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Activated T Cell Exosomes Promote Tumor Invasion via Fas Signaling Pathway

Zhijian Cai,*1 Fei Yang,*1 Lei Yu,* Zhou Yu,* Lingjing Jiang,* Qingqing Wang,* Yunshan Yang, ‡ Lie Wang,* Xuetao Cao,* and Jianli Wang*

Activated T cells release bioactive Fas ligand (FasL) in exosomes, which subsequently induce self-apoptosis of T cells. However, their potential effects on cell apoptosis in tumors are still unknown. In this study, we purified exosomes expressing FasL from activated CD8+ T cell from OT-I mice and found that activated T cell exosomes had little effect on apoptosis and proliferation of tumor cells but promoted the invasion of B16 and 3LL cancer cells in vitro via the Fas/FasL pathway. Activated T cell exosomes increased the amount of cellular FLICE inhibitory proteins and subsequently activated the ERK and NF-kB pathways, which subsequently increased MMP9 expression in the B16 murine melanoma cells. In a tumor-invasive model in vivo, we observed that the activated T cell exosomes promoted the migration of B16 tumor cells to lung. Interestingly, pretreatment with FasL mAb significantly reduced the migration of B16 tumor cells to lung. Furthermore, CD8 and FasL double-positive exosomes from tumor mice, but not normal mice, also increased the expression of MMP9 and promoted the invasive ability of B16 murine melanoma and 3LL lung cancer cells. In conclusion, our results indicate that activated T cell exosomes promote melanoma and lung cancer cell metastasis by increasing the expression of MMP9 via Fas signaling, revealing a new mechanism of tumor immune escape.

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Fas (also known as CD95/Apo-1) is a transmembrane protein belonging to the TNF/nerve growth factor receptor superfamily, which transmits an apoptotic signaling in susceptible cells once triggered by its natural ligand FasL (1). Fas-mediated apoptosis plays important roles in various biological processes, including activation-induced cell death in T lymphocytes or cell-mediated cytoxotoxicity against virus infection (2, 3). Almost all kinds of tumor cells express Fas, and the Fas/FasL system plays an important role in controlling survival or growth of tumor cells (4). Although Fas signaling was reported to induce tumor apoptosis and inhibit tumor growth in vivo when triggered by FasL (5–7), accumulated evidence also demonstrated that, in the presence of certain levels of FasL, Fas signaling promotes, not inhibits, tumor growth in Fas-resistant tumor cells (8, 9). In addition to inducing tumor cell proliferation, Fas ligation was proved to advance cell cycle and increase tumor motility and invasiveness (10–14). All of these studies suggest that Fas signaling plays an important role in tumor progress in Fas-resistant tumors.

AAbbreviations used in this article: c-FLIP, cellular FLICE inhibitory protein; COX2, cyclooxygenase-2; DC, dendritic cell; EXOcont, exosome derived from naive CD8+ OT-I T cell; EXOcd, exosome derived from Con A-activated CD8+ T cell of gld mice; EXOuri, exosome derived from Con A-activated CD8+ T cell of gld mice; EXOgld, exosome derived from Con A-activated CD8+ T cell of gld mice; PDTC, ammonium pyrrolidinedithiocarbamate; siRNA, small interfering RNA; WT, wild-type.

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Materials and Methods

Mice and cell lines

C57BL/6−/− Tg (Tcra Tcrb) 1100Mjb/J (OT-I) mice were obtained from the Institute of Immunology, National Key Laboratory of Medical Immunology, Second Military Medical University (Shanghai, China). Female C57BL/6J (8–8–8-wk-old) mice were purchased from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). Tnfsf6g/d mice on a C57BL/6 background were provided by Drs. Jianli Wang and Yuanzhi Shi (Zhejiang University, Hangzhou, China). All animal experiments were performed under the approved guidelines of the Animal Care Committee of Zhejiang University.

Activiation of T cells is a pivotal step in the process of host antitumor immunity. During this course, T cells release large numbers of membrane vesicular bodies termed exosomes (15), which were initially described by Johnstone et al. (16). Exosomes are nanoscale membrane vesicles (50–100 nm) released by various live cells, which have a lipid bilayer structure (17). They are formed by membrane budding into the lumen of an endocytic compartment, leading to the formation of multivesicular bodies. Fusion of multivesicular bodies to the plasma membrane leads to the extracellular release of exosomes (18). The exosomes secreted by activated T cells contain bioactive FasL and can induce the apoptosis of Jurkat T cells (15). Exosomes purified from CD8+ T cells, which are generated by cultivation of OVA-pulsed dendritic cells (DCs) with naive CD8+ T cells derived from OT-I mice, also express FasL and can inhibit DCs to stimulate CD8+ CTL responses (19). However, the potential effects of FasL in activated T cell exosomes on Fas-resistant tumor cells are not well understood.

Matrix metalloproteinase (MMP9) plays a critical role in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as tumor metastasis (20). MMP9 was reported to be an important factor in facilitating lung cancer invasion and metastases in the B16 melanoma model (21). It was also demonstrated that the activation of tumor Fas signaling can induce the expression of MMP9 (22). Therefore, we hypothesize that FasL in activated T cell exosomes probably upregulates MMP9 expression and promotes the invasive ability of Fas-resistant tumor cells, which may provide a novel view for understanding tumor escape from the immune system.
background (henceforth termed gld) were obtained from The Jackson Laboratory, bred under specific pathogen-free conditions, and used at 6–8 wk of age. B16 melanoma cell line and 3LL Lewis lung cancer cell line originating from C57BL/6 mice were purchased from American Type Culture Collection (Manassas, VA).

**Peptides, reagents, and Abs**

OVA357–364 peptide was synthesized by Chinese Peptide (Hangzhou, China). Matriplex matrix basement membrane was from BD Biosciences (San Diego, CA). DAPI was from BIOMOL Research Laboratories (NJ, CA). INTERFRein siRNA transfection reagent was from Polyplus (NY, CA). NF-κB inhibitor ammonium pyrrolidinedithiocarbamate (PDTC) and ERK/MAK inhibitor U0126 were from Sigma-Aldrich (St. Louis, MO). Abs against FasL, allophycocyanin (MFL3), CD8 PE (53-6.7), CD9 PE (eBioKMC8), CD11c PE (N418), and TCR FITC (B2.1) were from eBioscience (San Diego, CA). Abs against HSP70 (3A3), CD8 (YTS169.4), FasL (C-178), and Fas (5F9) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against phospho–NF-κB (Ser536) (93H1) and NF-κB (C-2), as well as HRP-linked secondary Abs, were from Cell Signaling Technology (MI, CA).

**Preparation and purification of CD8+ T cell-released exosomes**

A total of 2 × 10^6 cells/ml naive lymphocytes derived from OT-1 mice was cultured in RPMI 1640 complete medium (containing 50 mM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES, and 10 U/ml IL-2) and stimulated or not with OVA357–364 peptide (2 μg/ml) for 48 h. Active CD8+ T cells were purified using CD8+ Dynabeads (Invitrogen, Carlsbad, CA). Purified OVA-specific, active CD8+ T cells were then cultured in exosome-free RPMI 1640 complete medium for 24 h. Exosomes in T cell supernatants were isolated, as previously described (23). Briefly, collected culture supernatants were differentially centrifuged at 300 × g for 10 min, 1,200 × g for 20 min, and 10,000 × g for 30 min at 4°C. The supernatant from the final centrifugation was ultracentrifuged at 100,000 × g for 1 h at 4°C. After removing the supernatant, the exosome pellets were washed in large volume of ice-cold PBS and centrifuged at 100,000 × g for another 1 h at 4°C. To isolate the FasL+ exosomes released by CD8+ T cells of C57BL/6 and FasL-deficient gld mice, CD8+ T cells were stimulated with 50 μg/ml Con A for 15 min and then washed and cultured in exosome-free RPMI 1640 complete medium for 4 h. Supernatant in T cell culture supernatants were isolated, as previously described (23). Exosomes in the pellet were resuspended in PBS. Exosomes from CD8+ T cells, stimulated or not with OVA357–364 peptide, were termed EXO or EXOcont; exosomes from Con A-activated CD8+ T cells of C57BL/6 or gld mice were termed EXOwt or EXOgld, respectively.

**Electron microscopy**

For electron microscopy observations, exosome pellets were fixed in 4% paraformaldehyde at 4°C for 1 h. Then, the pellets were loaded onto electron microscopy grids coated with formvar, contrasted, and embedded in a mixture of uranyl acetate and methylcellulose. Sections were observed with a Philips Tecnai-10 transmission electron microscope operating at 80 kV (Phillips Electronic Instruments, Mahwah, NJ).

**FACS analysis of exosomes**

For FACS analysis, exosomes were coated onto 4-μm-diameter aldehyde/sulfate latex beads, using a previously described method (24). Briefly, 20 μg exosomes was incubated with 5 μl 4-μm-diameter aldehyde/sulfate latex beads for 15 min at room temperature in PBS, with 20 μl final volume. The mixture was then transferred to 1 ml PBS with gentle shaking for 1 h. After centrifugation, the pellet was blocked by incubation with 20 μl FCS for 30 min. Exosome-coated beads were washed thrice in PBS and resuspended in 50 μl PBS. Afterwards, beads were incubated with corresponding fluorescent Abs for 1 h at room temperature in the dark. Beads were analyzed by flow cytometry using a FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo software.

**Western blot**

A total of 50 μg exosomes or crude proteins extracted from cell lysates was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membrane was blocked with 5% BSA in TBST and then incubated with corresponding primary Abs overnight at 4°C. After incubating with HRP-coupled secondary Ab for 1 h, the membranes were scanned using Tanon 4500 (Shanghai, China), according to the manufacturer’s instructions.

**Tumor invasion assay in vitro**

After rehydration using a 6-fold volume of serum-free media, 50 μl Matriigel was added on a 8-μm polycarbonate membrane in 24-well Transwell plates, and the Matrigel was solidified at 37°C. A total of 1 × 10^6 cells/ml was stimulated with exosomes for 2 h in cryovials under constant rotation at 37°C. Then 2 × 10^5 B16 or 2 × 10^3 3LL tumor cells in 100 μl serum-free media were seeded into the top chamber. The bottom chamber was filled with 800 μl media containing 10% serum. Forty-eight hours after culture at 37°C, cells were fixed with methanol for 20 min and washed with PBS thrice for 20 min each. The fixed cells were stained with 10 μg/ml DAPI for 30 min and washed with PBS. The stained cells were examined using a fluorescence microscope. To confirm the role of Fas signal, MMP9, NF-κB, and ERK signal in the tumor invasion, 20 μg/ml anti-mouse FasL-neutralized Ab, 10 nM MMP9 inhibitor, 10 μM PDTC, or 10 μM U0126 was added to the culture medium.

**Detection of cell apoptosis**

For detection of apoptosis induced by exosomes, thymocytes (1 × 10^6/ml) were stimulated with 20 μg/ml EXOcont or EXOgld for 8 h. For detection of B16 or 3LL cell apoptosis induced by exosomes, viable tumor cells (2 × 10^5/ml) were stimulated with 5, 10, or 20 μg/ml EXOcont or EXO for 24 h. The apoptotic cells were stained with FITC Annexin V (BD Pharmin-gen, San Diego, CA) and propidium iodide (Sigma-Aldrich) for 5 min at 4°C in the dark. The stained cells were analyzed by FACS, as described previously (25).

**Cell proliferation assay**

Cells were stimulated with 5, 10, or 20 μg/ml exosomes for 24, 48, or 72 h in a 96-well plate, and 20 μl alamarBlue (Invitrogen) was added per well for 6 h. The fluorescent intensity was detected using a DTX 880 multimode detector (Beckman Coulter, Palo Alto, CA).

**RNA interference assay**

For transient silencing of cellular FLICE inhibitory protein (c-FLIP), 21-nt sequences of small interfering RNA (siRNA) duplexes were synthesized (GenePharma, Shanghai, China): 5′-CCUCUCUGAUAGCUAAGUTT-3′ (sense) and 5′-ACUAUGCUAUCCAGAGGF3′ (antisense). The following primers for MMP9 (sense) and 5′-AACGAACGUGCCAGAATT-3′ (antisense) were synthesized as siRNA scramble control. A total of 40 nM siRNA duplexes was transfected into cells (2 × 10^5/well) using 3 μl INTERFRein siRNA transfection reagent on a 24-well plate. The efficiency of c-FLIP, transient silencing was confirmed by Western blot.

**Real-time PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. The specific primers for B-actin for real-time PCR were 5′-CGTGTGACATCCTGTAACGACC-3′ (antisense) and 5′-ACCA-GTCCGGCTAGAAGAC-3′ (sense). The specific primers for MMP9 for real-time PCR were 5′-CGGCACGCTTGTGGTGA-3′ (sense) and 5′-GGCGCAGACGCCATATT-3′ (antisense). The following PCR conditions were used: 1 cycle at 95°C for 30 s and then 40 cycles of 5 s at 95°C and 34 s at 60°C. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR system (Foster City, CA).

**Lung invasion assay of B16 tumor cells in vivo**

A total of 5 × 10^5 B16 tumor cells with 30 μg EXOcont, EXO, 20 μg/ml anti-FasL-preincubated EXO, or PBS was injected into 8-wk-old C57BL/6 mice via the tail vein. Two weeks after injection, mice were sacrificed, and the lungs were detached. After the number of lung nodules was counted, the excised lungs were photographed and fixed in 10% formaldehyde for further H&E staining.

**Purification of an in vivo counterpart of EXO from tumor-activated CD8+ T cells**

A total of 1 × 10^6 B16 tumor cells was injected s.c. into 8-wk-old C57BL/6 mice. The mice were sacrificed 10 d after tumor inoculation. Lymphocyte suspensions from normal and tumor mice were prepared by crushing the lymph nodes and spleens between two pieces of ground glass. For purification of tumor tissue suspensions, tumor tissues were detached and dissociated enzymatically for 2 h with 1 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO) in the presence of 50 U/ml RNase and DNase.
Exosomes from all kinds of suspensions were filtrated using a 0.22-μm-filter device and then purified by differential centrifugation, as mentioned above. For purification of exosomes from CD8+ T cells, the exosomes were sorted through CD8+ Dynabeads (Dynal; Invitrogen). Briefly, 200 μg exosomes was mixed with 100 μl Dynabeads at 4°C by gentle shaking overnight, and the exosome-coated beads were detached with 200 μl DETACHaBEAD at room temperature for 45 min. Bead-free supernatant was collected after precipitating the beads via a magnet. CD8+ exosomes were washed in a large volume of ice-cold PBS and centrifuged at 100,000 × g for 1 h.

Statistical analysis

Experimental data were analyzed using one-way ANOVA and the t test using SPSS 11.5. Differences were considered significant when the p values were < 0.05.

Results

FasL+ EXO showed no effect on B16 tumor cell apoptosis and proliferation

To evaluate the isolated EXO integrity from activated CD8+ T cells, we inspected EXO morphology by electron microscopy. The observation showed that isolated EXO displayed a typical round morphology, with a diameter of 50–100 nm (Fig. 1A). Like their parent CD8+ T cells, EXO also expressed CD8+ T cell–specific molecules, including CD8 and TCR. In addition, they expressed specific EXO markers, such as CD9, HSP70, and TSG101, whereas endoplasmic reticulum-residing protein GRP94 was absent in EXO. Moreover, EXO, but not EXOcont, showed positive expression of FasL (Fig. 1B, 1C). These results demonstrate the integrity of isolated positive EXO from activated CD8+ T cells in this study. With the successful isolation of Fas+ EXO, we first examined whether EXO could induce apoptosis of thymocytes. Apoptosis assay by FACS showed that EXO, but not EXOcont, could significantly induce the apoptosis of thymocytes, and the ability of EXO to induce the apoptosis of thymocytes was greatly inhibited when preneutralized by FasL mAbs (Fig. 1D), which suggest the biological activity of FasL in EXO. To further confirm the relationship between apoptosis induction and FasL in EXO, we isolated exosomes from activated CD8+ T cells of C57BL/6 and gld mice and confirmed their expression of FasL (data not shown). After treatment with EXOwt or EXOgld, increasing apoptosis was found in EXOwt-treated, but not EXOgld-treated, thymocytes (Fig. 1E). These results indicate that the apoptosis-inducing function of EXO is FasL dependent. Then we detected whether EXO could also induce the apoptosis of Fas-expressing B16 or 3LL tumor cells. The results showed that FasL+ EXO has...
little effect on B16 apoptosis (Fig. 1F) or 3LL apoptosis (data not shown). Because it was demonstrated that Fas signaling can promote the proliferation of thyroid carcinomas (11), we next investigated whether EXO could promote the proliferation of B16 or 3LL tumor cells. The results showed that, with prolonged treatment time or increased concentration, EXO did not affect the proliferation of B16 (Fig. 1G) or 3LL (data not shown) compared with control.

**EXO promoted tumor cell invasion via Fas signaling in vitro**

Despite the observation that FasL+ EXO showed little effect on apoptosis and proliferation of B16 and 3LL tumor cells, we further investigated whether EXO can cause other functional changes in tumor cells. Because it was reported that activation of Fas signaling can induce motility and invasion of apoptosis-resistant tumor cells (13), we reasoned that EXO may increase the invasive ability of tumor cells in vitro. To validate this, invasiveness assays in vitro were performed. In this assay, B16 or 3LL tumor cells were placed in the top chamber in serum-free media with stimulus (EXO, EXOcont, or PBS) for 2 h. The bottom chamber contained 20% FCS. Forty-eight hours after culture, we observed an increasing number of B16 or 3LL tumor cells in the bottom chamber in the EXO group but not in the EXOcont or PBS group (Fig. 2A). Interestingly, once preneutralized by FasL mAbs, the ability of EXO to promote tumor cell invasion was completely abrogated (Fig. 2B). These results suggest that EXO may increase the invasive ability of tumor cells via Fas signaling in vitro.

**EXO-mediated activation of Fas signaling induced upregulation of MMP9 expression in tumor cells**

It was reported that CD95L mediates the migration of myeloid cells via MMP9 activation (26). We assume that the effect of EXO on tumor cell invasion is also related to MMP9 activation. As shown in Fig. 3A, after incubation with EXO, but not EXOcont, the mRNA and protein levels of MMP9 in B16 were significantly increased; the optimal concentration to stimulate was 10 μg/ml (Fig. 3A, 3B). After blockage of Fas signaling by anti-FasL, the expression of MMP9 induced by EXO decreased greatly (Fig. 3C), which provides solid evidence that upregulation of MMP9 induced by EXO is Fas/FasL dependent. To further confirm the relationship between EXO-mediated promotion of tumor cell invasion and MMP9 upregulation, MMP9 inhibitor was added to B16 tumor cell-invasiveness assays in vitro. A total of 10 nM of MMP9 inhibitor completely ablished the ability of EXO to promote tumor cell invasion (Fig. 3D). These results indicate that the tumor cell invasion induced by FasL+ EXO was associated with MMP9 expression.

**EXO promoted MMP9 expression and tumor cell invasion through ERK and NF-κB pathways**

The stimulation of CD95L on tumor cells can activate the NF-κB and ERK pathways (13), and the activation of NF-κB and ERK increases MMP9 expression (8, 27). Similarly, EXO stimulation activated the NF-κB and ERK pathways in B16 cancer cells, which was demonstrated by the increased phosphorylation of NF-κB and ERK in response to EXO stimulation but not EXOcont stimulation (Fig. 4A). Nevertheless, it seems that the expression of ERK and NF-κB is in parallel pathways, because when ERK expression was disrupted by its specific inhibitor U0126, little change in the expression of NF-κB was observed (data not shown). To further reveal the roles of activated NF-κB and ERK in MMP9 expression and tumor cell invasion, specific inhibitors for the NF-κB- and ERK-signaling pathways were used to treat B16 cancer cells before EXO stimulation; MMP9 expression was subsequently examined. We found that both NF-κB and ERK inhibitors (PDTC and U0126) markedly suppressed EXO-induced MMP9 expression and tumor cell invasion (Fig. 4B, 4C). Collectively, these results showed that FasL+ EXO-activated NF-κB- and ERK-signaling pathways were responsible for the increased MMP9 expression and tumor cell invasion.

**c-FLIP was critical to the increase in MMP9 expression and tumor invasion induced by EXO**

c-FLIP is an endogenous inhibitor of death receptor-induced apoptosis that acts through the caspase-8 pathway and is widely expressed in tumors (28, 29). c-FLIP exists as a long (c-FLIPL) or a short (c-FLIPS) variant (30, 31). c-FLIPS shows overall structural homology to caspase-8, containing two death effector domains that interact with Fas-associated death domain protein, as well as an inactive caspase-like domain. c-FLIPS contains only the two death effector domains and has lower antiapoptotic capacity (32). In B16 tumor cells, we found that Fas engagement with EXO led to the increased accumulation of c-FLIPL but not c-FLIPS (Fig. 5A). The increase in c-FLIPL was detected at 5 min after EXO stimulation, and the high level was maintained for 24 h (Fig. 5A). It was reported that c-FLIP can promote activation of the ERK-and NF-κB-signaling pathways (32). Therefore, we investigated whether their activation in B16 tumor cells is mediated by c-FLIPL upon EXO stimulation. We inhibited c-FLIPL expression by siRNA and confirmed the inhibitory efficiency by Western blot. c-FLIPL expression in c-FLIPL siRNA-transfected B16 cells decreased ~50% compared with c-FLIPL expression in control cells (Fig. 5B). Then we wanted to know whether the knock down of c-FLIPL leads to the apoptosis of B16 cells stimulated by EXO. We found that the apoptosis of B16 cells with c-FLIPL knock down showed few differences in comparison with control siRNA knockdown.
EXO increased the tumor cell lung invasion via Fas signaling in vivo

To further clarify the effect of EXO on tumor invasion, we performed tumor invasive analysis with the B16 tumor cell-induced murine lung cancer model. We found that EXO greatly increased the tumor cell lung invasion, as indicated by gross morphology of the lungs (Fig. 6A, left panels) and the number of invasive nodules (Fig. 6A, right panel). Histological assessment of lungs showed few tumor colonies in EXOcont and PBS groups but more numerous tumor colonies in EXO groups (Fig. 6B). After preincubation with FasL mAbs, the ability of EXO to promote tumor lung invasion was markedly decreased (Fig. 6). These results strongly support that EXO increase tumor cell lung invasion via Fas signaling in vivo.

Identification of a counterpart of EXO in vivo from tumor-activated CD8+ T cells

To further confirm the existence of CD8 and FasL+ exosomes and their effects on tumor metastasis in tumor mice, we isolated CD8+ exosomes from spleens and draining lymph nodes of control and tumor mice using CD8+ magnetic beads. The CD8+ exosomes from tumor tissues were also isolated. As shown in Fig. 7A, exosomes from 3LL and B16 tumor mice, but not control mice, expressed FasL; its expression level in exosomes from tumor tissues was greater than that in tumor lymphocytes. All exosomes expressed CD8 molecules and exosomal mark proteins, such as HSP70 and TSG101 (Fig. 7A). After stimulation with exosomes isolated from tumor mice, but not control mice, 3LL and B16 tumor cells showed increase expression of MMP9 and enhanced invasive ability. Consistent with their expression of FasL, exosomes from tumor tissue showed stronger stimulatory effect than did those from tumor lymphocytes (Fig. 7B, 7C). Based on our results, it can be predicted that the number of lung tumor nodules in the B16 tumor model of wild-type (WT) mice will be greater than that in gld mice. We examined the difference in the number of lung tumor nodules between WT and gld mice; as expected, the number was greater in WT mice (Fig. 7D). These results indicate that CD8+ and FasL+ exosomes are naturally generated with tumor progression and exert an exacerbated effect in tumor invasion.

FIGURE 3. EXO-mediated activation of Fas signaling induced upregulation of MMP9 expression in tumor cells. (A) B16 tumor cells were treated with the indicated doses of EXO for 24 h or 10 μg/ml EXO for the indicated times. mRNA expression levels of MMP9 were assayed by quantitative PCR. (B) Western blot analysis of MMP9 expression in B16 tumor cells stimulated with 10 μg/ml of EXO for the indicated times. (C) Western blot analysis of MMP9 expression in B16 tumor cells stimulated with 10 μg/ml of EXO, EXO preincubated with 20 μg/ml of FasL-neutralized mAbs, or isotype mAbs (ISO) for 2 h. (D) Invasive assay of B16 tumor cells after stimulation with 10 μg/ml of EXO or EXO plus MMP9 inhibitor (MMP9i) (n = 5). Data are representative of three independent experiments. *p < 0.05, **p < 0.01.

FIGURE 4. EXO promoted MMP9 expression and tumor cell invasion through ERK and NF-κB pathways. (A) B16 tumor cells were stimulated with 10 μg/ml of EXO or EXOcont for the indicated times and harvested; the extracted crude proteins were subjected to Western blot with Abs against NF-κB, phospho–NF-κB, ERK, or phospho-ERK. (B) B16 tumor cells were pretreated with NF-κB-specific inhibitor PDTC or ERK1/2-specific inhibitor U0126 for 30 min and then stimulated with 10 μg/ml EXO for 24 h. Western blot analysis of MMP9 expression in B16 tumor cells was detected by Western blot. (C) Using the same experimental conditions as in (B), the number of cells that migrated through Matrigel and adhered to the bottom of Transwell filter was quantified (n = 5). Data are representative of three independent experiments. **p < 0.01.
Discussion
Stimulation of FasR normally induces an apoptotic death signal. However, the interaction between Fas and FasL in tumor cells does not always induce a death signal, and conversely, promotes tumorigenesis (11). Activated CD8⁺ T cells secrete FasL⁺ exosomes, which can induce DC apoptosis and inhibit their ability to stimulate CD8⁺ CTL responses (19). Until now, the effect of FasL + exosomes from activated CD8⁺ CTLs on Fas-resistant tumors was unknown. In this study, we found that FasL + EXO did not affect the apoptosis and proliferation of tumor cells but accelerated their invasion in vivo via upregulation of MMP9 expression. Moreover, we identified a counterpart of EXO in tumor-bearing mice that also possessed the ability to increase MMP9 expression and cancer invasion.

Increasing the expression of FasL during activation is one of the properties of T lymphocytes. Tumor Ag-specific CTLs and CD8⁺ T cells activated by tumor Ag are generally the most important effector cells to attack tumor cells (34). Activated CD8⁺ T cells are present within the tumor site, but after being educated by tumor cells, they are in an unresponsive state (35). Our results show that CD8 and FasL + EXO could be isolated from tumor tissues, suggesting that, in the tumor environment, CTLs continuously secrete FasL⁺ EXO to promote tumor growth rather than eliminate tumor tissues (i.e., CTLs in the tumor site are converted from...
a guard into an accessory of tumor progression under such circumstances). In the FACS results in Fig. 1A, EXO is adsorbed onto latex with an intact membrane structure, and if FasL can be detected, it is probably membrane-bound. Moreover, in the results from Western blotting, we detected a band at ∼40 kDa, which is similar to the molecular mass of membrane-bound FasL, whereas we detected no signal at 26 kDa, which is similar to the molecular mass of soluble FasL (data not shown). CTLs themselves also express FasL and may trigger Fas signal in tumor cells, which probably promotes MMP9 expression and the invasive capability of tumor cells. However, there are very limited numbers of CTLs in tumor sites (35). Furthermore, unlike exosomes, whose diameters are <100 nm, allowing them to freely pass through basement membrane (36), immigration of CTLs from spleen and lymph nodes into the tumor site is more complicated. It can be presumed that more FasL* EXO will accumulate in the tumor location than CTLs, which will probably exert a stronger effect of invasive promotion on tumors than their parent CTLs.

Many molecules were reported to be associated with tumor invasion. Among them, MMP2, MMP9, cyclooxygenase-2 (COX2), β-catenin, and urokinase plasminogen activator, are considered the primary tumor-invasive effectors (13, 20, 37–39). In our study, we found that, after stimulation by EXO, expression of the mRNA levels of MMP2, MMP9, and COX2 was significantly elevated in 3LL and B16 tumor cells. However, we could not detect any change in the protein expression of MMP2 in either cell type (data not shown). Although the expression of COX2 mRNA increased in both cells, almost no PGE2 secretion could be detected with or without EXO stimulation in B16 tumor cells (data not shown). Interestingly, urokinase plasminogen activator, which is reported to be related to tumor invasion mediated by Fas signaling (13), showed no increase in either cell type after EXO stimulation (data not shown). Among those tumor-invasive effectors, we detected only elevated mRNA and protein levels of MMP9 in both cancer cell lines. It is likely that MMP9 is a universal effector molecule that promotes tumor invasion induced by FasL in EXO.

Although increasing tumors have been found to possess Fas resistance, the signaling mechanisms of tumor Fas resistance remain unknown. c-FLIP is an endogenous inhibitor of death receptor-induced apoptosis, and it plays a critical role in tumor-apoptosis resistance via the caspase-8 pathway (28, 40). In this study, we found that, only 5 min after FasL* EXO stimulation, the amount of c-FLIPb increased markedly, and this was sustained for ≥24 h. c-FLIP is extremely unstable and can be quickly degraded by the ubiquitination pathway (41, 42). The early increase in c-FLIPb probably results from the inhibition of its degradation by ubiquitin. Although it was reported that protein kinase C-mediated phosphorylation regulates c-FLIP ubiquitylation and stability (43), the mechanism underlying the ubiquitylation inhibition of c-FLIPb induced by EXO remains to be elucidated. As a result of positive feedback, NF-kB and ERK activation triggered by c-FLIPb probably promotes the synthesis of c-FLIPl, which may explain why it remains at high levels for a long time after EXO stimulation. Although the Fas-mediated apoptosis after c-FLIP siRNA inhibition in B16 cells showed no difference compared with control siRNA, we cannot exclude that c-FLIP plays a role in the Fas resistance of B16 cells, because the expression of c-FLIP knock down is mild, and the expression of c-FLIP decreases only ∼50%.

It was reported that activation of the ERK pathway and subsequent induction of antiapoptotic proteins, such as Bcl-2, play important roles in the survival of CD8+ T and NK cells (44, 45) In B16 tumor cells stimulated by FasL* EXO, we found that Bcl-2 was upregulated after EXO stimulation, and specific inhibitors of ERK and NF-kB activation could decrease the expression of Bcl-2. But there was no increasing apoptosis in the B16 cells treated with Bcl-2 inhibitor compared with the group treated with DMSO or control, which suggests that Bcl-2 may not be involved in the Fas resistance of B16 tumor cells induced by FasL-expressed EXO (data not shown). Tumor cells were also demonstrated to synthesize a protective protein to resistance apoptosis induced by Fas signal (46). A high level of a protein tyrosine phosphatase, Fas-associated phosphatase-1, which can interfere with transduction of the apoptotic signal by Fas (47) upon interaction of its third PDZ domain with the C-terminal three amino acids of Fas (48), has been implicated as a possible agent causing preferential survival of human pancreatic adenocarcinomas stimulated by FasL (46). It is possible that Fas-associated phosphatase-1 contributes to the Fas resistance in B16 tumor cells stimulated by EXO, but this needs to be explored further.
In conclusion, we report that CTLS are diverted from tumor inhibition to tumor promotion through secreting FasL, TNF-α by EXO. Our data showed that EXO could activate c-FLIP, c-FLIP-L, and ERK- and NF-κB(signaling pathways to increase MMP9 expression in tumor cells, which is Fas/FasL dependent. The upregulation of MMP9 leads to exacerbated tumor invasion, and these tumor-promoting exosomes are proved to exist in tumor-bearing mice. Our results reveal a novel mechanism of tumor immune escape and suggest that strategies to inhibit, rather than to promote, Fas activity should be considered during cancer therapy.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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