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Fas Signal Promotes Lung Cancer Growth by Recruiting Myeloid-Derived Suppressor Cells via Cancer Cell-Derived PGE$_2$

Yongliang Zhang,$^{2*}$† Qiuyan Liu,$^{2}$† Minggang Zhang,* Yizhi Yu, † Xia Liu, † and Xuetao Cao$^{3*}$†

Fas/FasL system has been extensively investigated with respect to its capacity to induce cellular apoptosis. However, accumulated evidences show that Fas signaling also exhibits nonapoptotic functions, such as induction of cell proliferation and differentiation. Lung cancer is one of cancer’s refractory to the immunotherapy, however, the underlying mechanisms remain to be fully understood. In this study, we show that Fas overexpression does not affect in vitro growth of 3LL cells, but promotes lung cancer growth in vivo. However, such tumor-promoting effect is not observed in FasL-deficient (gld) mice, and also not observed in the immune competent mice once inoculation with domain-negative Fas-overexpressing 3LL cells, suggesting the critical role of Fas signal in the promotion of lung cancer growth in vivo. More accumulation of myeloid-derived suppressor cells (MDSC) and Foxp3$^+$ regulatory T cells is found in tumors formed by inoculation with Fas-overexpressing 3LL cells, but not domain-negative Fas-overexpressing 3LL cells. Accordingly, Fas-ligated 3LL lung cancer cells can chemoattract more MDSC but not regulatory T cells in vitro. Furthermore, Fas ligation induces 3LL lung cancer cells to produce proinflammatory factor PGE$_2$, by activating p38 pathway, and in turn, 3LL cells-derived PGE$_2$ contribute to the Fas ligation-induced MDSC chemoattraction. Furthermore, in vivo administration of cyclooxygenase-2 inhibitor can significantly reduce MDSC accumulation in the Fas-overexpressing tumor. Therefore, our results demonstrate that Fas signal can promote lung cancer growth by recruiting MDSC via cancer cell-derived PGE$_2$, thus providing new mechanistic explanation for the role of inflammation in cancer progression and immune escape. The Journal of Immunology, 2009, 182: 3801–3808.

Tumor cells consistently release many kinds of immunosuppressive and proinflammatory factors such as TGF-β, IL-10, PGE$_2$, and vascular endothelial growth factor (VEGF), which facilitate tumor immune escape and tumor growth. Soluble mediators produced by cancer cells can recruit and activate immune suppressive cells, which further stimulate tumor progression. In recent years, more attention has been paid for immune suppressive cells infiltrating in the tumor tissue, they facilitate the constitution of immune suppressive network that inhibits the host immunity and supports the tumor growth. Of the immune suppressive cells, myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) attach more attraction in this field and have been found to play important roles in promoting tumor immune escape.

MDSC represent a heterogeneous population of myeloid cells comprising immature macrophages, granulocytes, dendritic cells (DCs), and other myeloid cells at earlier stages of differentiation that can be identified in mice by expression of CD11b and Gr-1. Tumor-derived factors mobilize MDSC from bone marrow into peripheral blood, and then MDSC accumulate and expand in tumor tissues, where MDSC exert their tumor-promoting effect through inhibiting T and NK cell proliferation and activation (6–8), facilitate tumor metastasis, and even contribute to the tumor angiogenesis by directly incorporating into the tumor endothelium (9). Treg cells represents a small fraction of the overall CD4$^+$ T cell population, expressing a high level of GITR and Foxp3 molecules, and mediating immune suppression through a cell-cell contact mechanism (10). In tumor-bearing mice, Treg cells are recruited and predominantly accumulated at tumor sites. Removal of CD4$^+$CD25$^+$ T cells by injecting anti-CD25 Ab into tumor-bearing mice can enhance antitumor responses (4). Therefore, accumulation of MDSC and Treg in tumor tissue is crucial for tumor immune escape and supposed to be the target of immunotherapy (11). However, little is known about the natural signals in the tumor microenvironment responsible for inducing accumulation and maintaining residence of MDSC and Treg cells in the tumor tissues.

Death receptor Fas (CD95/APO-1) transmits death signals in susceptible cells once triggered by its natural ligand FasL, or agonist Ab (Jo2). Fas/FasL system has been demonstrated to play important roles in various biological processes, including cytolytic T cell-mediated cytotoxicity against virally infected or transformed cells, immune privilege of tumor and the mediation of some therapeutic drugs for cancer (2, 12, 13). However, other than inducing cell death, Fas also transmits proliferation and activation signals (14). For example, in the presence of certain level of FasL, Fas signal in tumor is proposed to convert from tumor suppressor to tumor promoter (15), and this phenomenon has been proved in various human cancer cells and tumor-bearing mice (16–20). Tumor-promoting effect of Fas ligation has been reported to be mediated by inducing tumor cell proliferation (17,
advancing the cell cycle (19), increasing tumor motility, and invasiveness (20). These studies focused on the direct effect of Fas signaling on the tumor cells, and most of conclusions have been made on the basis of the in vitro experiments, however, the influence of Fas signal in tumor cells on tumor-bearing host was ignored.

In this study, we demonstrate that mouse Lewis lung cancer 3LL cells are resistant to Fas-induced apoptosis. Fas signal in 3LL lung cancer cells can promote lung cancer growth in vivo and increase the MDSC and Treg accumulation in tumor. Furthermore, Fas ligation can promote in vitro chemoattraction of MDSC by 3LL cells via inducing PGE2 production. Thus, our results demonstrate that Fas signal can promote lung cancer growth in vivo by recruiting immunosuppressive cells MDSC and Treg cells into tumor microenvironment, therefore providing new mechanistic explanation for the tumor promoting effect of Fas signaling in tumor microenvironment, and the role of inflammation in tumor progression and immune escape.

Materials and Methods

Mice, cell line, and reagents

Female C57BL/6J mice (6–8 wk) were obtained from Joint Ventures Sipper BK Experimental Animal. Tnfsf6<sup>gld</sup> mice on a C57BL/6 background (henceforth termed, gld) were obtained from The Jackson Laboratory and bred under specific pathogen-free conditions, used at age of 6–8 wk. The 3LL Lewis lung

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Fas signal promotes lung tumor growth in vivo. A, Lung cancer cells 3LL were detected for Fas expression at mRNA (left) and protein (right) levels. B, Susceptibility of 3LL cells to Fas-induced apoptosis was measured by staining with Annexin V-FITC and PI after 3LL cells were treated with Jo-2 or isotype Ab (ISO) at the indicated concentrations for 12 h. C, Quantification of Fas expression on stable 3LL transfectants of WT Fas (3LL/Fas WT), or dominant negative Fas (3LL/Fas DN) and parental 3LL cells (3LL) by flow cytometry. The solid lines represent control, dotted lines and shaded curves represent immunostaining with isotype and PE-Jo2 respectively. D, In vitro growth of 3LL cells overexpressing Fas or dominant-negative Fas was observed. Cell number was assayed by MTT, and data are presented as mean value. E and F, Five × 10<sup>5</sup> 3LL/Fas WT, 3LL/Fas DN, and parental 3LL cells were s.c. inoculated into C57BL/6 mice (E) and FasL-deficient gld mice (F). Then tumor growth and survival of tumor-bearing mice were monitored and analyzed as described in the Materials and Methods. Data points represent the mean value ± SEM from eight mice per group for E and F, respectively. The values of tumor size were compared at various time points and p values were denoted. 3LL/Fas-WT VS parental 3LL (∗, p < 0.05; ∗∗, p < 0.01), 3LL/Fas-WT VS 3LL/Fas-DN (∗, p < 0.05; ∗∗, p < 0.01), parental 3LL VS 3LL/Fas-DN (∗, p < 0.05; ∗∗, p < 0.01). Survival data were analyzed by log-rank statistics, and the p value was denoted, ∗, p < 0.05; ∗∗, p < 0.01.
FIGURE 2. Fas signal promotes the infiltration of MDSC and Foxp3+ Treg cells in the tumor tissue. A and C, 3LL/Fas-WT, 3LL/Fas-DN, and parental 3LL cells (10^6) were s.c. inoculated into C57BL/6 mice (A) and gld (C) mice. Seven and 14 days later, tumor tissues from the tumor-bearing mice were excised, fixed by 4% paraformaldehyde, and stained with anti-Gr1 and anti-Foxp3 Ab for analyzing MDSC and Treg cell infiltration by immunohistochemistry assay. B and D, TIL were isolated from C57BL/6 mice (B) and gld (D) mice 14 days after tumor inoculation. The ratio of MDSC in TIL and Foxp3+ cells in gated CD4+ cells were analyzed by FACS, and the results represent mean value ± SEM of three independent experiments with similar results. Original magnification, ×200.

3LL lung cancer cells were stimulated with Jo-2 or isotype Ab in the indicated concentrations for 12 h, then the apoptotic cells were stained with FITC-Annexin V (BD Pharmingen) and propidium iodide (Sigma-Aldrich) and analyzed by FACS as described previously (22).

Assay for cytokines

MCP-1, VEGF, PGE2, IL-1β, TGF-β, and IL-10 in the supernatants of 3LL cells stimulated with or without Jo-2 Ab were assayed using ELISA kits according to the manufacturer’s instructions (R&D Systems).

Assay for cell chemoattraction

Cell chemoatraction was performed in 3.0-μm pore size polyethylene terephthalate track-etched membrane cell-culture inserts (BD Labware). The supernatants of 3LL cells stimulated with or without 1 μg/ml Jo2 or isotype Ab for 12 h (600 μl) were five-fold diluted with RPMI 1640 medium. In the experiment to confirm the role of PGE2 in the cell chemoatraction, anti-PGE2 Ab (5, 10, 20, or 50 μg/ml) was added into the supernatants of 3LL cells stimulated with 1 μg/ml Jo2. Then CD11b+Gr1+ MDSC (2 × 10^5) and CD4+CD25+ Treg cells (2 × 10^5) purified from spleen of tumor-bearing mice using FACSDiVa sorting system (Becton Dickinson) and Miltenyi regulatory T cell isolation kit, respectively, were placed into transwell inserts. Four hours after incubation at 37°C, the cells that had migrated into low chambers were harvested, suspended in 200 μl PBS, and counted by FACSCalibur in low rate within 90 s and analyzed by CellQuest software (BD Biosciences).
Preparation and observation of tumor-bearing mice

One × 10^6 3LL cells, 3LL cells expressing Fas-WT (3LL/Fas-WT), or 3LL cells expressing Fas-DN (3LL/Fas-DN) suspended in 200 µl of HBSS were s.c. inoculated into the flank of C57BL/6 cells or gld mice, respectively. In some experiments, 250 µg RB6–3C5 (anti-Gr1 Ab) and control Isotype (rat IgG) were i.p injected on day 1, and injected repeatedly on the following day 3, 6, 9, 12, 15, 18, and 21 for depleting MDSC, or simultaneously i.p injected with COX-2 specific inhibitor SC58125 (5 mg/kg) or control DMSO once a day during 18 days. Then tumor mass were measured with a caliper after tumor inoculation, and the tumor size was calculated by multiplying vertical length by horizontal length. The survival of the tumor-bearing mice was monitored daily. Mice were sacrificed when the transplanted tumors reached 3 cm in diameter or severe ulceration developed. Experiments were performed three times and each group contained eight mice.

Phenotype analysis of tumor infiltrating lymphocytes

For analyzing the ratio of Gr1^+ CD11b^+ MDSC and CD4^+ Foxp3^+ Treg in tumor-infiltrating lymphocytes (TIL), 14 days after inoculation with parental 3LL cells, 3LL/Fas-WT, or 3LL/Fas-DN, TIL were isolated as described previously (23). Then the TIL were stained with FITC conjugated CD4 with PE conjugated Foxp3 for Treg cells analysis, and FITC conjugated CD4 with PE conjugated Foxp3 for Treg cells analysis, respectively by flow cytometry.

RT-PCR

Total RNA of 3LL cells and MDSC isolated with anti-Gr1 Beads (MACS, Germany) was extracted with TRIzol reagent (Invitrogen). cDNA was prepared using M-MLV reverse transcriptase (Promega). cDNA was synthesized from 1 µg of the total RNA preparing with AMV reverse transcriptase (Promega) using an oligo(dT)15 primer. The sequences of the PCR primers synthesized by Sangon and used in this study were: 5'–CTG CGA TGA AGA GCA TGG TTT-3' and 5'–CCA ATA GGC GAT TTC TGG CAC-3' for Fas; 5'–CCA CGA TGC TCC TGC TGC TT and 5'–TCC ACA AAG GTC AGT CTG TTT for EP2; 5'–GGT CAT CTT ACT CAT CGC CAC CTC TC-3' and 5'–TCC CAC TAA CCT CAT CGC GAC G-3', for EP4; 5'–CGT AAA GAC CTC TAT GCC AAC AC-3' and 5'–TCA GTA ACA GTC CGC CTA GAA GCA C-3' for β-actin. Cycling conditions for Fas and actin were 94°C for 30 s, annealing at 55°C for 30 s, and primer extension at 72°C for 45s, the optimum numbers of cycles were 34 cycles for Fas, 29 cycles for actin, and 90 cycles for β-actin. Cycling conditions for EP2/EP4 were 94°C for 30 s, annealing at 65°C for 30 s, and primer extension at 72°C for 60 s for 38 cycles.

Immunohistochemistry

Immunohistochemical staining was performed by using anti-mouse Gr1, or anti-Foxp3 (eBioscience), as described previously (22). Images were viewed with a microscope (DMIRB; Leica Microsystems) and processed with Adobe Photoshop software (Adobe Systems).

Western blot

Activation of ERK, JNK, p38, Iκ-B, and expression of COX-2 in 3LL lung cancer cells stimulated with or without Jo-2 Ab were detected using the assay kit according to manufacturer’s instructions. Blots were probed for 1 h with 1/1000 diluted, then incubated with 1/2000 diluted HRP-conjugated anti-rabbit or goat Ig G. Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate, as instructed by the manufacturer (Pierce).

Statistical analysis

The comparison of mean values between groups was determined by the Student’s t test. Statistical analysis of survival data was performed by the Kaplan-Meier method and analyzed by using the log-rank test, p < 0.05 was considered to be statistically significant.

Results

Fas signal in lung cancer cells promotes lung cancer growth in vivo

As shown in Fig. 1A. 3LL Lewis lung cancer cells constitutively express Fas at mRNA and protein levels. Upon stimulation of 3LL cells with agonistic anti-Fas Ab Jo-2, no significant apoptotic cells were observed, even high concentration of Jo2 (5 µg/ml) was used (Fig. 1B). These data indicate that 3LL lung cancer cells are resistant to Fas-induced apoptosis. To investigate the effects of Fas signal on the lung cancer growth, we first detect the in vitro 3LL growth in the presence of Jo2 and anit-FasL Ab by MTT assay, but both Jo2 and anti-FasL Ab does not affect the 3LL growth (data not shown). To observe Fas-induced 3LL growth in vivo, we transfected Fas-WT or Fas-DN into 3LL cells, and obtained stable 3LL transfectant clones expressing Fas-WT (3LL/Fas-WT) or Fas-DN (3LL/Fas-DN). Forced overexpression of Fas-WT or Fas-DN did not affect the in vitro growth of 3LL cells (Fig. 1D). However, when 3LL/Fas-WT, 3LL/Fas-DN, or parental 3LL cells were s.c. inoculated into C57BL/6/J mice, we found the accelerated growth of tumor formed by inoculation with 3LL/Fas-WT cells, as compared with that of parental 3LL cells or 3LL/Fas-DN cells. Accordingly, the survival of the mice bearing 3LL/Fas-WT cells was reduced more significantly than that of mice bearing parental 3LL cells or 3LL/Fas-DN cells (Fig. 1E). So, the results indicate that Fas signal in lung cancer cells can promote lung cancer growth in vivo. To confirm the role of Fas signal in the promotion of in vivo growth of lung cancer, we s.c. inoculated 3LL/Fas-WT cells, 3LL/Fas-DN cells, or parental 3LL cells into FasL-deficient gld mice,
and found there was no significant difference in the tumor growth and survival of tumor-bearing mice among these groups (Fig. 1F). Collectively, Fas signal in lung cancer cells promotes lung cancer growth in vivo, which is dependent on the Fas/FasL interaction.

**Increased MDSC and subsequent Foxp3+ Treg cell accumulation in Fas-overexpressing tumor**

Next, we wanted to know what’s the mechanism for the promotion of lung cancer growth in vivo by Fas signal in lung cancer cells. Because MDSC and Treg accumulation in tumor are involved in the tumor immune escape and tumor progression, we analyzed the ratio and the absolute numbers of CD11b+ Gr1+ MDSC in TIL and Foxp3+ Treg cells in CD4+ T cells by FACS. The average ratio of MDSC in TIL isolated from 3LL/Fas-WT, 3LL, and 3LL/Fas-DN tumors were 32.8, 24.5, and 18.8%, respectively, and the average ratio of Foxp3+ T cells in CD4+ T cells were 13.5, 10.2, and 7.8%, respectively in C57BL/6 mice (Fig. 2B). We also counted the absolute numbers of MDSC (Gr1+ CD11b+) and Tregs (CD4+ Foxp3+) in TIL. There were \( \sim 5.3 \times 10^5 \), \( 1.8 \times 10^5 \), and \( 1.0 \times 10^5 \) MDSC and \( 2.3 \times 10^5 \), \( 8 \times 10^4 \), and \( 5 \times 10^4 \) Treg in TILs derived from 3LL/Fas-WT, 3LL, or 3LL/Fas-DN in C57BL/6 mice, respectively. Similar to that in 3LL/Fas-DN from C57BL/6 mice, the absolute numbers of MDSC and Tregs were \( \sim 1.2 \times 10^5 \) MDSC and \( 5.5 \times 10^4 \) Tregs in TILs isolated from 3LL/Fas-WT, 3LL, or 3LL/Fas-DN in C57BL/6 mice, respectively.

To confirm above results, we isolated TIL from the tumors formed 14 days later by inoculation with 3LL/Fas-WT, 3LL, or 3LL/Fas-DN into C57BL/6 mice and FasL-deficient gld mice, and analyzed the ratio and the absolute numbers of CD11b+ Gr1+ MDSC in TIL and Foxp3+ Treg cells in CD4+ T cells by FACS. The average ratio of MDSC in TIL isolated from 3LL/Fas-WT, 3LL, and 3LL/Fas-DN tumors were 32.8, 24.5, and 18.8%, respectively, and the average ratio of Foxp3+ T cells in CD4+ T cells were 13.5, 10.2, and 7.8%, respectively in C57BL/6 mice (Fig. 2B). We also counted the absolute numbers of MDSC (Gr1+ CD11b+) and Tregs (CD4+ Foxp3+) in TIL. There were \( \sim 5.3 \times 10^5 \), \( 1.8 \times 10^5 \), and \( 1.0 \times 10^5 \) MDSC and \( 2.3 \times 10^5 \), \( 8 \times 10^4 \), and \( 5 \times 10^4 \) Treg in TILs derived from 3LL/Fas-WT, 3LL, or 3LL/Fas-DN in C57BL/6 mice, respectively. Similar to that in 3LL/Fas-DN from C57BL/6 mice, the absolute numbers of MDSC and Tregs were \( \sim 1.2 \times 10^5 \) MDSC and \( 5.5 \times 10^4 \) Tregs in TILs isolated from 3LL/Fas-WT, 3LL, or 3LL/Fas-DN in C57BL/6 mice, respectively. Therefore, more MDSC and Tregs were accumulated in 3LL/Fas-WT tumor tissue, but not in tumors in gld mice (Fig. 2D). Together with above data, we conclude that Fas signal in lung cancer cells can recruit more MDSC and Foxp3+ Treg cells into tumor tissues.

MDSC have been shown to be expanded rapidly in the tumor-bearing host. Considering that MDSC can induce generation of Treg cells (24) and the Treg accumulation in the Fas-overexpressing tumor was later than MDSC accumulation as observed above,
we wondered whether the rapid accumulation of MDSC could induce subsequent accumulation of Treg cells in the Fas-overexpressing tumor. By in vivo depleting Gr-1<sup>+</sup> cells in the mice bearing Fas-overexpressing tumor, we found the number of tumor-infiltrating Treg cells significantly decreased (Fig. 2E), consistent with the observations of the reduced tumor growth and prolonged survival of tumor-bearing mice (Fig. 2, F and G).

**Fas-ligated lung cancer cells chemoattract MDSC but not Treg cells in vitro**

As more accumulation of MDSC and Treg cells was observed in Fas-overexpressing tumor, we examined whether Fas-ligated 3LL lung cancer cells can chemoattract more MDSC or Treg cells in vitro. For parental 3LL cells, we found the supernatant of Jo2-stimulated-parental 3LL cells was more effective in chemoattracting CD11b<sup>+</sup>Gr1<sup>+</sup> MDSC than the control supernatant derived from isotype-treated parental 3LL cells, but did not chemoattract more CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells than controls (Fig. 3A). However, for 3LL/Fas-DN cells, Fas ligation did not enhance the chemoattraction effect of the supernatants on MDSC (Fig. 3B). Furthermore, compared with the supernatants from parental 3LL cells and 3LL/Fas-DN cells, supernatant derived from 3LL/Fas-WT cells exhibited more potent ability to chemoattract MDSC (Fig. 3C). So, Fas signal can enhance the ability of 3LL lung cancer cells to chemoattract MDSC but not Treg cells.

**PGE<sub>2</sub> derived from the Fas-ligated lung cancer cells contributes to the enhanced chemoattraction of MDSC**

Then, we went further to look for which factor(s) derived from Fas-ligated 3LL lung cancer cells responsible for the enhanced chemoattraction for MDSC. As reported, Fas signaling is associated with inflammation by increasing chemokine secretion and chemoattracting inflammatory cells (25, 26). VEGF and MCP-1 have been shown to be crucial for MDSC migration (27, 28). So, we detected the expression of VEGF and MCP-1 in the supernatant of 3LL lung cancer cells stimulated by Jo2, however, no increase of MCP-1 and VEGF secretion was found (data not shown). Then, we detected the proinflammatory cytokines IL-1β, IL-10, TGF-β, and PGE<sub>2</sub> in the supernatant of Jo2-stimulated 3LL lung cancer cells. No secretion of IL-1β, IL-10, in 3LL cells and no significant increase of TGF-β were observed in Fas-ligated 3LL cells (data not shown). As shown in Fig. 4A, we found that ligation could significantly induce 3LL lung cancer cells to produce PGE<sub>2</sub> in a dose- and time-dependent manner. Accordingly, Fas ligation also significantly increased COX-2 expression in 3LL cells (Fig. 4B).

As PGE<sub>2</sub> can promote migration of cells including DC (29, 30) and endothelial cells (31). Then we tested whether the increased production of PGE<sub>2</sub> by Fas-ligated cancer cells was responsible for the increased chemoattraction of MDSC. We found that the anti-PGE<sub>2</sub> Ab profoundly reduced the chemoattraction of MDSC by the supernatant of Fas-ligated 3LL lung cancer cells (Fig. 4C). Furthermore, COX-2 selective inhibitor SC-58125 (32) was used to treat the Fas-ligated 3LL cancer cells, then we found the increased chemoattraction of MDSC by Fas-ligated 3LL cancer cells was also abrogated (Fig. 4D). To further confirm the conclusion in vivo, we detected COX-2 expression in tumor tissues formed by 3LL/Fas-WT and 3LL/Fas-DN, and found that COX-2 expression in 3LL/Fas-WT tumor tissue was higher than that in 3LL/Fas-DN tumor tissue (data not shown). In vivo i.p. injection of COX-2 inhibitor SC58125 significantly reduced accumulation of MDSC in the tumor tissue of 3LL/Fas-WT-bearing mice (Fig. 4E) and inhibited tumor growth and prolonged survival of tumor-bearing mice (data not shown). Furthermore, we analyzed the apoptosis of 3LL cells stimulated with Jo2 in different concentrations and found

**FIGURE 5.** Fas signal increases PGE<sub>2</sub> production in lung cancer cells through activation of p38 signaling pathway. A, Western blot analysis of MAPK and NF-kB pathways in 3LL cells cultured in medium alone (Ctrl) or stimulated with Jo2, isotype Ab (ISO) for the indicated times. B, p38 signaling pathway is responsible for the Jo2-induced PGE<sub>2</sub> production. 3LL cells were pretreated with NF-kB inhibitor pyrrolidine dithiocarbamate, JNK/SAPK inhibitor SP600125, p38 MAPK inhibitor SB203580, and MER1/2 inhibitor U0126, respectively, for 30 min before Jo2 stimulation. PGE<sub>2</sub> expression was detected 24 h later by ELISA. Results were showed as mean value ± SD of the data obtained from three independent experiments. ***, p < 0.01.

Jo2 combined with SC58125 (70 nM, 700 nM) did not induce 3LL cell apoptosis, thus excluding the possibility that cell death induction may account for the abrogation (Fig. 4F). These data suggest that Fas signal can induce lung cancer cells to secrete more PGE<sub>2</sub>, which in turn contributes to the enhanced chemoattraction of MDSC by Fas-ligated lung cancer cells.

The biological effects of PGE<sub>2</sub> in the target cells are mediated by four different G protein-coupled receptor subtypes, EP1, EP2, EP3, and EP4 (33). Of PGE<sub>2</sub> receptors, EP2 and EP4 were known to be essential for PGE<sub>2</sub>-mediated cell migration (30, 31), then we detected EP2/EP4 expression in MDSC and 3LL cells. RT-PCR results showed that EP2/EP4 were expressed in both MDSC and 3LL cells, especially highly expressed in MDSC (Fig. 4G).

**Fas-activated p38 signaling pathway is responsible for the increased PGE<sub>2</sub> production by Fas-ligated lung cancer cells**

Finally, we wanted to know which pathway(s) is responsible for the increased COX-2 expression and PGE<sub>2</sub> production in Fas-ligated lung cancer cells. Activation of MAPK and NF-κB pathways has been shown to contribute to the Fas-mediated proinflammatory factor production (34, 35) and to be associated with tumor growth and invasion (20). As shown in Fig. 5, Jo2 stimulation can activate the ERK, p38, and NF-κB pathways in 3LL lung cancer cells (Fig. 5A). To elucidate which pathway(s) was essential for the increased production of PGE<sub>2</sub> by Fas ligation, specific inhibitors for signaling pathways were used to pretreat 3LL lung cancer cells before Jo2 stimulation. We found p38 MAPK-specific inhibitor SB203580 could markedly suppress the Jo2-induced PGE<sub>2</sub> production (Fig. 5B), indicating Fas-activated p38 MAPK signaling
pathway is responsible for the increased PGE$_2$ production by Fas-ligated lung cancer cells.

**Discussion**

Fas (CD95/APO-1) belongs to the TNF receptor superfamily and is expressed in a variety of normal and neoplastic cells (14). Triggering of Fas signaling by FasL or agonistic Ab Jo2 results in rapid induction of apoptosis in susceptible cells. Although, other than apoptosis induction, Fas also can transduce activating signal and induce cell proliferation, differentiation, and inflammation (17–20, 36, 37). Hepatocytes are very sensitive to apoptosis induction by agonistic Ab against Fas, however, in vivo administration of agonistic Ab against Fas after partial hepatectomy could promote the proliferation of mouse hepatocytes and contribute to the liver regeneration and healing (38). In addition, similar phenomena have been observed in neurite outgrowth and neuron regeneration following injury (39). It is thus evident that Fas signaling can promote the cell growth in some tissues and conditions. For tumor cells, previous studies demonstrate Fas activation promotes tumor growth in vitro, and their promoting effect results from the direct influence of Fas on cancer cells (17–20). In this study, we demonstrate for the first time that Fas signal promotes lung cancer growth in vivo by enhancing the MDSC and Treg accumulation in tumor, contributing to the immune escape of lung cancer.

Cancer can grow and progress by disrupting the surveillance of host immune system and escaping from the attack of immune cells. During this progress, induction of MDSC and Treg generation, triggering of MDSC and Treg accumulation in tumor microenvironment are tightly associated with tumor progression. In our study, we found Fas signal in lung cancer can promote the accumulation of MDSC and Foxp3$^+$ Treg cells in tumor tissues, and depletion of MDSC in vivo reduces tumor growth and prolongs survival of 3LL/Fas-WT-bearing mice, indicating that the accumulation of MDSC and Foxp3$^+$ Treg cells may contribute to Fas signal-induced lung cancer growth in vivo. Immunoochemical staining showed that MDSC appeared in the tumor 7 days after 3LL inoculation, while no Foxp3$^+$ Treg cells was found until 14 days later. Jo2 stimulation enhanced the chemotraction for MDSC but not Tregs in vitro, suggesting that MDSC were recruited to the tumor tissues earlier than Treg cells and then may facilitate the differentiation of Treg cells from the T lymphocyte precursor. MDSC have been shown to be able to mediate Treg cell development in tumor-bearing host (24). Depleting MDSC by anti-Gr1$^+$ Ab could decrease the Treg infiltration in Fas-overexpressing tumors confirmed that the increased accumulation of Treg cells in tumors by Fas signal may be due to the secondary effect of MDSC on Treg cell induction.

Tumors consistently produce the so-called tumor derived mediators and mobilize and induce MDSC and Treg accumulation in tumor tissue. We investigated which soluble mediator(s) is responsible for the Fas signal-mediated accumulation of MDSC and Foxp3$^+$ Treg cells in tumors. By screening the immunosuppressive and proinflammatory factors derived from tumor cells, we found Fas-ligated 3LL cells produce higher level of PGE$_2$, which was shown to subsequently facilitate migration of MDSC, just as described in the previous observation that PGE$_2$ can promote the MDSC accumulation in tumor (40). In vivo blockade of COX-2/PGE$_2$ with SC58125 not only decreased MDSC and Treg accumulation in 3LL/Fas-WT tumor tissues, but also reduced the tumor growth and prolonged the survival of 3LL/Fas-WT-bearing mice, thus further indicating that PGE$_2$ plays an important role in the promotion of 3LL lung cancer growth by Fas signal.

PGE$_2$ receptors EP2 and EP4 are involved in the PGE$_2$-mediated DC migration (30, 31). We confirmed that both EP2 and EP4 were expressed in MDSC by RT-PCR, also EP2/EP4 were expressed in 3LL cells. So, we predict that PGE$_2$ may promote chemotraction for MDSC by Fas-ligated 3LL cells through EP2/EP4 via different mechanism such as up-regulation of chemokine receptor expression (29), activation of migration signal (41) and enhancement of MMP9 expression (42). There is also possibility that autocrine PGE$_2$ may also induce 3LL cells to produce chemotactic factors which may also contribute to the increased chemotraction for MDSC by Fas-ligated 3LL cells. Whether or not such possibility exists needs to be investigated in the future.

Tumor-derived PGE$_2$ has been proved to play an essential role in tumorigenesis and progression. PGE$_2$ can activate signaling pathways controlling cell proliferation, migration, apoptosis, and angiogenesis (43). More importantly, PGE$_2$ is involved in the suppression of host immune response (44–46), for example, 3LL cell-derived PGE$_2$ was required for increased expression of Arginase I in MDSC (44), Foxp3 expression in Treg cells (45), and IDO expression in DC (46). Systemic administration of COX-2 selective inhibitor can markedly inhibit tumor growth and MDSC infiltration in tumors (40). In our study, we found Fas signals promote PGE$_2$ production in 3LL, and tumor-derived PGE$_2$ is required for MDSC chemotraction, providing new mechanistic explanation for inflammation and tumor immune escape. Our results also further confirm that blockade of COX-2/PGE$_2$ is a powerful strategy to the design of new approaches to cancer treatment.

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**Disclosures**

The authors have no financial conflict of interest.

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