE were analyzed using ELISA, indicating that CCL22 protein level in MPE was obviously elevated than that in NMPE ($P < 0.05$, Fig. 1C). The relationship between CCL22 expression and survival of MPE patients with lung cancer was further analyzed. For the concentration of CCL22 production in MPE, patients were grouped as "high" or "low" using the respective median (1500 pg/mL) as a cut-off point. The result showed that patients with high level of CCL22 in MPE presented a significantly worse overall survival rate ($P < 0.01$, Fig. 1D). Taken together, these results indicate that the level of CCL22 is increased in MPE.

3.2. CCL22 is dominantly derived from TAMs in MPE

Next, we wanted to determine which immunosuppressive cell population is responsible for CCL22 production in MPE. Our previous study showed that TAMs were enriched in MPE [12], so we further used flow cytometry to compare the percentages of TAMs in MPE and NMPE, and found that the percentage of TAMs in MPE was significantly higher than that in NMPE ($P < 0.001$, Fig. 2A). Then, the relationship between CCL22 level and TAM frequency was analyzed, showing that CCL22 level was closely correlated with TAM frequency in MPE ($P < 0.05$, Fig. 2B). Then, CCL22 expression was analyzed in TAMs, Tregs, monocyte-like MDSCs (M-MDSCs) and granulocyte-like MDSCs (G-MDSCs) sorted from MPE. The results showed that mRNA expression of CCL22 in purified TAMs was obviously higher than that in other purified immunosuppressive cells ($P < 0.05$, Fig. 2C). Using flow cytometry assay, we found that the amount of CCL22 production was significantly higher in MPE-derived TAMs compared to MPE-derived Tregs, M-MDSCs, and G-MDSCs ($P < 0.05$, Fig. 2D). An immunofluorescence assay showed that CCL22 was mainly found in $CD163^+$ macrophages from MPE-derived mononuclear cells (Fig. 2E). In summary, these findings suggest that CCL22 is mainly secreted by TAMs in MPE.

3.3. TGF-β secreted from TAMs induces CCL22 expression in TAMs via c-Fos

Our previous study indicates that TGF-β is mainly secreted from TAMs and involves in T cell dysfunction in MPE. So we wanted to understand whether TAMs-derived TGF-β could affect CCL22 production from TAMs in MPE. Firstly, we successfully induced THP-1 cells to M2

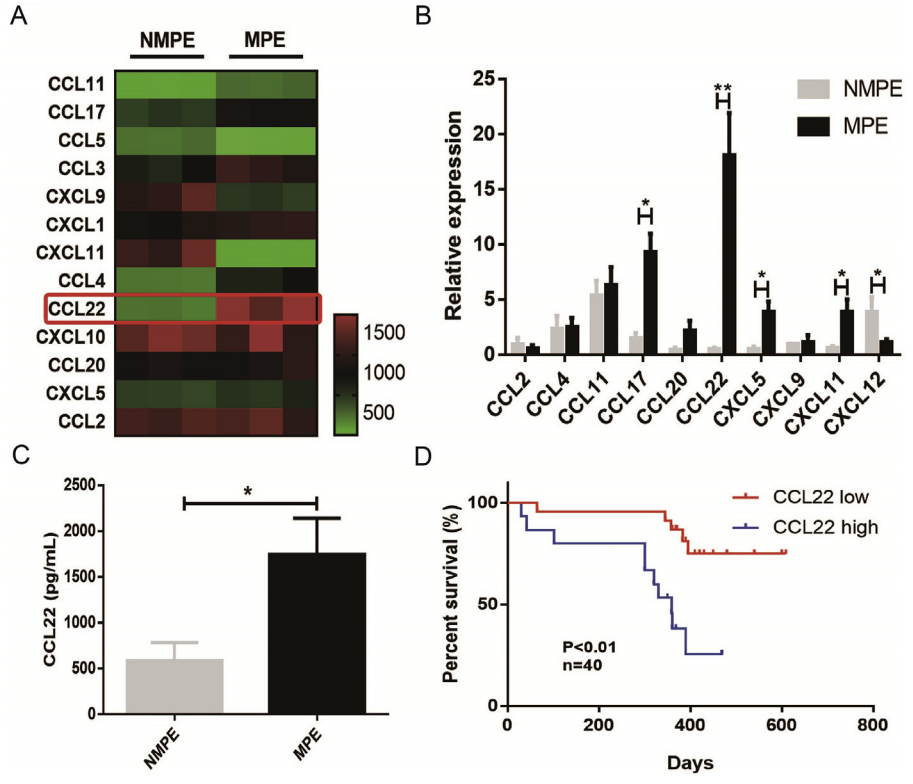

Fig. 1. The level of CCL22 is elevated in MPE. A. Heatmap showing the concentration of 13 chemokines (pg/mL) in supernatants obtained from MPE and NMPE measured by multiplex assay. **B.** Relative expression of CCL22 in mononuclear cells from MPE and NMPE was analyzed by qPCR. **C.** Concentration of CCL22 (pg/mL) in supernatants obtained from MPE and NMPE was measured by ELISA. **D.** Kaplan-Meier survival curves for 40 MPE patients with lung cancer. The other 10 MPE patients went missing. Data are represented as means ± SD. * = $P < 0.05$, ** = $P < 0.01$.

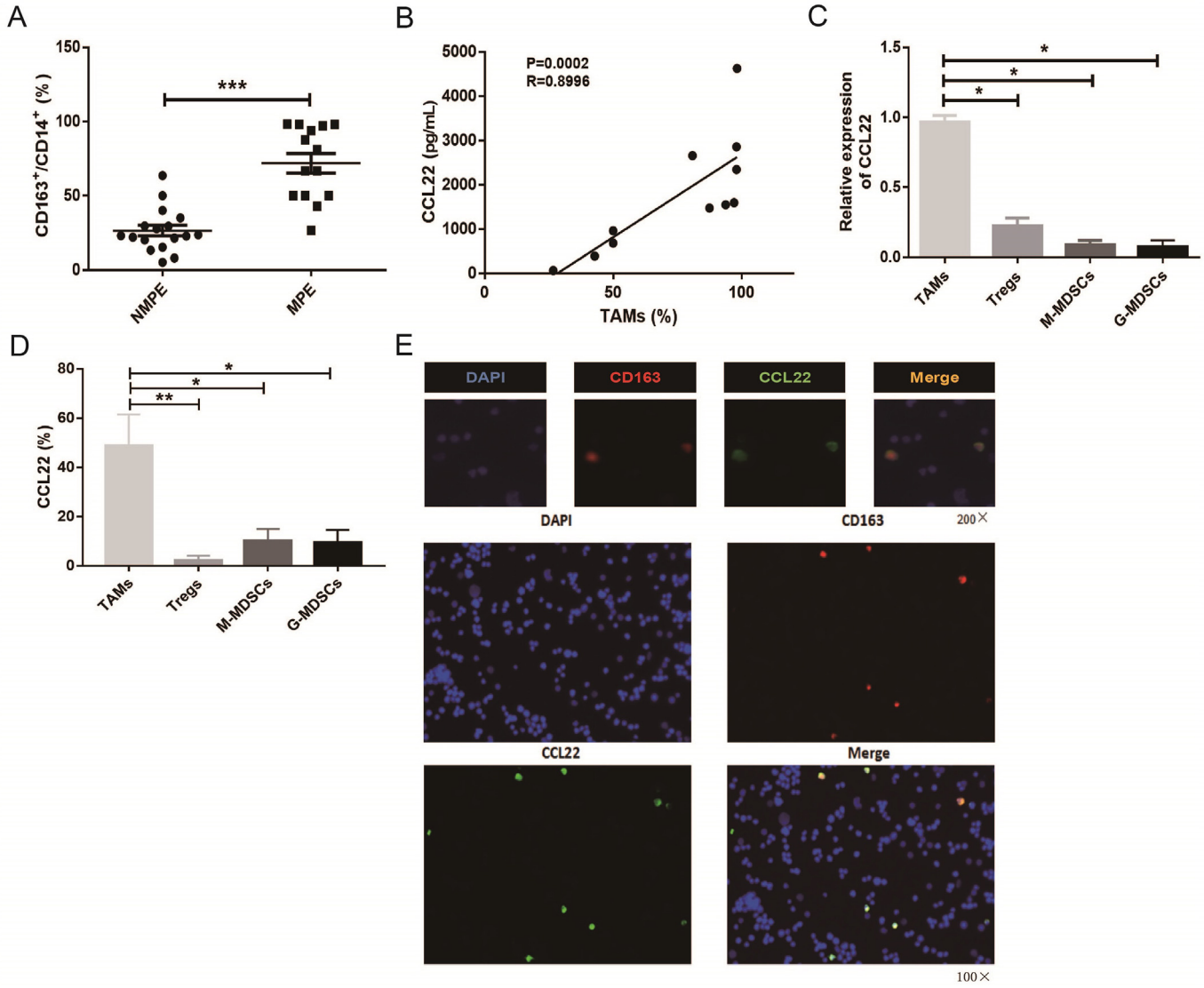

Fig. 2. CCL22 is mainly produced by TAMs in MPE. A. The percentage of $CD163^+CD14^+$ cells (TAMs) in MPE and NMPE was analyzed by flow cytometry. **B.** The relationship between CCL22 level and TAMs frequency in MPE. **C.** Relative expression of CCL22 in purified TAMs, Tregs, M-MDSCs and G-MDSCs from MPE was analyzed by qPCR. **D.** The percentage of $CCL22^+$ cells in purified TAMs, Tregs, M-MDSCs and G-MDSCs from MPE was analyzed by flow cytometry. **E.** MPE-derived mononuclear cells subjected to double immunofluorescence for CD163 (red), CCL22 (green) and DAPI (blue). One representative micrograph is shown. Data are represented as means ± SD. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

macrophages *in vitro* (Supplementary Figs. S1, S2, S3). After the treatment with rhTGF-β *in vitro*, the level of CCL22 from M2 macrophages was dramatically increased, and in a dose-dependent way ($P < 0.05$, Fig. 3A). With the usage of anti-TGF-β receptor (anti-TGF-βR) antibody, CCL22 production was significantly decreased compared to single usage of TGF-β group ($P < 0.01$, Fig. 3B).

To further evaluate which signaling molecules are involved in mediating CCL22 expression in TAMs induced by TGF-β, we performed RT^2 Profiler PCR Arrays to analyze the different transcription genes between $CD163^+$ and $CD163^-$ macrophages sorted from MPE. The results showed that c-Fos expression in $CD163^+$ macrophages was obviously higher than that in $CD163^-$ macrophages (Fig. 3C). Furthermore, induced M2 macrophages were infected with si-c-Fos to inhibit c-Fos expression. After downregulation of c-Fos expression in M2 macrophages, CCL22 production level was significantly decreased compared to control ($P < 0.001$, Fig. 3D). To investigate whether c-Fos physically bound to the promoter region of CCL22 or not, qCHIP assay was performed in induced M2 macrophages. And the results showed that the CCL22 promoter region had more enrichment of c-Fos in M2 macrophages compared to that in M1 macrophages ($P < 0.05$), revealing that c-Fos indeed located in the promoter region of CCL22 in M2 macrophages (Fig. 3E–G). Lastly, we performed qPCR to investigate the changes of c-Fos and CCL22 mRNA expression before and after the treatment with TGF-β and anti-TGF-βR antibody. Indeed, TGF-β enhanced the expression of c-Fos and CCL22 in M2 macrophages (Fig. 3H). However, blockade of TGF-β significantly downregulated TGF-β-induced c-Fos and CCL22 expression in M2 macrophages ($P < 0.05$, Fig. 3H). These results suggest that TAM-derived TGF-β induces CCL22 expression in TAMs via c-Fos.

3.4. CCL22 promotes the recruitment of Tregs in MPE

It has been demonstrated that CCL22 can recruit Tregs from peripheral blood to tumor tissues [14,15]. So we wanted to determine whether Tregs could be recruited to MPE by CCL22. Firstly, the relationship between CCL22 level and Treg frequency was analyzed. The result showed that CCL22 level was closely correlated with Treg frequency in MPE ($P < 0.0001$, Fig. 4A). A study in The Cancer Genome

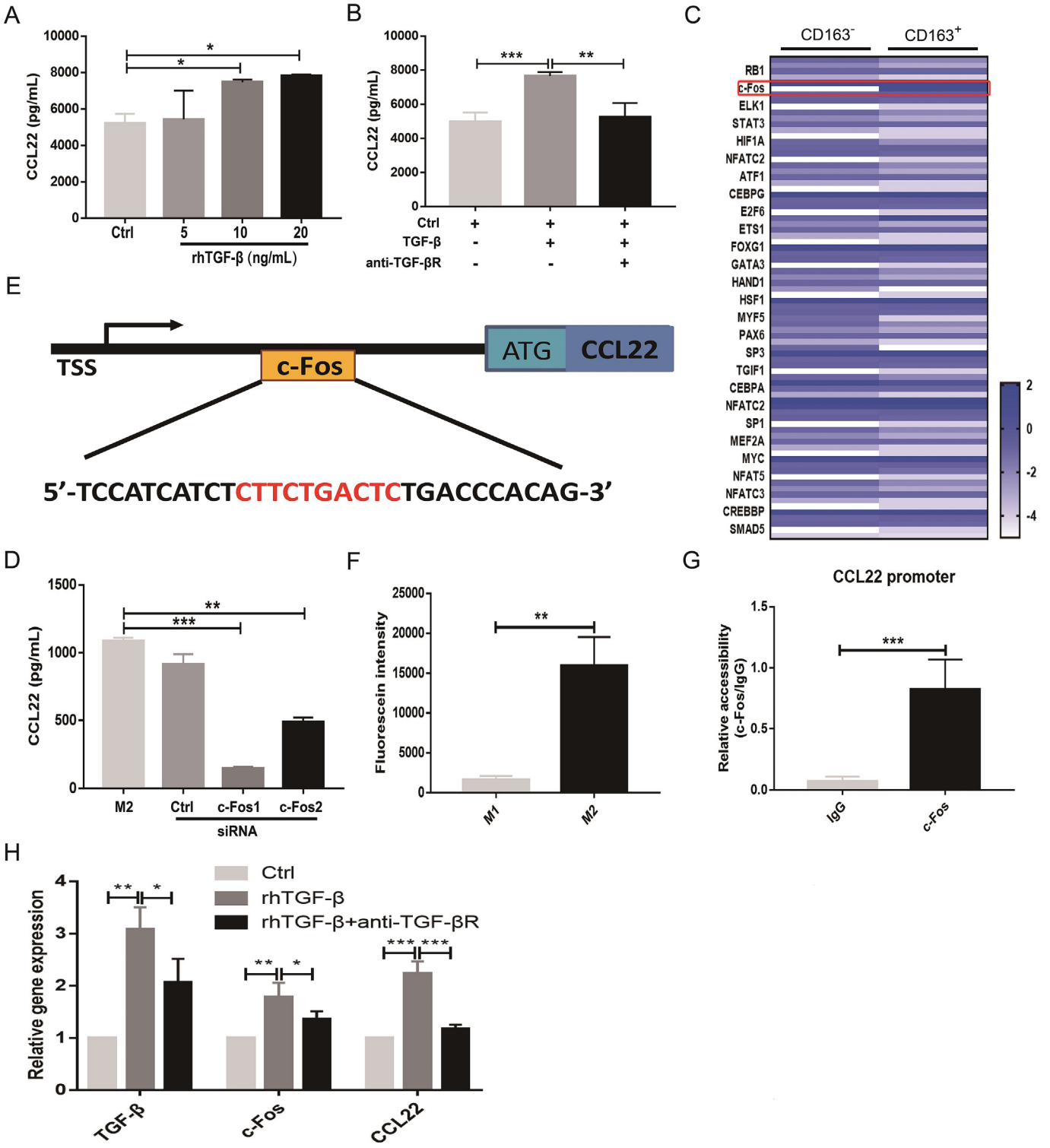

Fig. 3. TGF-β secreted from TAMs induces CCL22 expression in TAMs via c-Fos. A. Concentration of CCL22 (pg/mL) in supernatants obtained from induced M2 macrophages before and after TGF-β treatment (5, 10, 20 ng/mL) was measured by ELISA. **B.** Concentration of CCL22 (pg/mL) in supernatants obtained from induced M2 macrophages before and after anti-TGF-βR antibody treatment was measured by ELISA. **C.** Heatmap showing the expression of transcription-related genes in $CD163^+$ and $CD163^-$ macrophages measured by RT^2 Profiler PCR Arrays. **D.** Concentration of CCL22 (pg/mL) in supernatants obtained from si-c-Fos M2 macrophages was measured by ELISA. **E.** Schematic representation of the CCL22 promoter region. c-Fos indicates the location of primer on the CCL22 promoter. **F.** Fluorescein intensity of c-Fos was measured in induced M1 and M2 macrophages. **G.** qChIP assay was performed to evaluate c-Fos enrichment in the CCL22 promoter region of M2 macrophages. Normal goat anti-rabbit IgG served as a negative control. **H.** Relative expression of TGF-β, c-Fos and CCL22 in M2 macrophages before and after TGF-β or anti-TGF-βR antibody treatment was analyzed by qPCR. Data are represented as means ± SD. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

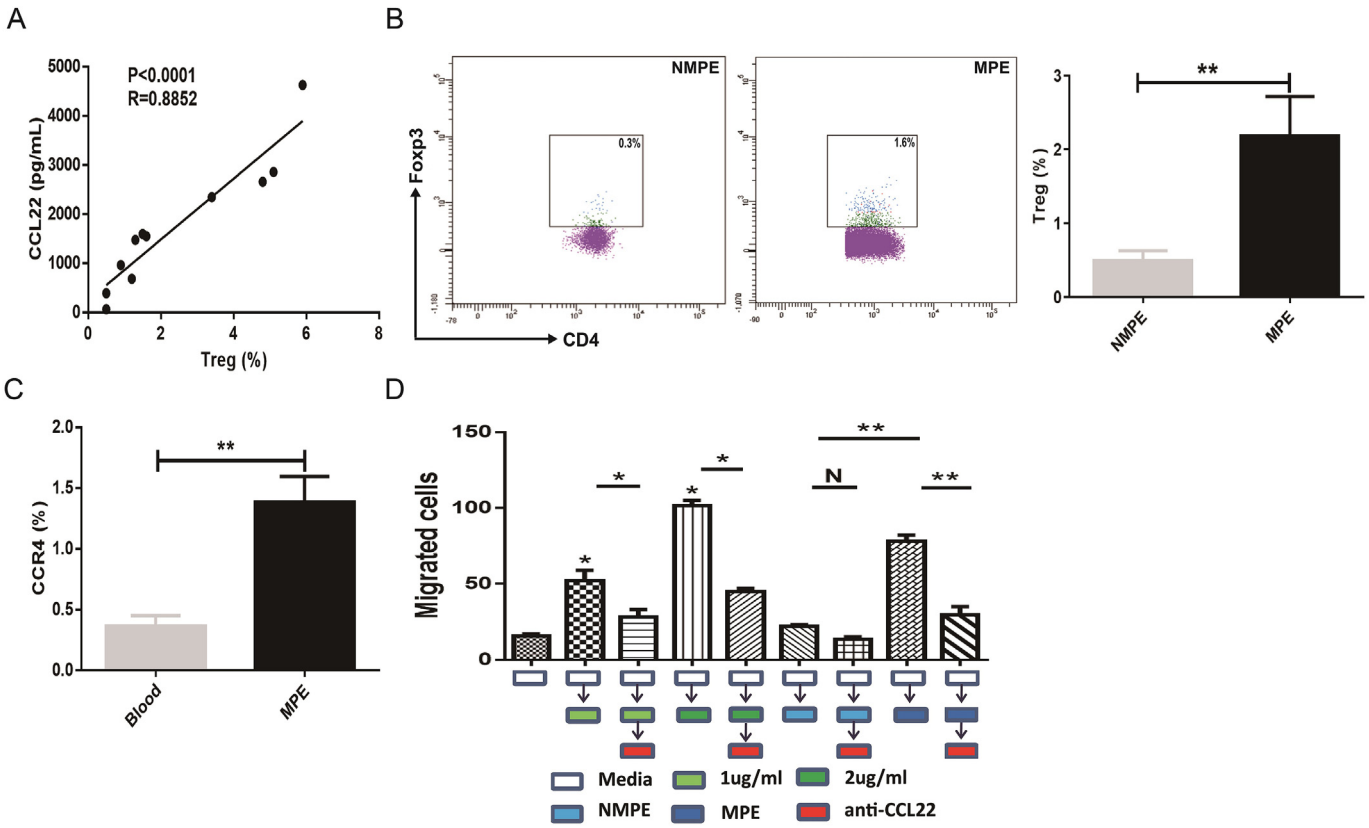

Fig. 4. TAM-derived CCL22 recruits Tregs in MPE. A. The relationship between CCL22 level and Treg frequency in MPE. **B.** The percentage of Tregs in MPE and NMPE was analyzed by flow cytometry. Representative analysis from one experiment is shown. The results are presented as a histogram. **C.** The percentage of $CCR4^+$ cells in Tregs from MPE and peripheral blood was analyzed by flow cytometry. **D.** Cell migration of Tregs sorted from MPE co-cultured with rhIL-8 (1, or 2 μg/mL), supernatants of MPE or NMPE, or anti-CCL22 antibody was analyzed by transwell assay. The results are presented as a histogram. Data are represented as means ± SD. * = $P < 0.05$, ** = $P < 0.01$, N = non-significant.

Atlas (TCGA) dataset revealed that there was a close correlation between CCL22 and Foxp3 expression in lung cancer tissues ($P < 0.0001$, Supplementary Fig. S4). Furthermore, by using flow cytometry assay, we found that the percentage of Tregs in MPE was significantly higher than that in NMPE ($P < 0.01$, Fig. 4B). To investigate the effect of CCL22 on the recruitment of Tregs, we detected CCR4 (the receptor of CCL22) expression on Tregs derived from MPE and paired peripheral blood, showing that $CCR4^+$ Treg frequency in MPE was dramatically higher than that in blood ($P < 0.01$, Fig. 4C). Transwell assay results showed that the migration rate of purified Tregs from MPE was increased after rhCCL22 treatment, in a dose-dependent manner ($P < 0.05$, Fig. 4D). Moreover, the migration rate of purified Tregs from MPE in the supernatants of MPE was significantly higher than that in the supernatants of NMPE ($P < 0.05$, Fig. 4D). Furthermore, after treatment with anti-CCL22 antibody, the migration rate of Tregs was decreased compared to the untreated group ($P < 0.05$, Fig. 4D). These data identify that CCL22 plays an important role in the recruitment of Tregs in MPE.

3.5. Tregs produce a high level of IL-8 in MPE

To further evaluate the relationship between TAMs and Tregs recruited by TAM-derived CCL22 in MPE, multiplex assay was performed to analyze the levels of different cytokines in MPE and NMPE. IL-6, IL-8 and IL-10 levels in MPE and NMPE were significantly different (Fig. 5A). Furthermore, IL-6, IL-8 and IL-10 protein levels analyzed by ELISA showed that IL-8 protein level in MPE was significantly higher than that in NMPE ($P < 0.05$, Fig. 5B). Next, we wanted to know which immunosuppressive cell was responsible for the secretion of IL-8 in MPE. qPCR was performed to analyze the mRNA expression of IL-8 in immunosuppressive cells including TAMs, Tregs, M-MDSCs and G-MDSCs. We found that IL-8 mRNA expression in purified Tregs was dramatically higher than that in other purified immunosuppressive cells (TAMs, M-MDSCs and G-MDSCs) in MPE ($P < 0.05$, Fig. 5C). Moreover, an immunofluorescence assay was performed to evaluate the co-localization of IL-8 and the Treg biomarker Foxp3. The result showed that IL-8 was dominantly found in $Foxp3^+$ Tregs from MPE-derived mononuclear cells (Fig. 5D). These findings demonstrate that IL-8 is mainly secreted by Tregs in MPE.

3.6. IL-8 derived from Tregs further induces TGF-β upregulation in M2 macrophages

To further investigate the correlation between Treg-derived IL-8 and TAMs in MPE, we analyzed the effect of IL-8 on TGF-β and CCL22 expression in M2 macrophages. After treatment with rhIL-8 *in vitro*, the mRNA expression of TGF-β in M2 macrophages ($P < 0.01$, Fig. 6A) and protein level of TGF-β secreted from M2 macrophages ($P < 0.05$, Fig. 6B) were significantly increased compared to control group. After treatment with anti-IL-8 antibody, TGF-β mRNA expression in M2 macrophages ($P < 0.01$, Fig. 6A) and TGF-β production level ($P < 0.05$, Fig. 6B) were significantly decreased compared to untreated group. Furthermore, with the treatment of Treg supernatants *in vitro*, TGF-β level in TAMs was also increased ($P < 0.05$, Fig. 6A and B). Blockade IL-8 signaling inhibited TGF-β expression in TAMs (Fig. 6A and B). In addition, considering TAM-derived TGF-β induced CCL22 expression in TAMs, we further analyzed CCL22 level in TAMs with the treatment of rhIL-8, Treg supernatants or anti-IL-8 antibody. The results showed that rhIL-8 and Treg supernatants also increased CCL22 expression in TAMs, and usage of anti-IL-8 antibody induced CCL22

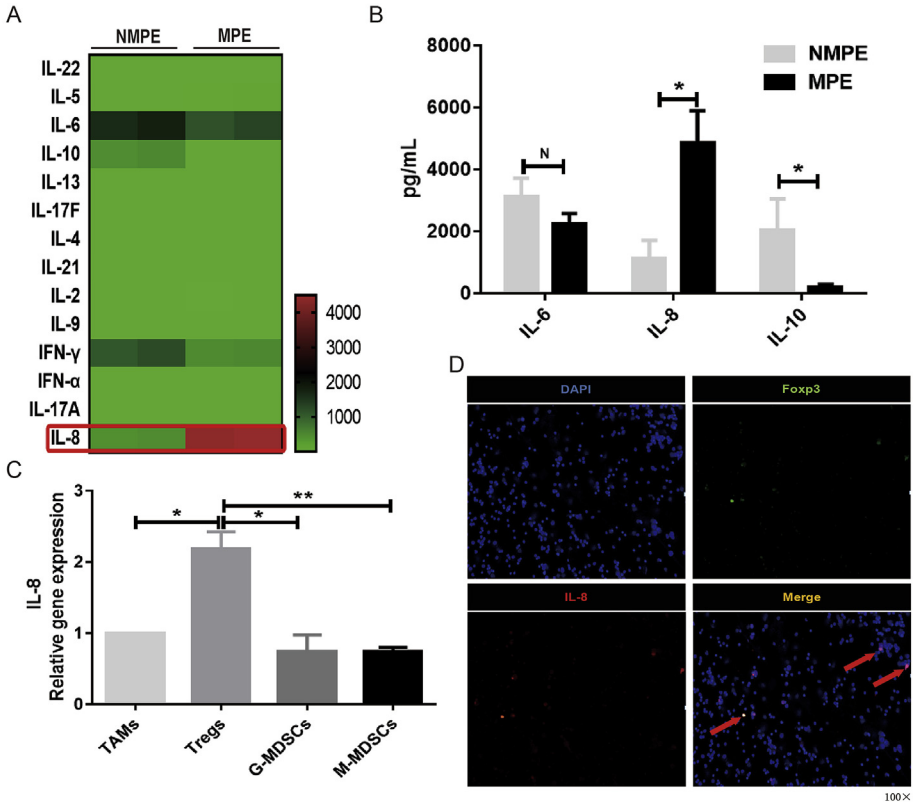

Fig. 5. Tregs produce a high level of IL-8 in MPE. **A.** Heatmap showing the concentration of 13 cytokines (pg/mL) in supernatants obtained from MPE and NMPE measured by multiplex assay. **B.** Concentration of IL-6, IL-8 and IL-10 (pg/mL) in supernatants obtained from MPE and NMPE was measured by ELISA. **C.** Relative expression of IL-8 in purified TAMs, Tregs, M-MDSCs and G-MDSCs was analyzed by qPCR. **D.** MPE-derived mononuclear cells subjected to double immunofluorescence for IL-8 (red), FoxP3 (green) and DAPI (blue). One representative micrograph is shown (100 ×). Data are represented as means ± SD. * = $P < 0.05$, N = non-significant.

downregulation in TAMs (Fig. 6C and D). All these data indicate that Treg-derived IL-8 induces TGF-β upregulation in TAMs, and further mediates CCL22 production from TAMs, which promotes an immunosuppressive tumor microenvironment in MPE.

4. Discussion

Increasing evidences show that the tumor microenvironment is responsible for T cell dysfunction [16–18]. Studying the local immune response in MPE will further our understanding of how the microenvironment of a malignancy promotes tumor escape from immune surveillance. In our previous study, T cells in MPE displayed a series of alterations that were not seen in paired peripheral blood, including an increase in inhibitory molecules and impairment of cytotoxic activity, both of which appeared to be affected by TGF-β released from TAMs. Therapeutic strategies that target the TGF-β pathway could therefore represent an effective method for MPE treatment [12]. However, the mechanism how TGF-β produced by TAMs, whether other immunoregulatory cells and factors mediated TGF-β secretion in MPE requires further investigation, which was mainly focused in this study.

Qin et al. reported that the concentration of CCL22 in MPE was significantly higher than that in the corresponding serum. Pleural fluid from lung cancer patients was chemotactic for Tregs, and this activity was partly blocked by an anti-CCL22. Intrapleural administration of CCL22 of patients produced a marked progressive influx of Tregs into pleural space [19]. The increased $CD4^+CD25^+$ T cells found in MPE expressed high levels of Foxp3 transcription factor and potently suppressed the proliferation of $CD4^+CD25^-$ T cells, and cytotoxic lymphocyte-associated antigen-4 wass involved in the suppressive activity of pleural $CD4^+CD25^+$ T cells [20]. In addition, Wu et al. found that CCL22 appeared to be increased in tuberculous pleural effusions compared with bacterial pleural effusions or transudates. CCL22 may be responsible for the infiltration of $CD4^+CD25^{high}$ T cells into the pleural space of patients with tuberculous pleurisy [21]. In this study, we also found that CCL22 seemed to be increased in MPE, and could directly induce Treg infiltration into the pleural space in patients with lung cancer.

It is demonstrated that tumor cells and tumor-infiltrating macrophages produce the chemokine CCL22, which attracts Tregs into the tumor microenvironment, decreasing anticancer immunity [22]. CCL17 and CCL22 within the tumor are related to the increased population of $Foxp3^+$ Tregs, with such an observation occurring in early gastric cancer [23] and esophageal squamous cell carcinoma [24]. In recently, Wiedemann et al. identified tumor cell-derived IL-1α as a major inducer of the Treg attracting chemokine CCL22 in human cancer cells. Therapeutic blockade of the IL-1 pathway could represent a promising strategy to inhibit tumor-induced immunosuppression [25]. Type I IFN can block the Treg-attracting chemokine CCL22 and thus help limit the recruitment of Treg to tumors and block cancer progression [26]. Thus, targeting CCL22-mediated signal transduction in M2 macrophages may provide a novel approach to controlling tumor growth. Furthermore, fucoidan inhibited tumor cells migration and $CD4^+$ T lymphocytes, especially Treg cells, recruitment induced by M2 macrophages conditioned medium through suppression of CCL22. CCL22 in M2 macrophages via NF-κB-dependent transcription may be a novel and promising mechanism for tumor immunotherapy [27]. In addition, Osabe et al. reported that fucoidan could inhibit tumor cell migration and lymphocytes recruitment by suppressing CCL22 in M2 macrophages via NF-κB-dependent transcription, which may be a novel and promising mechanism for tumor immunotherapy [28]. In the current study, we also indicate that CCL22 derived from M2 macrophages plays an important role in the recruitment of Tregs in MPE, which may serve as a potential therapeutic target for MPE treatment from lung cancer patients.

A study identified $Foxp3^+CD4^+$ Tregs as an additional intrahepatic source of IL-8 in chronic hepatitis C acting on hepatic stellate cells [29]. Our results also showed that IL-8 was dominantly produced from Tregs in MPE. IL-8 level has been observed to be associated with advance stage cancer for several types of cancer and a poor prognostic maker for malignant disease [30]. IL-8 induced neo-angiogenesis through

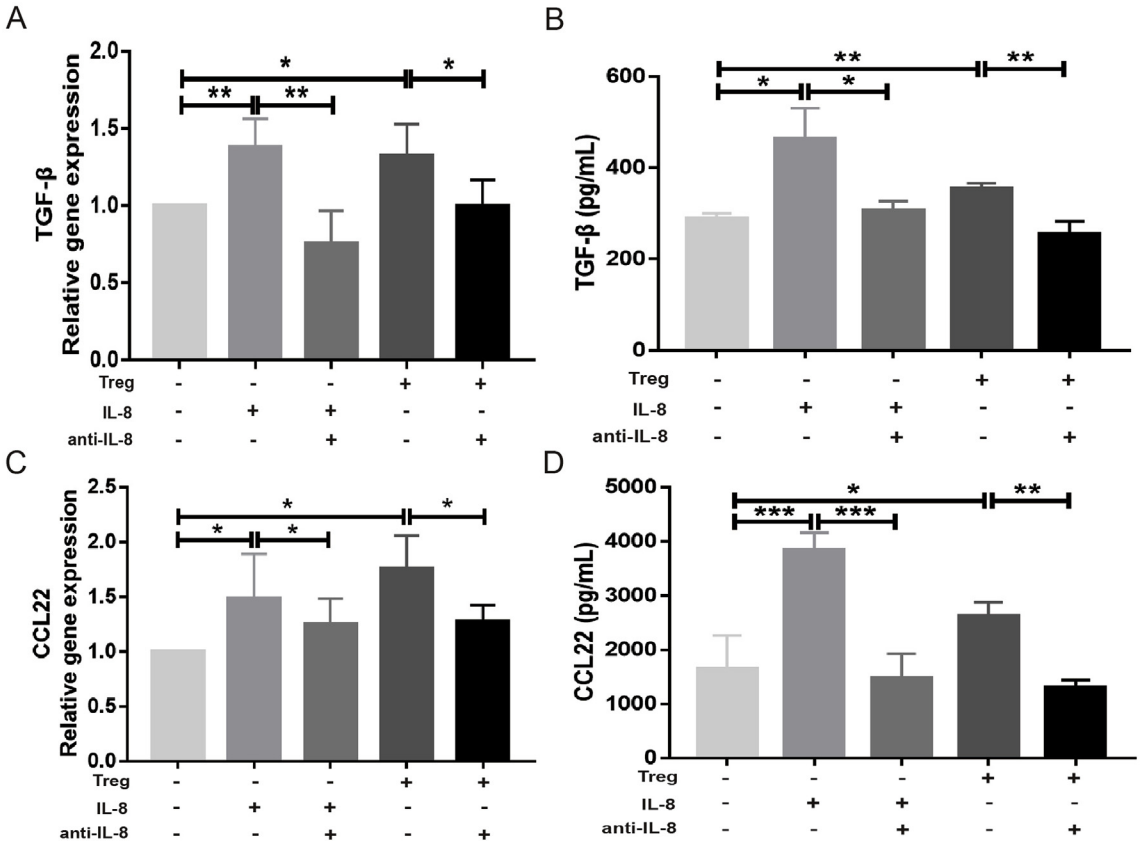

Fig. 6. Treg-secreted IL-8 induces TGF-β upregulation in M2 macrophages. Induced M2 macrophages were used in all experiments. **A.** Relative expression of TGF-β in induced M2 macrophages before and after treatment of rhIL-8, supernatants of Tregs, and anti-IL-8 antibody was analyzed by qPCR. **B.** Concentration of TGF-β (pg/mL) in supernatants obtained from induced M2 macrophages before and after treatment of rhIL-8, supernatants of Tregs, and anti-IL-8 antibody was measured by ELISA. **C.** Relative expression of CCL22 in induced M2 macrophages before and after treatment of rhIL-8, supernatants of Tregs, and anti-IL-8 antibody was analyzed by qPCR. **D.** Concentration of CCL22 (pg/mL) in supernatants obtained from induced M2 macrophages before and after treatment of rhIL-8, supernatants of Tregs, and anti-IL-8 antibody was measured by ELISA. Data are represented as means ± SD. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

activation of the vascular endothelial growth factor pathway. Additionally, IL-8 enhanced the activity of matrix metalloproteinase-2 and -9 which in turn increased the metastatic activity of the underlying malignancy. Inhibition of IL-8 production could be a potential treatment both for chronic inflammatory diseases and tumor modulation [31]. Increasing evidences show that the potential role of the IL-8-IL-8 receptor axis on the induction and/or maintenance of tumor EMT and its ability to remodel the tumor microenvironment [32–34]. In this study, we found that Treg-derived IL-8 induces TGF-β upregulation in TAMs, and further mediates CCL22 production from TAMs, which promotes an immunosuppressive tumor microenvironment in MPE.

Targeting IL-8-mediated tumor progression may provide a novel approach to controlling tumor growth. IL-8 signaling pathway and the potential of combining CXCR1/2 inhibitors with other treatments such as HER2-targeted therapy as a novel approach to eliminate cancer stem cells and improve breast cancer patient survival were reviewed [35]. Anti-cancer drug-induced IL-8 secretion increased the expression of ATP-binding cassette transporters and side population cells, promoting the growth of human hepatocellular carcinoma *in vitro*. Knockdown of IL-8 significantly reduced tumor size *in vivo*. Thus IL-8 may be a potential therapeutic target in the treatment of human hepatocellular carcinoma [36]. IL-8 knockdown inhibited tamoxifen-resistant cell growth and invasion, which highlighted a role of IL-8 signaling as a potential therapeutic target in FOXA1-overexpressing estrogen receptor α-positive tumors [37]. In this study, we found that Treg-derived IL-8 induced TGF-β upregulation in TAMs, and further mediated CCL22 production from TAMs, which promoted an immunosuppressive tumor microenvironment in MPE. Blockade IL-8 signaling inhibited TGF-β expression in TAMs, which could be served as a potential therapeutic target for MPE patients with lung cancer.

In conclusions, TAM-derived TGF-β induces CCL22 expression in TAMs via c-Fos in MPE. CCL22 further promotes the recruitment of Tregs in MPE. Tregs produce a high level of IL-8, which further induce TGF-β upregulation in TAMs, and promote an immunosuppressive tumor microenvironment in MPE (Fig. 7). These findings point to a key role of CCL22 and IL-8 in immune escape of MPE, and provide a rationale for therapeutic targeting of the CCL22/IL-8 pathway.

Authors' contributions

Conception and design: L. Yang, Y. Zhang.

Development of methodology: D. Wang, D. Yue, D. Wang, Y. Zheng.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Cao, Y. Ping, L. Wang.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Wang, L. Li.

Writing, review, and/or revision of the manuscript: L. Yang, Y. Zhang.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Li, Z. Shen.

Study supervision: Y. Zhang.

Conflicts of interest statement

The authors declare no potential conflicts of interest.

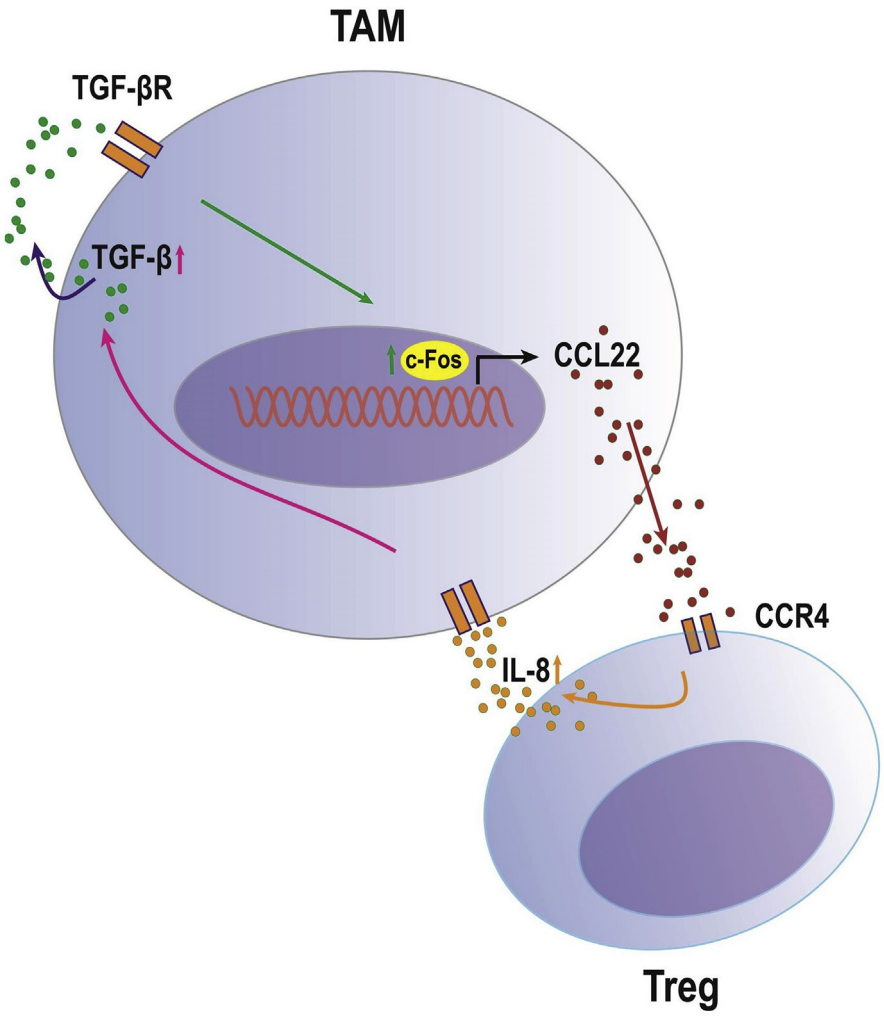

Fig. 7. Graph showing the proposed mechanism that macrophage-derived CCL22 promotes an immunosuppressive tumor microenvironment via IL-8 in MPE. TAM-derived TGF-β induced CCL22 expression in TAMs via c-Fos in MPE. CCL22 further promoted the recruitment of Tregs in MPE. Tregs produced a high level of IL-8, which further induced TGF-β upregulation in TAMs, and promoted an immunosuppressive tumor microenvironment in MPE.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (No.81771781, No.81602024, No.81872410), Funding from State's Key Project of Research and Development Plan (No. 2016YFC1303501), International Research Cooperation Grant from Science and Technology Department of Henan Province (No.162102410059), Research Grant from the Ministry of Public Health (No.201501004).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.040.

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