Blockade of Fas Signaling in Breast Cancer Cells Suppresses Tumor Growth and Metastasis via Disruption of Fas Signaling-initiated Cancer-related Inflammation

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Background: The non-apoptotic functions of Fas signaling have been proposed to play an important role in promoting tumor progression.

Results: Blockade of Fas signaling suppresses tumor growth and metastasis via disruption of Fas signaling-initiated cancer-related inflammation.

Conclusion: Fas signaling-initiated cancer-related inflammation in breast cancer cells may be a potential target for cancer treatment.

Significance: This study provides mechanistic insight into the role of Fas signaling in cancer-related inflammation.

Mechanisms for cancer-related inflammation remain to be fully elucidated. Non-apoptotic functions of Fas signaling have been proposed to play an important role in promoting tumor progression. It has yet to be determined if targeting Fas signaling can control tumor progression through suppression of cancer-related inflammation. In the current study we found that breast cancer cells with constitutive Fas expression were resistant to apoptosis induction by agonistic anti-Fas antibody (Jo2) ligation or Fas ligand cross-linking. Higher expression of Fas in human breast cancer tissue has been significantly correlated with poorer prognosis in breast cancer patients. To determine whether blockade of Fas signaling in breast cancer could suppress tumor progression, we prepared an orthotopic xenograft mouse model with mammary cancers cells 4T1 and found that blockade of Fas signaling in 4T1 cancer cells markedly reduced tumor growth, inhibited tumor metastasis in vivo, and prolonged survival of tumor-bearing mice. Mechanistically, blockade of Fas signaling in cancer cells significantly decreased systemic or local recruitment of myeloid derived suppressor cells (MDSCs) in vivo. Furthermore, blockade of Fas signaling markedly reduced IL-6, prostaglandin E2 production from breast cancer cells by impairing p-p38, and activity of the NFκB pathway. In addition, administration of a COX-2 inhibitor and anti-IL-6 antibody significantly reduced MDSC accumulation in vivo. Therefore, blockade of Fas signaling can suppress breast cancer progression by inhibiting proinflammatory cytokine production and MDSC accumulation, indicating that Fas signaling-initiated cancer-related inflammation in breast cancer cells may be a potential target for treatment of breast cancer.

Fas (also known as CD95/Apo-1) is a transmembrane protein belonging to the tumor necrosis factor (TNF) receptor superfamily known to transmit an apoptotic signal in susceptible cells once triggered by its natural ligand, FasL. Fas is widely expressed in normal and neoplastic tissues. Fas-mediated apoptosis plays an important role in various biological processes, including activation-induced cell death (AICD), T-cell-induced cytotoxicity, immune privilege, and tumor surveillance (1). However, accumulating evidence suggests that activation of the Fas signal cannot only induce apoptosis but can also mediate a variety of non-apoptotic activities especially during tumorigenesis and tumor progression in Fas-resistant tumor cells (2–4). First, the Fas signal has a growth-promoting role during tumorigenesis, evidenced by the observation that a loss of Fas in mouse models of liver cancer and ovarian cancer reduces cancer incidence as well as the size of the tumor (5). Second, the Fas signal is proposed to convert from tumor suppressor to tumor promoter (6, 7), directly promoting apoptosis-resistant cancer cell growth and invasion (8–14). Additionally, Fas signal activation can induce secretion of proinflammatory cytokines and chemokines in different cell lines and tissues (15–18) and, consequently, can recruit peripheral myeloid cells into the inflammatory site (19). Our previous studies showed that Fas signaling could promote lung cancer growth by recruiting myeloid-derived suppressor cells (MDSCs) in vivo (20), and the bioactive
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MATERIALS AND METHODS

Mice, Cell Lines, and Reagents—Female BALB/c mice (6–8 weeks) were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. Mouse mammary cancer cell lines 4T1 and EMT6 (derived from BALB/c origin), murine embryonic liver cells (BNL CL.2), and human breast cancer cell line MCF-7 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI1640 complete medium (PAA Laboratories, Linz, Austria) supplemented with 10% FCS (PAA Laboratories) at 37 °C in a 5% CO2 atmosphere. ELISA kits for murine IL-6, G-CSF, M-CSF, vascular endothelial growth factor (VEGF), MCP-1, CXCL2, CXCL1, TNF-α, PGE2, IL-1β, the recombinant mouse Fas ligand (6128-SA), cross-liking Ab mouse anti-HA (AB6060), the recombinant human Fas ligand, polyhistidine antibody, and neutralizing Ab to IL-6 were obtained from R&D Systems (Minneapolis, MN). Fluorescein-conjugated mAbs to CD3, CD4, CD8, CXCR3, CD11b, Gr1, and isotype control mAbs were from eBioscience (San Diego, CA). Purified mouse anti-human CD11b/Mac-1, anti-mouse CD95 (Io-2), rat anti-mouse CD8α, rat anti-mouse CD4, rat anti-mouse Gr1, and Alexa Fluor 488 rabbit anti-rat IgG were obtained from BD Pharmingen. 2-(4-Aminophenyl)-6-indolecarbamidine dihydrochloride was from Molecular Probes (Leiden, The Netherlands). Purified mouse anti-CD95 was obtained from BD Transduction Laboratories™. The DAKO Envision System (Hamburg, Germany) was used for immunohistochemistry (IHC). SC58125, a specific inhibitor of COX2, was from Calbiochem. 3,3-Diaminobenzidine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma. Phospho-Abs against extracellular signal-regulated kinase p44/p42 (ERK1/2, Thr-202/Tyr-204), c-Jun N-terminal kinase/stress-associated protein kinase (JNK/SAPK, Thr-183/Tyr-185), p38 (Thr-180/Tyr-182), JNK, SB203580, an inhibitor of p38 MAPK, and U0126, an inhibitor ERR MAPK, were obtained from Calbiochem. Cholesterol-conjugated Fas siRNA for in vivo RNA delivery and negative control siRNA were from Ribobio Co., Ltd (Guangzhou, China). Human breast cancer tissue microarrays (HBre-Duc159sur-01, Cohort1; HBre-Duc060CS-1, Cohort2) were from Shanghai Outdo Biotech Co., Ltd (Shanghai, China).

Preparation of Stably Transfected 4T1 Cell Clones with Over-expressed or Silenced Fas—TA Cloning Expression Vectors encoding full-length wild-type Fas (Fas-WT) or dominant negative Fas (Fas-DN), which does not contain the intracellular signaling domain of Fas, were kindly provided by Dr. J.-K. Lee (Seoul, Korea). The vectors Fas-WT and Fas-DN were confirmed by sequencing. The vectors were transfected into 4T1 cells, and the transfected 4T1 cells were selected in 800 μg/ml carbamoyl (FasL) released by activated T cells in exosomes could promote melanoma and lung cancer cell metastasis through Fas signaling (21). Furthermore, Fas ligation promotes not only survival but also an increase in maturation of dendritic cells (DCs) and induces DCs to rapidly produce both CXC and CC chemokines, which results in enhanced chemoattraction of neutrophils and T cells in vivo or in vitro (22, 23). In some instances cytokines and chemokines can recruit antitumor immune effector cells so as to control tumor progression (24, 25); however, cytokines and chemokines in the tumor microenvironment usually recruit immunosuppressive cells to help tumor escape immunological attack and promote angiogenesis (20–21, 26). Therefore, targeting Fas signaling or Fas signal-initiated cancer-related inflammation may be useful as a cancer therapeutic, which needs to be explored further (27).

Cancer-related inflammation has many tumor-promoting effects, promoting cancer cell proliferation and survival, facilitating angiogenesis and cancer metastasis, and suppressing immune responses against cancer (28). Increasing evidence demonstrates that proinflammatory cytokines and immunosuppressive cells are crucial for cancer-related inflammation, which can directly or indirectly promote tumor development and progression (29, 30). MDSCs are characterized by the expression of Gr1 and CD11b, which represent a heterogeneous population of immunosuppressive, incompletely differentiated myeloid progenitor cells originally identified in tumor-bearing mice (31). Various tumor-derived cytokines are found to drive MDSC mobilization and recruitment from bone marrow into peripheral blood, spleen, lymph nodes, and tumor tissue of tumor-bearing animals and patients (32) where MDSCs inhibit innate and adaptive immunity, promote tumor immune escape, facilitate tumor metastasis, and even contribute to tumor angiogenesis by directly incorporating into the tumor endothelium (33–35). An increase in circulating MDSCs correlates with clinical cancer stage and metastatic tumor burden, confirming that targeting MDSCs may be a potential mechanism for cancer treatment (36).

To determine whether Fas signal-mediated inflammation is involved in promoting cancer progression and whether blockade of Fas signaling can inhibit cancer progression by suppressing cancer-related inflammation, we investigated the effect of Fas signal on breast cancer growth and metastasis. Using an orthotopic xenograft breast cancer mouse model, we found that Fas signaling in breast cancer cells could promote breast cancer progression by enhancing cancer-related inflammation including increased production of IL-6 and PGE2 and recruitment of MDSCs. Accordingly, blockade of Fas signaling in breast cancer cells or tumor tissue could significantly reduce primary tumor growth, inhibit tumor metastasis, and prolong the survival of tumor-bearing mice. Furthermore, clinical data showed that higher expression of Fas in human breast cancer tissue significantly correlated with poorer prognosis of breast cancer patients. Therefore, our data suggest that blockade of Fas signaling-initiated cancer-related inflammation may be a useful approach for the treatment of breast cancer.
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Geneticin for 14 days. The resistant 4T1 clones were selected by limiting dilution, and the stably transfected 4T1 clones that expressed a high level of full-length or dominant negative Fas were characterized by flow cytometry and Western blot. The 4T1 cell clone expressing a high level of Fas-WT was designated as 4T1/Fas-WT, and the clone expressing a high level of Fas-DN was designated as 4T1/Fas-DN. The shRNA plasmid vector (pGPU6/GFP/Neo) of the mouse Fas gene (Fas shRNA-1 (+109), Fas shRNA-2 (+364), and Fas shRNA-3 (+802)) and negative control shRNA plasmid vector (pGPU6/GFP/Neo) were designed and synthesized by Gene Pharma Co. (Shanghai, China). The vectors were transfected into 4T1 cells using jetPEI (Illkirch, France) and then selected in 800 μg/ml Geneticin. The resistant GFP+ 4T1 clones were sorted by flow cytometry. The expression of Fas in stable GFP+ 4T1 cell clones was further confirmed by flow cytometry and Western blot as previously described (37). Fas-silenced 4T1 cell clones were designated as 4T1/Fas shRNA, and the negative control 4T1 cell clone was designated as 4T1/NC.

Detection of Cell Apoptosis—For the induction of cell apoptosis, 4T1, EMT6, or BNL CL.2 cells were incubated with or without Jo-2 or recombinant mouse Fas ligand/TNFSF6 at the indicated concentrations in the presence of 2.5 μg/ml cross-linking Ab mouse anti-HA for 24 h. Apoptotic cells were stained with FITC-annexin V (BD Pharmingen) and propidium iodide (Sigma) according to the manufacturer’s instructions and examined by flow cytometry. The data were analyzed using CellQuest software (BD Biosciences) as previously described (38).

Assay for Cytokines—MCP-1, VEGF, CXCL1, CXCL2, G-CSF, M-CSF, TNF-α, IL-1β, PGE2, and IL-6 in the supernatants of 4T1, 4T1/Fas-WT, and 4T1/Fas-DN cells stimulated with or without Jo-2 antibody were assayed using ELISA kits according to the manufacturer’s instructions and examined by flow cytometry. The data were analyzed using CellQuest software (BD Biosciences) as previously described (38).

Reverse-transcription PCR and Real-time PCR—Gr1+ CD11b+ MDSCs were sorted by flow cytometry (BD FACS Vantage) from primary tumor tissue, including liver, lung, peripheral blood, and spleen derived from 4T1-bearing mice after subcutaneous inoculation into the flank of BALB/c mice for 40 days. Total cellular RNA was extracted using TRIzol reagent (Invitrogen). 2 μg of total RNA was used in a 20-μl reverse transcription reaction using the First Strand cDNA Synthesis kit (Toyobo); the cDNA was then diluted into 160 μl as the template for the next quantitative PCR. Real-time qRT-PCR assay was performed as previously described (37). For evaluation of Fas, IL-6, COX2, EP2, EP4, and IL-6Rα mRNA expression, the primers used for PCR amplification were 5′-TAT CAA GGA GGC CCA TTT TGC-3′ and 5′-TGT TTC CAT CCC TAC TTC TAA ACC AGC CTG-3′ for Fas, 5′-TAG GCC ATC TCC CAA CTA CTT CC-3′ and 5′-TGG GGC AGC AAC TCC TCG GCC-3′ for IL-6, 5′-TTC AAC ACA CTC TAT CAC TGG C-3′ and 5′-AGA AGC GGT TGC GGT ACT CAT-3′ for COX2, 5′-GGA GGA GCG CAA GAG GAC TCG TC-3′ and 5′-GAC GGG ATG ATG AGC CCC AGA ACC-3′ for EP2, 5′-ACC ATT CCT AGC TCG AAC CGT-3′ and 5′-CAC CCC CCC CAA GAT GAA CAT-3′ for EP4, and 5′-CCT GAG ACT CAA GCA GAA ATG G-3′ and 5′-AGA AGG AAG GTC GGC TTC AGT-3′ for CD126 (IL-6Rα).

Preparation and Observation of Tumor-bearing Mice—5 × 105 tumor cells suspended in 200 μl of Hanks’ balanced salt solution were subcutaneously (s.c.) inoculated into the flank (4th mammary gland) of BALB/c mice. The tumor mass was measured with a caliper after tumor inoculation every 2–3 days, and tumor volume was determined by measuring the maximal (a) and minimal (b) diameters using a caliper and calculated by using the formula a × b^2/2. Survival of the tumor-bearing mice was monitored daily as previously described (38). In some experiments, 40 days after inoculation with 4T1/NC and 4T1/ΔFas, mice were humanely killed by cervical dislocation; the lung, liver, and draining lymph node (DLN) were photographed and resected, fixed in formalin, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E). Tumor metastasis to the lung, liver, and DLN was detected by H&E staining. The percentage of mice that developed primary tumor metastasis to a distant organ (liver, lung, and DLN) was calculated as incidence of metastasis (%) = numbers of mice that developed distant organ metastasis/numbers of mice injected with a primary tumor × 100%. Experiments were performed independently three times, and each group contained at least eight mice.

Flow Cytometry or Immunofluorescence of T Cells and MDSCs in Tumor-bearing Mice—Peripheral blood lymphocytes, splenocytes, and tumor-infiltrating lymphocytes were isolated for analysis of the percentage and number of Gr1+CD11b+ MDSCs, CD3+CD4+CXCR3+ T cells, and CD3+CD8+ T cells in peripheral blood, spleen, and tumor-infiltrating lymphocytes 14 days after inoculation with parental 4T1, 4T1/Fas-WT, and 4T1/Fas-DN, as previously described (20). Then 1 × 106 splenocytes, peripheral blood lymphocytes, and tumor-infiltrating lymphocytes were stained with FITC-conjugated CD11b in combination with PE-conjugated Gr1 for MDSCs analysis, FITC-conjugated CD3 with PE-Cy5-conjugated CD4, and APC-conjugated CXCR3+ for activated CD4+ T cells analysis and FITC-conjugated CD3 with PerC-conjugated CD8, for CD8+ T cells analysis by flow cytometry. In some experiments 5 × 105 4T1 cells were inoculated s.c. into BALB/c mice, and 125 μg of anti-IL-6 Ab or isotype control (mouse IgG) or COX2 inhibitor SC58125 (5 mg/kg) or PBS were injected intraperitoneally once a day for 14 days. Then the cells were stained with FITC-CD11b and PE-Gr1 for MDSCs and FITC-CD3, PE-Cy5-CD4, PerC-CD8, and APC-CXCR3 for CD3+CD4+CXCR3+ T cells and CD3+CD8+ T cells and analyzed by flow cytometry. Gr1+ MDSCs in tumor tissue, liver, lung, and DLN from the tumor-bearing mice were analyzed further by immunofluorescence staining. Tumor mass was measured with a caliper after tumor inoculation every 2 days, and tumor volume was determined by measuring the maximal (a) and minimal (b) diameters using a caliper and calculated by using the formula: a × b^2/2.

IHC—Paraffin-embedded tumor tissue sections from tumor-bearing mice or human breast cancer tissue microarrays were analyzed by IHC staining using the DAKO Envision System. Gr1, Fas, and COX2 antibodies were dilution 1:50, 1:200, and 1:50, respectively. Rabbit polyclonal IgG (DAKO) replaced the primary antibody and was used as the negative control. Digital imaging was performed using the software LAS V3.7 (Leica DM
Cox proportional hazard regression was conducted using SPSS. Measurements were performed independently three times. Control for detecting apoptosis induction (body, Jo2, or cross-linked Fas ligand) were used as a positive control. Embryonic liver cells (BNL CL.2) treated with anti-Fas antibody suppressed cancer growth and metastasis in vivo (40). Briefly, the cells were lysed, and protein concentration was determined by the BCA Protein Assay kit (Pierce). Cell lysates were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were then blotted with the indicated antibodies. Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate, as instructed by the manufacturer (Pierce).

**Western Blot Analysis**—Western blot was performed as previously described (40). Briefly, the cells were lysed, and protein concentration was determined by the BCA Protein Assay kit (Pierce). Cell lysates were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were then blotted with the indicated antibodies. Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate, as instructed by the manufacturer (Pierce).

**In Vivo Assay**—5×10^5 4T1 tumor cells suspended in 200 μl of Hanks’ balanced salt solution were inoculated s.c. into the flank (4th mammary gland) of BALB/c mice; 10 days after tumor inoculation, cholesterol-conjugated Fas gene siRNA (Fas siRNA), cholesterol-conjugated negative control siRNA (Ctrl siRNA) (10 nmol of RNA in 0.1 ml saline buffer), or PBS was injected intratumorally once every 3 days for 2 weeks as previously described (38). Tumor volume and tumor metastasis to the lung, liver, and DLN after tumor inoculation for 40 days were determined as described above.

**Statistical Analysis**—Results are provided as the mean ± S.E. or S.D. The comparison of mean values between groups was determined by Student’s t test. Statistical analysis of survival data were performed by the Kaplan–Meier method and analyzed using the log-rank test. p values <0.05 were considered statistically significant. Analysis of univariate or multivariate Cox proportional hazard regression was conducted using SPSS 17.0 with the hazard ratios and p values indicated. All experiments were performed independently three times.

**RESULTS**

Blockade of Fas Signaling in Mouse Mammary Cancer Cells Suppresses Cancer Growth and Metastasis in Vivo—Fas is found constitutively expressed in mouse (4T1 and EMT6) and human (MCF-7) breast cancer cells (Fig. 1A). When stimulated with agonistic anti-Fas antibody, Jo2, or cross-linked with Fas ligand, no significant apoptotic cells were observed in 4T1 (Fig. 1B) or EMT6 cells (data not shown). Simultaneously, murine embryonic liver cells (BNL CL.2) treated with anti-Fas antibody, Jo2, or cross-linked Fas ligand were used as a positive control for detecting apoptosis induction (supplemental Fig. 1F). These data indicate that breast cancer cells are resistant to Fas signal-induced apoptosis, which is consistent with previous reports (41). Next, it was determined whether Fas signaling could affect growth in Fas signal/apoptosis-resistant 4T1 cells. The stably transfected 4T1 clones expressing dominant negative Fas (4T1/Fas-DN) or full-length Fas (4T1/Fas-WT) (Fig. 1C and D) were observed for their proliferation in vitro after stimulation with or without Jo2; no difference was observed (data not shown), indicating that activation or blockade of Fas signaling does not significantly accelerate 4T1 breast cancer cell growth in vitro. To determine whether Fas signaling could accelerate breast cancer progression in vivo, parental 4T1, 4T1/Fas-DN, or 4T1/Fas-WT cells were inoculated s.c. into BALB/c mice. In vivo growth of 4T1/Fas-DN was reduced significantly when compared with that of parental 4T1 cells or 4T1/Fas-WT cells (Fig. 1E). Accordingly, the survival of mice bearing 4T1/Fas-DN cells was significantly prolonged compared with that of mice bearing parental 4T1 cells or 4T1/Fas-WT cells (Fig. 1F).

Furthermore, we investigated whether silencing Fas expression in 4T1 cells could inhibit tumor growth in vivo. The shRNA plasmid (pGPU6/GFP/Neo) of mouse Fas (Fas shRNA-1(+109), Fas shRNA-2(+364), or Fas shRNA-3(-802)) gene was transfected into 4T1 cells and screened with 800 μg/ml Geneticin. The resistant GFP^+ 4T1 cells were sorted by flow cytometry. Down-regulation of Fas expression in stable GFP^+ 4T1 cell clones was detected by Western blot and FACS. Fas expression in 4T1/Fas shRNA-2 cell clones decreased by nearly 70% compared with the 4T1 clones stably transfected with negative control plasmid (4T1/NC) (supplemental Fig. 2, A and B), confirming the down-regulated expression of Fas shRNA-2 transfection. Therefore, the stably transfected 4T1/Fas shRNA-2 cell clones were selected as 4T1/Fas shRNA cell clones in all subsequent experiments. 4T1/Fas shRNA, 4T1/NC, or parental 4T1 cells were then inoculated s.c. into the flank (fourth mammary gland) of BALB/c mice to examine primary tumor growth, tumor metastasis, and survival of tumor-bearing mice. As shown in Figs. 1, G and H, and 3A, primary tumor growth was significantly reduced, and tumor metastasis to the DLN, lung, and liver was markedly decreased in the mice bearing Fas-silenced 4T1 cells as compared with those bearing control 4T1 cells. Accordingly, survival of mice bearing Fas-silenced 4T1 cells was also significantly prolonged compared with that of mice bearing control 4T1 cells (Fig. 1I). Fas expression in tumor tissue derived from mice bearing Fas-silenced 4T1 cells significantly decreased when compared with that of mice bearing parental 4T1 cells or control 4T1 cells (supplemental Fig. 2C). Collectively, these results indicate that breast cancer cells are resistant to Fas signal-induced apoptosis and that blockade of Fas signaling in breast cancer cells can suppress breast cancer growth and metastasis in vivo.

Blockade of Fas Signaling in Breast Cancer Cells Reduces Systemic and Local Mobilization and Accumulation of MDSCs in Tumor-bearing Mice—Next, we investigated the mechanisms responsible for blockade of Fas signaling in 4T1 cells and if they could reduce tumor growth in vivo. We also sought to determine if cancer-related inflammation, such as the induction of proinflammatory cytokines or accumulation of MDSCs, is involved in this process? It is well known that mobilization and accumulation of MDSCs in the tumor microenvironment is closely associated with tumor progression and restraints tumor immunity (33–36). We determined that the ratio and absolute number of MDSCs markedly decreased in spleen, peripheral blood, and tumor tissues in mice bearing 4T1/Fas-DN cells compared with those bearing parental 4T1 cells or 4T1/Fas-WT cells (Fig. 2, A–C). Furthermore, we detected MDSC infiltration in tumor tissues derived from tumor-bearing mice or human breast cancer tissues by IHC through staining of Gr1.
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(supplemental Fig. 3A) or CD11b (supplemental Fig. 3B), respectively, and observed increased infiltration of MDSCs in tumor tissues that expressed higher levels of Fas.

Furthermore, we analyzed the recruitment of Gr1+ MDSCs in primary tumor tissue, liver, lung, and DLN from mice 40 days after inoculation with 4T1/NC and 4T1/siFas cells. For control 4T1 cells-bearing mice, the primary tumor or metastatic tumor sites (liver, lung, and DLN) contained abundant Gr1+ MDSCs; however, there was less Gr1+ MDSCs infiltration in Fas-silenced 4T1 cell-bearing mice (Fig. 3, A–B). Additionally, compared with the control 4T1 cells-bearing mice, MDSCs in peripheral blood and spleen also decreased significantly in the mice bearing Fas-silenced 4T1 cells (Fig. 3C). Collectively, these data demonstrate that blockade of Fas signaling in breast cancer cells decreases systemic and local mobilization and recruitment of MDSCs in vivo.

FIGURE 1. Blockade of Fas signaling in breast cancer cells suppresses tumor growth and metastasis in vivo. A, Fas expression on mouse and human breast cancer cells was detected by Western blot (upper) and RT-PCR (lower). B, susceptibility of 4T1 cells to Fas-induced apoptosis was measured by staining with annexin V-FITC and propidium iodide (PI) after treatment with or without agonistic anti-Fas antibody, Jo2, or cross-linking Fas ligand at the indicated concentrations for 24 h. C and D, expression of Fas in stably transfected 4T1 cell clones (4T1/Fas-WT and 4T1/Fas-DN) was identified by Western blot assay (C) and flow cytometry (D). Iso, isotype. E and F, 5 × 10^5 4T1/Fas-WT, 4T1/Fas-DN, and parental 4T1 cells were inoculated s.c. into the flank of BALB/c mice, then tumor growth and the survival of tumor-bearing mice were monitored and analyzed as described under “Materials and Methods.” G, H, and I, 5 × 10^5 Fas-silenced 4T1 cell clones (4T1/Fas shRNA), negative control 4T1 cell clone (4T1/NC), and parental 4T1 cells were inoculated s.c. into the flank of BALB/c mice. Tumor growth (G), quantification of the proportion of animals with liver, lung, and DLNs micrometastasis (H), and survival (I) were monitored and analyzed. Data points represent the mean ± S.E. from eight mice per group. Tumor sizes were compared at various time points, and p values are denoted. **, p < 0.01.
Blockade of Fas Signaling in Breast Cancer Cells Increases the Number of Activated T Cells in Tumor-bearing Mice—Next, we analyzed the recruitment of activated CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and NK cells in peripheral blood, spleen, and tumor tissues from tumor-bearing mice (42). The ratio and the absolute number of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup> T cells and CD3<sup>+</sup>CD8<sup>+</sup> T cells significantly increased in peripheral blood, spleen, and tumor tissues in the mice bearing 4T1/Fas-DN cells compared with those in mice bearing parental 4T1 cells or 4T1/Fas-WT cells. However, the ratio and the absolute number of NK cells remained unchanged between all groups (data not shown). Additionally, the ratio of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup> T cells and CD3<sup>+</sup>CD8<sup>+</sup> T cells increased significantly in peripheral blood, spleen, and tumor tissues of mice bearing Fas-silenced 4T1 cells as compared with that in mice bearing control 4T1 cells (Fig. 5, A–C). These results suggest that blockade of Fas signaling in breast cancer cells could increase the number of the activated T cells in tumor-bearing mice. Together, we conclude that blockade of Fas signaling in breast cancer cells can recruit more activated T cells but induce less mobilization and accumulation of MDSCs in tumor-bearing mice both locally (in the tumor microenvironment) and systemically (in the peripheral blood and spleen), benefiting the immune response against cancer.

Blockade of Fas Signaling in Breast Cancer Cells Reduces IL-6 and PGE2 Production and Subsequently Reduces MDSC Accumulation—Fas signaling has been associated with inflammation by increasing cytokine secretion and chemoattraction of inflammatory cells (15–19). Therefore, in the current study, IL-6, G-CSF, M-CSF, VEGF, MCP-1, CXCL2, CXCL1, TNF-α,
PGE2, and IL-1β were detected in the supernatants of 4T1/Fas-DN cells, parental 4T1 cells, or 4T1/Fas-WT cells after stimulation with or without Jo-2. As shown in Fig. 6, A and B, secretion of cytokines IL-6 and PGE2 markedly decreased in 4T1/Fas-DN cells as compared with that in parental 4T1 cells or 4T1/Fas-WT cells after Jo-2 stimulation. No significant difference in the production of other cytokines and chemokines including G-CSF, M-CSF, VEGF, MCP-1, CXCL1, CXCL2, TNF-α, and IL-1β was observed (data not shown). Furthermore, we investigated the signal pathway(s) that may be responsible for the decrease in IL-6 and PGE2 production in 4T1/Fas-DN cells once triggered by the Fas signal. Activation of MAPK and NF-κB pathways has been shown to contribute to proinflammatory cytokine production. As shown in Fig. 6, C–D, Jo2 stimulation could activate the ERK, p-38 MAPK, and NFκB signal pathways in 4T1 cells. To elucidate which pathway(s) was responsible for decreased production of IL-6 and PGE2, specific inhibitors for these signaling pathways were used to pretreat 4T1 cells for 30 min before Jo2 stimulation. p38 MAPK-specific inhibitor SB203580 markedly suppressed IL-6 production (Fig. 6E), p38 MAPK-specific inhibitor SB203580 and NF-κB-specific inhibitor pyrrolidine dithiocarbamate suppressed PGE2 production (Fig. 6F). The blocking effect of these signaling inhibitors was detected via Western blot assay (supplemental Fig. 4). Additionally, to further confirm whether the decrease in IL-6 and PGE2, shown responsible for the reduced expansion and accumulation of MDSCs in vivo, anti-IL-6 neutralizing antibody, or COX-2 inhibitor SC58125, were administered intraperitoneally into the 4T1 cell-bearing mice once a day for 14 days. As shown in Fig. 6G, administration of anti-IL-6 neutralizing antibody reduced MDSC accumulation only in tumor tissue, whereas the COX-2 inhibitor significantly reduced the recruitment of MDSCs in spleen, peripheral blood, and tumor tissue of 4T1-bearing mice (Fig. 6H). Accordingly,
administration of anti-IL-6 neutralizing antibody or COX-2 inhibitor SC58125 significantly inhibited tumor growth in vivo (supplemental Fig. 5).

Additionally, to determine whether tumor-derived IL-6 and PGE2 are key factors in local and systemic mobilization and accumulation of MDSCs in tumor-bearing mice, we detected IL-6, COX2 (PGE2), and expression of their receptors IL-6R/H9251 and EP2/EP4 in tumor tissue and MDSCs sorted from the liver, lung, spleen, peripheral blood, and tumor tissue in 4T1-bearing mice. COX2 was highly expressed in tumor tissue and tumor-infiltrating MDSCs but not in the other MDSCs; IL-6 was highly expressed in tumor tissue and MDSCs except for peripheral MDSCs (supplemental Fig. 6A). PGE2 receptors EP2 and EP4 were highly expressed in MDSCs, and the IL-6 receptor IL-6Ra (CD126) was highly expressed in MDSCs except spleen-derived MDSCs (supplemental Fig. 6B). These data suggest that tumor-derived IL-6 and PGE2 play an important role in MDSC mobilization and accumulation in vivo.

In Vivo Blockade of Fas Signaling Suppresses Tumor Growth and Metastasis in Tumor-bearing Mice—Furthermore, to address whether targeted intra-tumor Fas signaling could inhibit tumor growth, 4T1 tumor cells were inoculated s.c. into the flank of BALB/c mice. 14 days later peripheral blood, spleen, and tumor tissues from normal or tumor-bearing mice were collected, and then 1 × 10^6 cells were stained with FITC-CD3, PE-Cy5-CD4, and APC-CXCR3 for CD3^+ CD4^+ CXCR3^+ T cells and FITC-CD3 and PerCP-CD8 for CD3^+ CD8^+ T cells, respectively. The percentage and number of CD3^+ CD4^+ CXCR3^+ T cells and CD3^+ CD8^+ T cells in peripheral blood (A), spleen (B), and tumor tissues (C) were analyzed by FACS, and the results are represented as the mean value ± S.E. of three independent experiments with similar results. *, p < 0.05; **, p < 0.01.

In Vivo Blockade of Fas Signaling Enhances the Number of Activated T Cells in vivo—A–C, 5 × 10^5 4T1/Fas-WT, 4T1/Fas-DN, and parental 4T1 cells were inoculated s.c. into the flank of BALB/c mice. 14 days later peripheral blood, spleen, and tumor tissues were collected, and then 1 × 10^6 cells were stained with FITC-CD3, PE-Cys-CD4, and APC-CXCR3 for CD3^+ CD4^+ CXCR3^+ T cells and FITC-CD3 and PerCP-CD8 for CD3^+ CD8^+ T cells, respectively. The percentage and number of CD3^+ CD4^+ CXCR3^+ T cells and CD3^+ CD8^+ T cells in peripheral blood (A), spleen (B), and tumor tissues (C) were analyzed by FACS, and the results are represented as the mean value ± S.E. of three independent experiments with similar results. *, p < 0.05; **, p < 0.01.

Higher Expression of Fas in Human Breast Cancer Tissue Is Correlated with Poorer Prognosis in Breast Cancer Patients—Finally, to determine whether Fas is constitutively expressed in human breast cancer tissues and whether Fas expression in human breast cancer tissue is correlated with the survival of breast cancer patients, Fas expression in human breast cancer tissues derived from 119 (Cohort 1, with patient’s survival information) and 30 (Cohort 2) breast cancer patients (supplemental Table 1) was detected by IHC. Increased Fas expression was found in 52.9% (63/119, Cohort 1) and 66.7% (20/30, Cohort 2) of breast cancer patients; one representative image of lower or higher expression of Fas in human breast cancer tissue is shown in Fig. 8A. Furthermore, the correlation between Fas expression and survival in breast cancer patients from Cohort 1 was analyzed by Kaplan-Meier survival analysis of overall survival.
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**FIGURE 5. Silencing of Fas expression in breast cancer cells enhances the number of activated T cells in vivo.** (A–C) 5 × 10^5 4T1/Fas shRNA and 4T1/NC cells were inoculated s.c. into the flank of BALB/c mice. 14 days later, peripheral blood and spleen from tumor-bearing mice were collected and stained with FITC-CD3, PE-Cy5-CD4, and APC-CXCR3 for CD3^{+}CD4^{+}CXCR3^{+} T cells and FITC-CD3 and PE-Cy5-CD4 for CD3^{+}CD4^{+} T cells. The percentage of CD3^{+}CD4^{+}CXCR3^{+} T cells and CD3^{+}CD8^{+} T cells in peripheral blood (A) and spleen (B) was analyzed by FACS. Results represent mean value ± S.E. of 3 independent experiments with similar results. *p < 0.05 and **p < 0.01. C, infiltrated of CD4^{+}/CD8^{+} T cells were stained with rat anti-mouse CD8α or rat anti-mouse CD4 antibody followed by staining with Alexa Fluor 488 rabbit anti-rat IgG in tumor tissue as detected by immunofluorescence assay. The bar represents 100 μm.

As shown in Fig. 8B, the breast cancer patients with relative higher Fas expression had a poorer prognosis than found in patients with relative lower Fas expression. Cox proportional hazards regression analysis also determined that higher Fas expression in human breast cancer tissues was an independent predictor for reduced overall survival of breast cancer patients (supplemental Table 2). Fas expression in human breast cancer tissue was positively correlated with clinical TNM stages (supplemental Table 3). These results suggest that higher Fas expression in human breast cancer tissue may be involved in breast cancer progression.

**DISCUSSION**

Fas, as a quintessential death receptor, could rapidly induce apoptosis of susceptible cells once bound by FasL or agonistic antibody, Jo-2 (1–2). However, Fas is expressed in a variety of normal and neoplastic cells. Rather than apoptosis induction, Fas could also exhibit non-apoptotic functions depending on the tissue and the conditions involved (2–4). In hepatocytes, Fas was found constitutively expressed at a high level, and agonistic anti-Fas antibody, Jo-2, could cause massive apoptosis of hepatocytes. Additionally, Fas-induced apoptosis also contributed to viral hepatitis, liver cirrhosis, and Wilson’s disease. However, after partial hepatectomy, in vivo administration of agonistic Jo-2 could actually accelerate liver regeneration and healing in mice (43). These results strongly suggest non-apoptotic activity for Fas in the liver after liver damage. Similarly, Fas is widely expressed in the central nervous system, and after experimental sciatic nerve crush injury, injection of agonistic anti-Fas Ab into mice may actually accelerate functional recovery, and Fas ligation could induce neurite outgrowth in vitro (44). Collectively, these data suggest that Fas may exert different functions, apoptosis induction, or non-apoptotic activity, depending upon the physiological or pathological conditions.

It has been reported that almost all human tumors express Fas. It has also been demonstrated that Fas could directly or indirectly promote tumor growth and metastasis (8–14, 20, 21). In this study we found that the forced overexpression of wild-type Fas in 4T1 cells neither accelerates tumor growth nor does it decrease survival in tumor-bearing mice compared with that of mice inoculated with parental 4T1 cells. It is possible that Fas was already constitutively expressed in 4T1 cells at a high level, which was enough to exert their non-apoptotic function, and that artificially forced overexpression of wild-type Fas in 4T1 cells has no significant enhancing effect on tumor progression in vivo, which is consistent with previous studies (5). However, blockade of Fas signaling in 4T1 cells could significantly reduce tumor growth and prolong survival of tumor-bearing mice. Importantly, silencing of Fas expression in 4T1 cells also markedly decreased the tumor growth, inhibited tumor metastasis to liver, lung, and DLN, and significantly prolonged the survival of tumor-bearing mice. Additionally, in vivo blockade of Fas signaling by intra-tumor injection of cholesterol-conjugated Fas siRNA significantly inhibited tumor growth and markedly decreased tumor metastasis to liver, lung, and DLN in 4T1 tumor-bearing mice. Similar to our findings, other studies have shown that reducing Fas or Fas ligand expression in cancer cells significantly inhibited cell proliferation, and loss of Fas expression reduced cancer incidence as well as the size of the tumors in mouse models of liver cancer and ovarian cancer (5). Furthermore, we used a tissue array to show that breast cancer patients with relative higher Fas expression had a poorer outcome than patients with relative lower Fas expression. Furthermore, Fas expression in human breast cancer tissue was positively correlated with clinical TNM stages. Our results are consistent with a previous report that high Fas expression in renal cell carcinomas was negatively correlated with disease-specific survival and associated with lymph node metastasis in these patients (45). Collectively, these data suggest that high Fas expression in breast cancer could be used to identify high-risk patients with a poor clinical prognosis, and blockade of Fas signaling could inhibit breast cancer progression, which may benefit breast cancer therapy.
It is important to determine the mechanism for the inhibition of breast cancer progression \textit{in vivo} by blockade of Fas signaling in breast cancer cells. Cancer-related inflammation is crucial for promoting tumor development and progression, and proinflammatory cytokines and immunosuppressive cells are key components. Previous studies have illustrated that activation of the Fas signal could promote secretion of proinflammatory cytokines and chemokines (15–17) and recruitment of MDSCs to tumor tissue (19). Thereby, we speculated that blockade of Fas signaling maybe decrease cancer-related inflammation, thereby resulting in suppression of tumor progression \textit{in vivo}. In a 4T1 mammary cancer orthotopic xenograft model, blockade of Fas signaling markedly decreased the mobilization and recruitment of MDSCs in peripheral blood, spleen, liver, lung, DLN, and tumor tissue. It has been demonstrated that COX2, PGE2, G-CSF, IL-6, and VEGF were involved in MDSC mobilization and recruitment (46–49). In the current study blockade of Fas signaling in 4T1 cells signific-
cantly decreased PGE2 and IL-6 secretion, and systemic administration of a COX2/PGE2 inhibitor significantly reduced MDSCs expansion and accumulation in spleen, peripheral blood, and tumor tissue, resulting in significantly reduced tumor growth in vivo. Interestingly, in vivo administration of anti-IL-6 neutralizing antibody only reduced MDSC accumulation in tumor tissue of 4T1-bearing mice. These data indicate that tumor-derived PGE2 and IL-6 may play an important role in the mobilization and accumulation of MDSCs at both systemic and local levels, which may contribute to the promotion of tumor progression by the Fas signal. Besides the tumor tissue, MDSCs sorted from liver, lung, spleen, and tumor tissue
in 4T1-bearing mice expressed a high level of IL-6, and the tumor-infiltrating MDSCs also expressed a high level of COX2. We speculate that tumor-derived PGE2 and IL-6 could induce mobilization and accumulation of MDSCs, which express a high level of EP2/EP4 and IL-6R α (CD126) and further promote the expression of COX2 and IL-6, building a feedback loop, finally leading to accumulation of more MDSCs.

It has been demonstrated that cancer patients frequently have elevated levels of the physiological FasL (50), which was predominantly produced by tumor cells, and could drive Fas activation to promote tumor progression (5). Additionally, T cells derived from tumor-bearing mice could release bioactive FasL in exosomes, which promote tumor metastasis via Fas signaling (21). However, to our limited knowledge, Tnfsf6gld mice obtained from The Jackson Laboratory were on a C57BL/6 background, but the mouse mammary cancer cell lines 4T1 and EMT6 are both derived from BALB/c mice. Therefore, we do not have direct evidence to compare the growth of the altered 4T1 cells when implanted into Tnfsf6gld mice or WT mice.

Convincingly, our previous study demonstrated that the Fas signal in lung cancer cells promotes lung cancer growth in vivo, which is dependent of the Fas/FasL interaction, as confirmed in the tumor-bearing model in FasL-deficient Tnfsf6gld mice (20). However, the in vivo factors in breast cancer patients that can initiate Fas signaling to promote breast cancer progression needs to be further investigated.

In summary, our results indicate that the activated Fas signaling can induce production of proinflammatory cytokines IL-6 and PGE2, which then systemically and locally mobilize and accumulate MDSCs and, consequently, promote tumor progression (Fig. 9). Blockade of Fas signaling in breast cancer can significantly suppress tumor progression by inhibiting pro-inflammatory cytokine production and MDSC accumulation. Therefore, blockade of Fas signaling-initiated cancer-related inflammation in breast cancer cells may be useful in the treatment of breast cancer.

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