Cancer Cell-Derived Exosomes Induce Mitogen-Activated Protein Kinase-Dependent Monocyte Survival by Transport of Functional Receptor Tyrosine Kinases

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Running title: Cancer cell-derived exosomes promote monocyte survival

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Key words: exosomes (vesicle); survival; receptor tyrosine kinase; extracellular signal-regulated kinase; caspase; inflammatory environment.

ABSTRACT

Tumor-associated macrophages (TAM) play pivotal roles in cancer initiation and progression. Monocytes, the precursors of TAMs, normally undergo spontaneous apoptosis within two days, but can subsist in the inflammatory tumor microenvironment for continuous survival and generation of sufficient TAMs. The mechanisms underlying tumor-driving monocyte survival remain obscure. Here we report that cancer cell-derived exosomes were crucial mediators for monocyte survival in the inflammatory niche. Analysis of the survival-promoting molecules in monocytes revealed that cancer cell-derived exosomes activated Ras and extracellular signal-regulated kinases in the mitogen-activated protein kinase (MAPK) pathway, resulting in the prevention of caspase cleavage. Phosphorylated receptor tyrosine kinases (RTKs), such as phosphorylated epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2), were abundantly expressed in cancer cell-derived exosomes. Knockout of EGFR or/and HER-2, or alternatively, inhibitors against their phosphorylation significantly disturbed the exosome-mediated activation of MAPK pathway, inhibition of caspase cleavage and increase in survival rate in monocytes. Moreover, the deprived survival-stimulating activity of exosomes due to null expression of EGFR and HER-2 could be restored by activation of another RTK, insulin receptor. Overall, our study...
uncovered a mechanism of tumor-associated monocyte survival and demonstrated that cancer cell-derived exosomes can stimulate the MAPK pathway in monocytes through transport of functional RTKs, leading to inactivation of apoptosis-related caspases. This work provides insights into the long sought question on monocyte survival prior to formation of plentiful TAMs in the tumor microenvironment.

INTRODUCTION

Clinical and experimental evidences suggest that a particular type of macrophage population is present within the tumor microenvironment (1). Tumor-associated macrophages (TAMs) are derived from recruited monocytes or resident tissue macrophages (2), and play essential roles in tumor initiation, progression and metastasis (3). Monocyte recruitment, survival and differentiation are fundamental steps for continuous generation of TAMs. Previous studies are focused on understanding the complicated mechanisms of monocyte recruitment and differentiation within tumor tissues (2). However, investigations regarding how monocytes maintain survival before differentiation into sufficient TAMs have never been reported.

As key components of the innate immune system, monocytes are continuously produced by bone marrow but normally undergo spontaneous apoptosis within 48 h in blood circulation (4,5). Monocyte apoptosis can be prevented in vitro by serum at high concentrations (>20%), differentiation factors, and a wide range of inflammatory stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ), all of which reduce the activation of caspases and trigger the differentiation of monocytes into macrophages or dendritic cells (5-10). However, in the conflicting inflammatory environment that contains both pro- and anti-inflammatory factors, cell death is required for elimination of immune responses and maintenance of immune homeostasis (11). For example, when monocytes/macrophages are treated by pro-inflammatory factors, LPS and IFN-γ, and anti-inflammatory factor, interleukin-4 (IL-4), these cells undergo programmed cell death (12). Tumors have been reported to drive an inflammatory microenvironment with a range of pro- and anti-inflammatory factors which are regarded to induce monocyte death (13,14). However, tumor-associated monocytes can refrain from intrinsic apoptosis, possibly due to the dynamic interplay between cancer cells and monocytes. The mechanism underlying how monocytes escape from cell death in the tumor niche is still elusive.

Recently there have been considerable studies on understanding the roles of exosomes on promoting cell survival and controlling the crosstalk between cancer cells and their surrounding stroma (15,16). Exosomes are cell-derived and membrane-bound vesicles that carry a range of functional molecules including proteins, mRNAs and microRNAs (17). They are generated by the endosomal-sorting complex required for transport machinery and are released by membrane fusion between multi-vesicular bodies and plasma membrane (17,18). Since exosomes contribute to paracrine cellular communication within tumors (19), we thus investigated the possible roles of tumor cell-derived exosomes in promoting monocyte survival.

In the present study, we imitated a pro-inflammatory environment by treatment of human primary monocytes with a combination of LPS and IFN-γ, and a conflicting environment with LPS, IFN-γ and IL-4 (20). We found that exosomes isolated from various cancer cell lines improved monocyte survival in both inflammatory conditions. Functional proteins transported by
integral exosomes were imperative for this survival-promoting effect, which was achieved by activation of the mitogen-activated protein kinase (MAPK) pathway in monocytes. Phosphorylated receptor tyrosine kinases (RTKs) in cancer cell-derived exosomes were responsible for the stimulation of monocytes for circumventing apoptosis. Our study uncovered a molecular mechanism of tumor-associated monocyte survival by demonstrating the central roles of cancer cell-derived exosomes in this process.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human cytokines granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), IFN-γ, and IL-4 were purchased from PeproTech (Rocky Hill, USA). Sorafenib, PD0325901, SCH772984, gefitinib, CP-724714, lapatinib and protease inhibitor cocktail were purchased from SelleckChem (Houston, USA). LPS, puromycin, saponin, proteinase K, and lidocaine hydrochloride were purchased from Sigma-Aldrich (Shanghai, China). Lipofectamine 3000 transfection reagent, HEPES and TRIzol® were from Invitrogen (Carlsbad, CA, USA). Ficoll-paque was obtained from GE Healthcare (Shanghai, China). Radioimmune precipitation assay (RIPA) lysis buffer and trypan blue solution were purchased from Solarbio (Beijing, China). BsmBI, alkaline phosphatase, and T4-DNA ligase were from New England Biolabs (Beijing, China). Stbl3 chemically competent E. coli and pEASY-E1 (T vector) were purchased from Transgen (Beijing, China). Pronase was from M&C gene technology (Beijing, China).

Cell lines—Human lung adenocarcinoma cell line A549, human hepatocellular carcinoma cell line HepG2, human mammary epithelial cell line MCF10A, and human breast carcinoma cell line MCF-7 (an estrogen-independent sub-line) were obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China). The special line of MCF-7 is a unique sub-clone of the estrogen-responsive MCF-7 cell line after long-term growth in estrogen-free medium and cell proliferation is no longer estrogen dependent (21). Compared with estrogen-dependent MCF-7, the sub-line exhibits high cell viability in serum-deprived culture medium, and therefore, we utilized this sub-line for avoiding the generation of apoptotic bodies and isolating exosomes with optimal quantity and purity. Cell lines used were those frozen within 6 months of purchase from the cell bank (authenticated using short tandem repeat DNA profiling analysis). MCF10A cells were maintained in DMEM/F12K medium (Wisent, St-Bruno, Canada) with 100 ng/ml cholera toxin (Sigma-Aldrich, Shanghai, China). A549, HepG2, and MCF-7 cells were maintained in DMEM high glucose medium (Wisent, St-Bruno, Canada). Above cell culture medium was supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Wisent, St-Bruno, Canada). For exosome isolation, cells were maintained in serum-deprived culture medium supplemented with 10 mM HEPES. To induce the expression and activation of insulin receptor (InR) in MCF-7, cells were incubated in DMEM medium with 1 μg/mL of insulin (Novo Nordisk, Bagsvaerd, Denmark).

Isolation of primary human monocytes—Standard buffy coats were collected from healthy volunteer blood donors from Beijing Red Cross Blood Center (Beijing, China). Buffy coats were used anonymously after approval by the ethical committees of Chinese Academy of Sciences and Beijing Red Cross Blood Center based on the Chinese law. Experiments were carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association. Peripheral blood mononuclear cells were isolated
from the buffy coats by centrifugation in Ficoll-paque according to the manufacturer’s instructions. Monocytes were then isolated using a monocyte isolation kit II (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purified monocytes were cultured in macrophage serum-free medium (Gibco, Carlsbad, USA).

Plasmid construction and cell transfection—LentiCRISPR plasmid (pXPR_001, Addgene; a gift from Feng Zhang), a CRISPR-Cas9 site-specific genome editing system, was used to knock out human epidermal growth factor receptor (EGFR), epidermal growth factor receptor 2 (HER2) or both in MCF-7 cells (22). Oligonucleotides (Table 1) designed to target the first exon of either EGFR (NM_005228) or HER2 (NM_001005862) were inserted into the plasmid. MCF-7 cells were transfected with CRISPR-EGFR, CRISPR-HER2 or both plasmids using Lipofectamine 3000. Twenty-four hours after transfection, cells were treated with puromycin (2 μg/mL) for selection of stable transfectants. The culture medium was replaced daily until MCF-7 cells proliferated normally. To validate the effects of gene deletion, transfected cells were lysed using RIPA buffer with protease inhibitor cocktail, and analyzed by western blotting using anti-EGFR and anti-HER2 antibodies (Santa Cruz Biotechnology, Dallas, USA). MCF-7 cells without detectable EGFR and/or HER2, were further cultured and genomic DNA was extracted using a QIAamp DNA mini Kit (Qiagen, Hilden, Germany) for sequencing. To verify the knockout clones, genomic DNA from the transfected cells was amplified with the primers for the relevant genes (Table 1). The amplification products were cloned into pEASY-E1 and verified by DNA sequencing. Gene knockouts were confirmed by frameshift mutations at the designed sites in multiple colonies.

Cell treatment and exosome purification—Wild-type or transfected cancer cells were cultured in DMEM supplemented with 10% FBS until they were 60% confluent. Cells were washed three times by phosphate buffered saline (PBS) and cultured in serum-free DMEM medium for 20 h. To evaluate the effect of activated RTKs, cancer cells were incubated with phosphorylation inhibitors in DMEM-full medium until the cells were 50% confluent. Then cells were washed three times by PBS and further cultured in serum-free DMEM with phosphorylation inhibitors for 6 - 12 h. Cell viability was determined by trypan blue staining using Vi-Cell XR cell counter (Beckman Coulter). For exosome isolation, the medium was collected and subjected to differential centrifugation at 4 °C to remove cells and cell debris: 20 min at 500 × g (twice), 20 min at 2,000 × g (twice), and 40 min at 10,000 × g (once). The supernatant was collected, incubated with Exosome Isolation Reagent (Invitrogen, Carlsbad, USA) for over 12 h, and centrifuged at 10,000 × g for 2 h. The pellet that was considered as exosomes was suspended in ice-cold PBS and protein concentration was measured using a BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, USA). Exosomal RNA was extracted using TRIzol® and was transected into monocytes using Lipofectamine 3000. To obtain permeabilized exosomes, the exosome pellet was incubated in ice-cold PBS with 0.1% saponin at 37 °C for 30 min.

Transmission electron microscopy (TEM) analysis and nanoparticle tracking analysis (NTA) for exosomes—For TEM analysis, exosomes were fixed with 2% paraformaldehyde for 30 min at room temperature. About 8 μL of the mixture was dropped onto carbon-coated copper grids that had been pretreated with UV light to reduce static electricity. After air-drying for 10 min, exosomes
were stained twice (6 min each) with 1% uranyl acetate. The dried grids were examined using an HT7700 TEM (Hitachi, Japan) at 120 kV. NTA measurements were performed using the Nanosight LM10 system (Nanosight, Amesbury, UK). Exosome movement under Brownian motion was recorded and analyzed with NTA analytical software (Nanosight). The exosome concentration was normalized to harvested cell number.

**Western blotting**—GTP-Ras levels were quantified with an active Ras pulldown assay kit (ThermoFisher Scientific, Waltham, USA). Cells and exosomes were lysed using ice-cold RIPA buffer containing protease inhibitor cocktail. Equal amounts of proteins were prepared and analyzed by western blotting. Antibodies against pan-Ras (sc-166691), Janus-activated kinase 1 (JAK1, sc-7228), phospho-JAK1 (sc-16773), extracellular signal-regulated kinases (ERK1/2, sc-292838), phospho-ERK1/2 (sc-16982), NFkB (sc-7178), phospho-NFkB (sc-33022), protein kinase B (AKT, sc-8312), phospho-AKT (sc-33437), cleaved caspase-3 (sc-22140), total caspase-3 (sc-7272), cleaved caspase-8 (sc-6136), caspase-8 (sc-56070), cleaved caspase-10 (sc-7955), total caspase-10 (sc-134299), phospho-EGFR (sc-12351), phospho-HER2 (sc-101695), InR (sc-559), phospho-InR (sc-17196), Tsg101 (sc-7964) and β-Actin (sc-47778) were purchased from Santa Cruz Biotechnology. Analyses were carried out in triplicate and densitometry was performed using Image J for quantification.

**Cell counting**—Human primary monocytes were counted using the Vi-Cell XR Cell Counter, and then seeded into 96-well plates at a density of $5 \times 10^3$ per well with at least 6 replicates. Cultured monocytes were washed with ice-cold PBS for three times, incubated in PBS (Mg$^{2+}$ and Ca$^{2+}$ free) containing 4 mg/mL lidocaine hydrochloride and 5 mM EDTA (pH = 7.4) for 5 - 15 min on ice, and detached with gentle pipetting. Cell viability was immediately determined by trypan blue staining using either a Vi-Cell XR cell counter (large amount) or a hemocytometer under an inverted microscope (small amount).

**Statistical analysis**—All data represented at least three independent experiments and results were expressed as mean ± S.D. Statistical comparisons of monocyte survival were made using two-way analysis of variance (two-way ANOVA) followed by Bonferroni’s post hoc test. Densitometry data were analyzed using the Student’s t test. Values of $P < 0.05$ were considered significant.

**RESULTS**

**Characterization of cancer cell-derived exosomes and their interaction with monocytes**—To characterize cancer cell-derived exosomes, we first measured the morphology and size distribution of the exosomes purified from MCF-7 breast carcinoma cells which were cultured in the complete (ExoS+) or serum-free (ExoS-) medium. Electron microscopy images of the negatively stained vesicles revealed a typical shape of exosomes within the size range of 30 – 150 nm (Figure 1A and 1B). The vesicle size was further analyzed using the Nanosight system and the size distribution of ExoS+ was quite different from that of ExoS-. For ExoS+ samples, the peaks of the vesicle size appeared at ~40, ~100 and ~120 nm, while the ~40 nm peak was vanished in ExoS- samples (Figure 1C). Thus, exosomes distributed ~40 nm were likely derived from serum rather than cancer cells, although further investigation is needed to confirm this hypothesis. Next, we cultured MCF-7 cells in serum-free medium for different days to harvest cells with distinct viability. Vesicles were isolated from equivalent volumes of serum-free medium from MCF-7 cells with high viability (ExoHV, 95.4%
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Since cancer cell-derived exosomes are secreted into the tumor stroma, it is reasonable to evaluate their functions in a cancer-simulative inflammatory environment. The well-known role of LPS and IFN-γ combination treatment in the polarization of M1 macrophages prompted us to utilize these two reagents to establish a pro-inflammatory environment (2). IL-4 has been reported to induce the formation of anti-inflammatory M2 macrophages, and therefore, a cocktail of LPS, IFN-γ and IL-4 was applied for imitation of the complex inflammatory tumor milieu (12). To investigate the effects of cancer cell-derived exosomes on monocyte survival, monocytes were treated with serum-derived exosomes or exosomes isolated from MCF10A (MCF10AEexo), MCF-7 (MCF7Exo), A549 (A549Exo), or HepG2 (HepG2Exo) in normal or inflammatory environments. Viable cells were counted every day (Figure 2A). Without inflammatory stimuli, none of these exosomes prolonged monocyte survival. Stimulation of monocytes with LPS-IFN-γ combination or IL-4 alone slightly elevated the viability of untreated, MCF10A exosome-treated and serum exosome-treated monocytes. Strikingly, the monocyte survival rate was dramatically enhanced only after incubation with above cancer cell-derived exosomes in the pro-inflammatory (LPS + IFN-γ) and conflicting inflammatory (LPS + IFN-γ + IL-4) conditions, but not in the anti-inflammatory environment (IL-4). These observations indicate the necessity of inflammatory environment and cancer cell-derived exosomes for monocyte survival. Thus, we utilized the pro-inflammatory and conflicting inflammatory conditions for the following studies. Meanwhile, the survival-promoting effects were similar among exosomes from different cancer cells, suggesting that such activity of cancer cell-derived exosomes was universal among various cancer types. As MCF-7 cells were found to have the capacity of producing more exosomes than other cancer cell lines, we utilized the exosomes from MCF-7 cells for further studies. We then compared the activity of MCF-7-derived exosomes with human serum (20%) or CSFs (GM-CSF, 20 ng/mL; and M-CSF, 10 ng/mL) which were reported to improve monocyte survival (8). As indicated in Figure 2B,
exposure to MCF-7-derived exosomes (200 ng/mL) significantly prevented cell death over 7 days of culture in both inflammatory conditions. The magnitude of protection was similar to that seen with CSF treatment, but the effect of serum was much weaker. The limited amount of cell debris in the cultures suggested that the living monocytes retained a strong capacity for phagocytosis (data not shown).

The short life span of monocytes is determined by their constitutively programmed cell death. Persistent binding of the ligands to the tumor necrosis factor (TNF) receptors activates caspase-8 to eliminate monocytes (23). To further examine the role of exosomes in monocyte survival, we measured whether the activation of caspase-8 was suppressed by exosomes. After culture under various treatment conditions for 12 h, adherent monocytes were lysed and the proteins were analyzed by western blotting. Under both inflammatory conditions, exosomes, serum and CSFs all impaired caspase-8 activation (Figure 2C). Since caspase-10 is also activated in response to Fas apoptotic signals and, as a result, caspase-3 is subsequently activated, we measured the levels of cleaved caspase-10 and caspase-3. The results showed that the exosomes also impaired the activation of these caspases (Figure 2D). Together, these findings suggest that cancer cell-derived exosomes can promote monocyte survival in inflammatory microenvironments by inactivation of caspase-8, 3 and 10, while serum and CSFs showed much less potent to prevent caspase activation.

We next evaluated the relationship between concentration and functions of cancer cell-derived exosomes. Monocytes were incubated with MCF-7-derived exosomes at different concentrations (20, 40, 80, or 200 ng/mL) in both inflammatory environments and the viable monocytes were counted daily (Figure 2E). A positive correlation between monocyte survival rate and exosome dosage was observed, and the number of living monocytes reached a plateau when the exosome concentration reached 200 ng/mL.

Previous studies suggested that the efficiency of exosomes in transport of functional biomolecules relies on their vesicle integrity (24). To validate the necessity of exosome integrity for monocyte survival, we disrupted MCF-7-derived exosomes by repeated freezing and thawing or by permeabilization of the exosome membrane with 0.1% saponin (25). Disrupted exosomes were ineffective in promoting monocyte survival (Figure 2F). We also extracted exosome RNAs and transfected them into monocytes (26). Treatment with exosome RNAs was not effective to prevent monocyte survival at all. These results suggest that transport of protein molecules, such as membrane proteins, other than RNAs, from intact exosomes to monocytes may be required for the monocyte survival.

Cancer cell-derived exosomes activate the MAPK survival pathway in monocytes—Based on the roles of cancer cell-derived exosomes in promoting monocyte survival, we next studied the functional molecules in monocytes to trigger the cell survival machinery. ERK, NFXB-p50, JAK1 and AKT are key survival mediators which are activated in response to inflammation and have previously been shown to inhibit caspase activation and promote monocyte differentiation (27-30). Therefore, we examined the activation of these potential survival molecules by western blotting. MCF-7-derived exosomes showed marginal effects on the phosphorylation of JAK1, NFXB-p50 and AKT (data not shown), but strongly induced the phosphorylation of ERK1/2 and expression of GTP-Ras (Figure 3A). The MAPK pathway (Ras-Raf-MEK-ERK) is fundamental to cell survival (31), and activation
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of this pathway can override the apoptotic signaling from Fas, TNF, and TRAIL receptors (32). To determine the relationship between MAPK pathway and caspase-8, monocytes were treated with MCF-7-derived exosomes, together with the b/c-Raf inhibitor (sorafenib), the MEK inhibitor (PD0325901), or ERK inhibitor (SCH772984) for 24 h. All inhibitors significantly decreased the expression of phosphorylated ERK, and led to an increase in the levels of cleaved caspase-8 (Figure 3B). As these inhibitors suppressed the activity of Ras downstream molecules, they showed little effect on the expression of GTP-Ras (Figure 3B). These data suggest that activation of MAPK signaling by cancer cell-derived exosomes led to effective inhibition of caspase-8 activation in monocytes.

Activated RTKs in cancer cell-derived exosomes are indispensable for improving monocyte survival—Since RTKs have been reported to initiate the activation of MAPK pathway (32), we investigated the possible role of exosome RTKs in driving monocyte survival. We evaluated the effect of EGFR and HER2 in MCF-7-derived exosomes as they were important RTKs in breast carcinoma (33). We found that MCF-7-derived exosomes were rich in the phosphorylated forms of EGFR and HER2 (Figure 4A). We then used the CRISPR-Cas9 system to knock out EGFR (MCF-7EGFR), HER2 (MCF-7HER2), or both (MCF-7EGF-HER2) in MCF-7 cells. Alternatively, we incubated MCF-7 cells with the inhibitors against the phosphorylation of EGFR (gefitinib), HER2 (CP-724714), or dual EGFR/HER2 (lapatinib). Exosomes were then isolated from above transfected or inhibitor-treated cells, and were analyzed by western blotting for the expression of phosphorylated EGFR and HER2. Both strategies effectively decreased the amount of the activated EGFR and HER2 in the exosomes (Figure 4B and 4C).

Next, we examined whether inhibition of EGFR or HER2 in the exosomes influenced the activation of ERK and caspase-8 in monocytes. As shown in Figure 4D, compared with normal MCF-7-derived exosomes, exosomes from MCF-7EGFR- or gefitinib-treated MCF-7 cells had a reduced capacity to activate ERK phosphorylation, and consistently increased the cleavage of caspase-8 in monocytes. Similar results were observed when monocytes were incubated with exosomes from MCF-7HER2- or cells treated by CP-724714 (Figure 4E). Moreover, dual knockout or inhibition of EGFR and HER2 in exosomes led to comparable suppression of ERK phosphorylation and up-regulation of caspase-8 cleavage (Figure 4F). These results demonstrate that RTKs in cancer cell-derived exosomes were crucial for the activation of MAPK survival pathway and subsequent inhibition of caspase activity in monocytes.

In addition, we examined the viability of monocytes after treatment with the above exosomes lacking in phosphorylated EGFR, HER2 or both. Compared with wild-type exosomes, exosomes with null expression of EGFR or un-phosphorylated EGFR showed significantly decreased activity in preventing cell loss over 7 days of culture in both pro- and conflicting inflammatory conditions (Figure 5A). Similar phenomena were observed after exposure of monocytes to exosomes from MCF-7HER2-, MCF-7EGF-HER2-, CP-724714-treated or lapatinib-treated MCF-7 cells (Figure 5B and 5C). These data suggest that RTKs in cancer cell-derived exosomes stimulated monocyte survival.

To further demonstrate the essential roles of RTKs, we activated another RTK, InR, in MCF-7EGF-HER2- cells by incubation with insulin. The increased expression of phosphorylated InR
in MCF-7 cells and isolated exosomes was confirmed by western blotting (Figure 5D). Then, we incubated monocytes with the exosomes from MCF-7EGFR-HER2- cells with or without insulin treatment at different inflammatory conditions. To exclude the possible role of insulin in monocyte survival, monocytes treated with the lysates of InR-overexpressed exosomes served as a control. We observed that activation of InR in exosomes from MCF-7EGFR-HER2- cells enhanced the phosphorylation of ERK and attenuated the cleavage of caspase-8 in inflammatory environments (Figure 5E), further confirming the contribution of exosome RTKs to monocyte survival. Consistently, monocyte death was prevented after incubation with the exosomes from MCF-7EGFR-HER2+InR+ but not the exosomes from MCF-7EGFR-HER2- cells (Figure 5F). Taken together, these results strongly suggest that transport of RTKs from cancer cell-derived exosomes to monocytes play central roles in driving monocyte survival.

DISCUSSION

Monocytes are not fully differentiated cells and their fate can be modulated by a range of environmental stimuli (8). In most analyses of tissue macrophages, monocyte differentiation is separated into two independent processes: [1] monocytes are differentiated into M0 macrophages by M-CSF and GM-CSF treatment; [2] M0 macrophages are further polarized into M1 or M2 types by environmental factors (3,34). However, unpolarized macrophages have not been identified in vivo and, more importantly, it is doubtful whether the CSF-based model is the only mechanism for activating monocytes given the variety of tissues that host monocyte differentiation (35). Our present study is the first to demonstrate that malignant cell-derived exosomes can activate monocytes while inducing an anti-apoptotic state.

The inflammatory environment is a prerequisite for tumoral exosome-induced monocyte survival. In non-inflammatory environment, all types of exosomes showed no effect on improvement of monocyte survival (Figure 2A). This phenomenon is consistent with the fact that monocytes undergo spontaneous apoptosis in peripheral blood where exosomes secreted by blood cells and cancer cells are present. The pro-inflammatory stimuli, LPS and IFN-γ, led to continuous monocyte loss over 7 days of culture, with 20% cells still viable on day 7. However, after simultaneous incubation with a combination of LPS, IFN-γ and IL-4, almost all monocytes died within 48 h. Consistent with previous report [20], our observation supports that immune hemostasis requires programmed cell death in a conflicting inflammatory environment. In both pro-inflammatory and conflicting inflammatory environments, cancer cell-derived exosomes significantly increased monocyte survival rate, indicating the indispensability of exosomes and inflammatory environment for monocyte survival. Since the tumor stroma contains both pro-inflammatory and anti-inflammatory factors, the conflicting inflammatory environment is more simulative to the inflammatory tumor environment. Of several survival factors tested, cancer cell-derived exosomes proved the most effective at reducing cell loss (Figure 2B). The fact that monocytes survived without CSFs may suggest a new model for the origin of TAMs.

Exosomes mediate cell communication through fusion between the exosome membrane and the plasma membrane of the recipient cell, a process that requires the integrity of the exosome membrane (24). We confirmed this in our studies by showing that exosomes that had been disrupted had no effect on monocyte survival. These
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findings are also consistent with the possibility that fusion between the endosome membrane and the monocyte plasma membrane is responsible for transferring anti-apoptotic signals. Candidate exosome-derived factors include hydrophilic small molecules, proteins or RNAs. In view of this, we investigated whether exosome-derived RNAs (mRNA and microRNA) could prevent monocyte apoptosis, but no effect on monocyte survival was observed (Figure 2F). Consistent with our results, none of the microRNAs known to be secreted by MCF-7 cells have been shown to have anti-apoptotic effects (36,37).

Toll-like receptors (TLRs) are a class of proteins in the innate immune system [38]. Stimulation of TLR4 by LPS induces the release of critical pro-inflammatory cytokines and promotes monocyte survival by activating the NFκB, AKT and ERK signaling pathways (31,38). Interactions between IFN-γ and IFN-receptors, as well as between IL-4 and its receptor, can trigger the activation of the JAK signaling pathway (39). NFκB, AKT, ERK and JAK are key mediators of cell survival (28,40). When we investigated whether cancer cell-derived exosomes could influence these pathways, we observed that exosomes enhanced the activation of ERK and its upstream mediator Ras (Figure 3A), but showed no effects on NFκB, AKT or JAK. Therefore, cancer cell-derived exosomes activated the MAPK pathway (Ras-Raf-MEK-ERK) in monocytes. By using inhibitors of Raf, MEK and ERK, we observed activation of caspase-8 and inhibition of ERK, providing strong evidence that activation of the MAPK pathway by exosomes plays an important role in suppressing monocyte intrinsic apoptosis (Figure 3B). However, we cannot exclude the possibility that cancer cell-derived exosomes contained anti-apoptotic molecules such as survivin which may also contribute to monocyte survival. Since survivin is essential for maintenance of cell viability, in our experiments we could not acquire viable cancer cells and harvest eligible exosomes when survivin was knocked out.

Ras is a membrane-associated GTP binding protein that is activated in response to extracellular signals (such as hormones), activated RTKs or T-cell receptors (41). T-cell receptors are specifically expressed on the surface of T lymphocytes, but not on monocytes or cancer cells (42). While Ras was activated by exosomes, exosome Ras contents were much lower than monocyte Ras levels (Data not shown). These evidences suggest that exosome-derived RTKs could be the activators of Ras. RTKs are a major type of cell surface receptor, and family members, such as EGFR, HER2, and InR, are expressed on the plasma membrane of cancer cells (43). We found that phosphorylated forms of HER2 and EGFR were present in MCF-7-derived exosomes (Figure 4A), possibly due to the ligand-independent dimerization and activation of EGFR family members (44,45). To exclude the artificial effect of the Total Exosome Isolation Reagent on RTK activation, we also examined the expression of phosphorylated EGFR and HER2 in MCF-7-derived exosomes which were isolated using both traditional ultracentrifugation method and the isolation kit from Invitrogen. The levels of phosphorylated EGFR and HER2 in both types of exosomes were similar (Data not shown). When HER2 and EGFR were knocked out in MCF-7 cells using the CRISPR-Cas9 system, or their phosphorylation was inhibited using specific inhibitors, the effects of exosomes on monocyte survival were abrogated (Figure 4D - 4F, and Figure 5A - 5C). We noticed a drop in the viability of MCF-7 cells when they were treated with phosphorylation inhibitors, raising the possibility that apoptotic bodies or other vesicles associated with cell death were being produced (46). To
account for this, we adjusted the concentration of the inhibitors to ensure that most cells were viable. However, since it has been shown that these inhibitors can activate apoptotic signals in MCF-7 cells (47), we cannot exclude the possibility that exosomes isolated from these cells carried pro-apoptotic factors which influenced monocyte survival. Moreover, the similar effects of dual inhibition/knockout and single inhibition/knockout of EGFR and HER2 likely resulted from the quite similar downstream pathways of EGFR and HER2 (48). Meanwhile, EGFR and HER2 can form heterodimers to activate downstream molecules and the activity of the heterodimer is much higher than each monomer (49). Thus, inhibition of either EGFR or HER2 can block the formation of the heterodimer, which showed similar effect with dual inhibition, but no additive or synergistic effects.

In summary, we present strong evidences that cancer cell-derived exosomes prolonged monocyte survival in the inflammatory environment without CSFs. RTK family members are necessary for cancer cell-derived exosomes to activate the MAPK pathway in monocytes and consequently prevent monocyte apoptosis signaling (Figure 6). This study provides a novel perspective on the interaction of tumor cells and monocytes in the tumor microenvironment and provides new clues for the long sought question on monocyte survival prior to differentiation into sufficient macrophages in tumors.

ACKNOWLEDGEMENTS

We thank Dr. Feng Zhang from MIT for providing LentiCRISPR plasmid and Jian Shi for lab assistance.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

XS, YD and GL designed the project. XS, GL, XY, RZ, YZ, and XZ carried out experiments. XS, YD and GL analyzed the data. YD, XS, GJA and GN wrote the paper. GN supervised the experiments and provided advice. All authors revised and approved of the manuscript.

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**FOOTNOTES**

This work was supported by the grants from MoST 973 (2012CB934004 to G. Nie), the National Natural Science Foundation of China (31325010 to G. Nie, 31300822 to Y. Ding), and the Key Research Program of the Chinese Academy of Sciences (KGZD-EW-T06 to G. Nie). G.J.A. is a Senior Research
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FIGURE LEGENDS

FIGURE 1. Characterization of cancer cell-derived exosomes. (A, B) TEM examination of the morphology of MCF-7-derived extracellular vesicles. After MCF-7 cells (5 × 10^6) were cultured in DMEM with (ExoS+) or without (ExoS-) 10% FBS for 24 h, membrane vesicles were isolated and analyzed by TEM. (C) The size distribution and concentration of the vesicles from panel A (ExoS+) and B (ExoS-) were determined using Nanosight system. (D) The size distribution of exosomes purified from MCF-7 cells with distinct viability. MCF-7 cells (5 × 10^6) were cultured in serum-free medium which was changed every day. The vesicles were isolated 8 h after medium change at day 2 (ExoHV, 95.4 % viable cells), day 4 (ExoMV, 81.0% viable cells) and day 5 (ExoLV, 74.2 % viable cells) and analyzed by Nanosight system. (E) Expression of Tsg101 in the exosomes from human serum and the culture medium of MCF10A, MCF-7, A549, or HepG2 cells. GAPDH in corresponding buffy coat and cells served as loading control. (F) Flow cytometry analysis of the interaction between cancer cell-derived exosomes and monocytes. Exosomes were isolated from MCF-7 cells which were transfected with pEGFP-Tsg101, and were incubated with monocytes for 2 h (Exo+). Monocytes without exosome treatment served as a background control (Exo-). After monocytes were treated with pronase, the intensity of GFP fluorescent signals in monocytes was determined by flow cytometry.

FIGURE 2. Cancer cell-derived exosomes promote monocyte survival. (A) Monocyte survival rate was significantly improved by cancer cell-derived exosomes in pro- and conflicting inflammatory environments. Monocytes were treated by various cancer cell-derived exosomes in non-inflammatory (without any stimuli), anti-inflammatory (IL-4, 20 ng/mL), pro-inflammatory (LPS, 100 ng/mL; IFN-γ, 20 ng/mL), or conflicting inflammatory (LPS, 100 ng/mL; IFN-γ, 20 ng/mL; IL-4, 20 ng/mL) environment. Viable cells were stained by trypan blue and counted daily for 7 days. Control, without exosome; SerumExo, serum-derived exosome; MCF10AExo, MCF10A-derived exosome; A549Exo, A549-derived exosome; HepG2Exo, HepG2-derived exosome; MCF7Exo, MCF-7-derived exosome. Results are mean ± S.D. (n = 6). ***P < 0.001. (B) Comparison of the effects of cancer cell-derived exosomes, serum and CSFs on monocyte survival. In either pro- or conflicting inflammatory environment, monocytes were incubated with MCF-7-derived exosomes (Exo, 200 ng/mL), 20% serum or CSFs (GM-CSF, 20 ng/mL; M-CSF, 10 ng/mL). Viable cells were counted daily. Results are mean ± S.D. (n = 6). *P < 0.05. ***P < 0.001. (C) Western blotting analysis of cleaved caspase-8 (c-Casp8) in monocytes after treatment with CSFs, 20% serum or MCF-7-derived exosomes (Exo) for 12 h in both pro- and conflicting environments. The relative intensity of cleaved caspase-8 to total caspase-8 was analyzed by Image J. Results are mean ± S.D. (n = 3). *P < 0.05. **P < 0.01. Control, without exosome treatment. (D) Western blotting analysis of cleaved caspase-10 (c-Casp10) and caspase-3 (c-Casp3) in monocytes after incubation with (Exo+) or without (Exo-) MCF-7-derived exosomes (200 ng/mL) for 12 h in pro- and conflicting environments. The relative intensity of cleaved caspas to corresponding total caspases was analyzed by Image J. Quantified results were mean ± S.D. (n = 3). *P < 0.05, **P < 0.01. (E)
Dose-dependent effects of exosomes on monocyte survival. Monocytes were incubated with exosomes at different concentrations (0, 20, 40, 80, or 200 ng/mL) in inflammatory environments. Viable cells were counted daily. Results are mean ± S.D. (n = 6). **P < 0.01, ***P < 0.001. (F) Exosome integrity was required for the activity of promoting monocyte survival. Monocytes were treated with integral (Exo), permeabilized with saponin (ExoSaponin) or lysed (ExoLysates) MCF-7-derived exosomes (200 ng/mL), or exosome-derived RNA (ExoRNA). Living cells were calculated daily. Results are mean ± S.D. (n = 6). ***P < 0.001.

**FIGURE 3.** The effects of cancer cell-derived exosomes on survival-associated molecules in monocytes. (A) MCF-7-derived exosomes induced the expression of GTP-Ras and phosphorylation of ERK. The relative intensities of phosphorylated ERK (p-ERK) and GTP-Ras were quantified. (B) Inhibition of the MAPK pathway induced the expression of cleaved caspase-8 in monocytes treated with exosomes. Monocytes were incubated with MCF-7-derived exosomes together with 4 μM of b/c-Raf inhibitor sorafenib (Exo+Sora), 5 μM of MEK inhibitor PD0325901 (Exo+PD), or 5 μM of ERK inhibitor SCH772984 (Exo+SCH) for 24 h in pro- and conflicting inflammatory environments. The expression levels of GTP-Ras, cleaved caspase-8 and phosphorylated ERK1/2 were assessed by western blotting and quantified by normalization to their corresponding total proteins. All the blotting images were analyzed by Image J. Results are mean ± S.D. (n = 3). *P < 0.05, **P < 0.01.

**FIGURE 4.** Activated RTKs in cancer cell-derived exosomes drive the monocyte survival signal transduction. (A) Expression of phosphorylated EGFR (p-EGFR) and HER2 (p-HER2) in both MCF-7 cells and MCF-7-derived exosomes. Identical quantities (10 μg) of cell lysates and exosomes were used. (B) The expression levels of phosphorylated EGFR (p-EGFR) in exosomes from MCF-7 cells treated with various conditions. Exo, wild-type exosomes; Exo+Gefi, exosomes from MCF-7 cells treated with gefitinib; ExoEGFR-, exosomes from MCF-7EGFR clones; Exo+Lapa, exosomes from MCF-7 cells treated with lapatinib; Exo-/-, exosomes from MCF-7EGFR,HER2 clones. Tsg101 was used as an internal control. (C) The expression levels of phosphorylated HER2 (p-HER2) in the exosomes from MCF-7 cells treated with various conditions. Exo+CP, exosomes from MCF-7 cells treated with CP-724714; ExoHER2-, exosomes from MCF-7HER2 clones. (D) The effects of exosomal EGFR inhibition or knockout on monocyte survival signaling transduction. Monocytes were treated with the exosomes carrying inactivated EGFR or exosomes from MCF-7EGFR in the inflammatory environments. Phosphorylation of ERK and cleavage of caspase-8 (c-Casp8) in monocytes were examined by western blotting. (E) The effects of HER2 inhibition or knockout on monocyte survival in inflammatory environments. (F) The effects of EGFR and HER2 dual inhibition or knockout on monocyte survival signaling in inflammatory environments. All above blotting images were analyzed by Image J. Results are mean ± S.D. (n = 3). *P < 0.05, **P < 0.01.

**FIGURE 5.** RTKs in cancer cell-derived exosomes are the central players for improving monocyte survival. (A) MCF-7-derived exosomes with inactivated EGFR (Exo+Gefi) or exosomes from MCF-7EGFR (ExoEGFR-) showed decreased activity in promoting monocyte survival in the inflammatory environments. Results are mean ± S.D. (n = 6). **P < 0.01, ***P < 0.001. (B) The effects of HER2 inhibition (Exo+CP) or knockout (ExoHER2-) on exosome-mediated monocyte survival. Results are
mean ± S.D. (n = 6). **P < 0.01, ***P < 0.001. (C) Dual inhibition (Exo+Lapa) or knockout (Exo-/−) of EGFR and HER2 attenuated the effects of exosomes on monocyte survival. Results are mean ± S.D. (n = 6). **P < 0.01, ***P < 0.001. (D) The expression of phosphorylated InR (p-InR) in MCF-7 cells with or without insulin stimulation, and the exosomes isolated from these cells were also examined. Tsg101 served as loading controls. The relative intensity of p-InR or InR to Tsg101 was quantified. Results are mean ± S.D. (n = 3). **P < 0.01. (E) The effects of InR activation in exosomes EGFR-HER2- on phosphorylation of ERK and cleavage of caspase-8. MCF-7 EGFR-HER2- cells were stimulated with insulin for activation of InR. The exosomes isolated from these cells were indicated as Exo-/− InR. The lysates of Exo-/− InR were used to treat monocytes for excluding the possible effect of insulin on monocyte survival. Control monocytes were those without exosome treatment. The intensity ratio of p-ERK/ERK and c-Casp8/Casp8 was quantified. Results are mean ± S.D. (n = 3). *P < 0.05, **P < 0.01. (F) The effects of InR activation in exosomes EGFR-HER2- on monocyte survival in inflammatory environments. Results are mean ± S.D. (n = 6). **P < 0.01, ***P < 0.001.

**FIGURE 6.** Schematic illustration represents the potential signaling transduction mechanism by which cancer cell-derived exosomes prevent monocyte apoptosis. Malignant cell-derived exosomes transfer phosphorylated RTKs to monocytes through membrane fusion, and activate downstream MAPK (Ras-Raf-MEK-ERK) signaling pathway in monocytes. The activation of ERK prevents the cleavage of caspases and promotes monocyte survival.
TABLE 1. Sequence of oligonucleotides for plasmid construction and primers for gene sequencing

| Methods                    | Names        | Sequence (5’ - 3’)         |
|----------------------------|--------------|-----------------------------|
| CRISPR plasmid construction| EGFR-Oligo1  | CACCGAGTAACAAGCTCACGCAGTT   |
| CRISPR plasmid construction| EGFR-Oligo2  | AAACAACTGCGTGAGCTTGTTACTC   |
| CRISPR plasmid construction| HER2-Oligo1  | CACCGTGCCAGTCCCCGAGACCCACC  |
| CRISPR plasmid construction| HER2-Oligo2  | AAACGGTGGGTCTCGGGACTGGCAC   |
| Sequencing                 | EGFR forward | TTTTCATCTACCACCCACC         |
| Sequencing                 | EGFR reverse | CAAATTCCCAAGGACCAC          |
| Sequencing                 | HER2 forward | AGTGTCCTCTGACCCATCTG        |
| Sequencing                 | HER2 reverse | AATCCACCTTTCTCACCAGC        |
Figure 6

Malignant Cells ➔ RTKs

Exosome

Survival ➔ ERK1/2 ➔ P ➔ MEK1/2 ➔ P ➔ Raf ➔ GTP-Ras

Monocytes

Casp8 ➔ Casp3 ➔ Apoptosis

Casp10 ➔ Fas-FasL

Pro-Casp8 ➔ Pro-Casp10
