Novel Mechanism of Macrophage-Mediated Metastasis Revealed in a Zebrafish Model of Tumor Development

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Abstract

Cancer metastasis can occur at early stages of tumor development due to facilitative alterations in the tumor microenvironment. Although imaging techniques have considerably improved our understanding of metastasis, early events remain challenging to study due to the small numbers of malignant cells involved that are often undetectable. Using a novel zebrafish model to investigate this process, we discovered that tumor-associated macrophages (TAM) acted to facilitate metastasis by binding tumor cells and mediating their intravasation. Mechanistic investigations revealed that IL6 and TNFα promoted the ability of macrophages to mediate this step. M2 macrophages were particularly potent when induced by IL4, IL10, and TGFβ. In contrast, IFNγ-lipopolysaccharide–induced M1 macrophages lacked the capability to function in the same way in the model. Confirming these observations, we found that human TAM isolated from primary breast, lung, colorectal, and endometrial cancers exhibited a similar capability in invasion and metastasis. Taken together, our work shows how zebrafish can be used to study how host contributions can facilitate metastasis at its earliest stages, and they reveal a new macrophage-dependent mechanism of metastasis with possible prognostic implications. Cancer Res; 75(2); 1–10. ©2014 AACR.

Introduction

Metastatic disease is responsible for mortality of a majority of patients with cancer and metastatic lesions often exist upon cancer diagnosis, suggesting the early onset of the metastatic process (1). Clinically detectable metastases often represent the ultimate consequence of the complex metastatic cascade, which includes intravasation of malignant cells into the circulation, transport of tumor cells to distal organs, extravasation of tumor cells from the circulation, the formation of the initial metastatic nodules, and finally regrowth of microscopic lesions to clinically detectable masses (2, 3). Although detection of large metastatic masses can be achieved using advanced imaging techniques in combination of metabolic changes, such as PET-CT and MRI (4, 5), visualization and investigation of early events of cancer cell invasion including intravasation into the circulation remain challenging issues due to lack of appropriate and optimal preclinical models. These clinically relevant issues are particularly difficult to approach when primary tumors are small at the microscopic sizes. In a substantial number of patients with cancer, the malignant disease is initially diagnosed based on metastasis, and their primary tumors are not detectable (6). The existence of metastases without detectable primary tumors supports the fact that metastasis occurs when primary tumors are tiny.

We recently developed a zebrafish model to study the early events of metastasis when primary tumors are relatively small (2, 7). In that model, we investigated tumor cell behaviors in relation to tissue hypoxia. However, cancer metastasis often represents the ultimate consequences of interactions between tumor cells and various host cells. Particularly, tumor associated macrophages (TAM) have been demonstrated in mouse models to facilitate metastasis through a mechanism of intravasation (8, 9). Moreover, the M2 subtype of macrophages has been correlated with an invasive phenotype of cancers (10). Despite these claims, the detailed process of TAMs in promoting tumor cell invasion, interactions between TAMs and tumor cells, and the identity of molecular players in regulation of tumor invasion by macrophages are not fully understood. We aim to address these important issues in the current study by reconstituting the TAM-malignant cell–interactive microenvironment that recapitulates the clinical situation. We chose embryonic zebrafish as a model system by taking the following advantages: (i) immune privileges without causing rejections of human or mouse cells; (ii) the...
Materials and Methods

Zebrafish tumor model

All zebrafish experiments were approved by the Northern Stockholm Experimental Animal Ethical Committee. Zebrafish embryos of the transgenic strain expressing enhanced GFP (EGFP) under the flI promoter (Fli1:EGFP; ref. 11) were raised at 28°C under standard experimental conditions. Twenty-four hours after fertilization (hpf) zebrafish embryos were incubated in aquarium water containing 0.2 mmol/L 1-phenyl-2-thio-urea (PTU, Sigma) to prevent pigmentation. At 48 hpf, Fli1:EGFP zebrafish embryos were dechorionated with a pair of sharp-tip forceps and anesthetized with 0.04 mg/mL of tricaine (MS-222, Sigma). Anesthetized embryos were moved onto a modified agarose gel for microinjection. Before injection, tumor cells or macrophages were labeled in vitro with 2 g/mL of 1,1′-Diocadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Sigma) or Vybrant DiD cell-labeling solution (Life Technologies). Approximately 300–500 tumor cells or a mixture of equal numbers of 150–250 tumor cells and 150–250 macrophages were resuspended in serum-free DMEM (Hyclone) and 5 mL of tumor cell solution were injected into the perivitelline space (PVS) of each embryo by an Eppendorf microinjector (Femtojet 5247, Eppendorf and Manipulator MM33-Right, Marzhäuser Wetzlar). Nonfilamentous borosilicate glass capillaries needles were used for the injection. After injection, the zebrafish embryos were immediately transferred into PTU aquarium water. Injected embryos were kept at 28°C and were checked 4 days after injection for investigating tumor invasion and metastasis using a fluorescent microscope (Nikon Eclipse C1).

Tumor cell culture

Murine LLC (Lewis lung carcinoma) tumor cells transfected with TNFα and IL6 were kindly provided by Dr. Fumiaki Koizumi (The Japanese National Cancer Center Hospital, Tokyo, Japan). High expression levels of TNFα and IL6 were confirmed (PCR, ELISA) before experimental use. LLC cells were grown in DMEM supplemented with 20% FBS (HyClone) and cultured in DMEM supplemented with 10% FBS. All cell lines were not authenticated after purchase or transferred from other laboratories but were routinely tested negative for mycoplasma by using the Mycoplasma Detection Kit (Lonza).

Isolation, differentiation, and polarization of bone marrow–derived primary monocytes/macrophages

Bone marrow–derived monocytes/macrophages were isolated and cultured according to a previously published method (12). In brief, femoral bone marrow cells from C57BL/6 mice were collected, single-cell suspensions were prepared, and cultured in DMEM supplemented with 20% FBS and 20% macrophage colony-stimulating factor (M-CSF) conditioned supernatant from L929 cells for 10 days. Then, bone marrow-derived monocytes/macrophages were divided into 5 groups that received different treatments for 24 hours: (i) vehicle-stimulated cells; (ii) stimulated with 20 ng/mL TNFα; (iii) stimulated with 20 ng/mL IL6; (iv) stimulated with 50 ng/mL lipopolysaccharide (LPS) plus 20 ng/mL IFNγ; (v) stimulated with 20 ng/mL recombinant mouse IL4, IL10, and TGFβ1 (R&D Systems Inc.). Nonstimulated and stimulated cells were further analyzed by FACS.

Isolation, differentiation, and polarization of human monocytes

Human monocytes were isolated and purified from the mononuclear cell (PBMC) fraction of peripheral blood by buffy coats obtained from healthy volunteers (Clinical Immunology and Transfusion Medicine Department of Karolinska Institute, Sweden) using Ficoll–Hypaque (GE Healthcare) according to the manufacturer’s instructions. Monocytes/macrophages in the PBMC fraction were further purified using a CD14+ Selection Kit (Miltenyi Biotec), followed by culturing in the RPMI1640 medium (Gibco) containing 10% FBS. Monocytes were differentiated into macrophages for 6 days using a differentiation RPMI medium containing 10% FBS, 50 ng/mL of either M-CSF or granulocyte macrophage colony-stimulating factor (GM-CSF; BD Pharmingen). Then, human monocyte-differentiated macrophages were divided into 3 groups that received different treatments for 24 hours: (i) vehicle-stimulated cells; (ii) stimulated with 50 ng/mL LPS plus 20 ng/mL IFNγ; (iii) stimulated with 20 ng/mL recombinant human IL4, IL10, and TGFβ1 (R&D Systems Inc.). Nonstimulated and stimulated cells were further analyzed by FACS.

FACS analysis

Stimulated and nonstimulated macrophages were subjected to FACS using specific cell surface markers. Macrophages were detached from cell cultures by incubating with 2 mmol/L EDTA (Sigma) for 30 minutes at 37°C. Cells were stained with antibodies specific for surface markers: CD86, PDL1, PDL2, CD206, and isotypic IgG controls (all from BD Pharmingen). Cell samples were run in a Gallios flow cytometer (Beckman Coulter) and analyzed using the Kaluza v1.1 software (Beckman Coulter). Expression levels were quantified using median fluorescence intensity of the interested markers.

Mouse tumor experiments

All mice experiments were approved by the Northern Stockholm Experimental Animal Ethical Committee. C57BL/6 mice were bought from the breeding unit of the animal facility of Department of Microbiology, Tumor and Cell Biology, Karolinska Institute (Stockholm, Sweden). Approximately, 1 × 106 tumor cells in 100 μL PBS solution were subcutaneously implanted into the mid-dorsal region of each 6–8 weeks old male C57BL/6 mouse (n = 6–8 mice per group; refs. 13, 14). Before tumor implantation, mice were anesthetized by inhalation of isoflurane (Abbott Scandinavia). Tumor sizes were measured every other day, and tumor volumes were calculated according to a standard formula as previously described (15, 16). At the end of experiment, mice were euthanized, and tumor tissues were immediately used for the magnetic cell separation (MACS) sorting.
Macrophage-Dependent Metastasis in Zebrafish

Patient materials
Handling of fresh tumor tissue was performed at the Department of Clinical Pathology, Karolinska University Hospital (Stockholm, Sweden). Surgically resected fresh specimens (Table 1 and Supplementary Table S1) with tumors of sufficient size were selected for biopsy. A surgical pathologist punched 1–2 10 mm diameter tissue cores from viable tumor areas. The tumor core was directly used for MACS separation. Subsequently, tumor content within the biopsy area was confirmed by microscopic evaluation of hematoxylin/eosin–stained tissue sections. Handling of human tumor tissues for research purposes was approved by the Karolinska Biobank Review Board. Accordingly, all patient materials were anonymized before transferring to research laboratories.

Isolation TAMs by MACS separation
Subcutaneous wild-type (wt), TNFα- and IL6-LLC tumors and human tumor tissues were prepared as single-cell suspensions, followed by incubation for 40 minutes at 37°C with a PBS solution containing 0.15% collagenase I and collagenase II. Cells in suspensions were stained for 15 minutes at 4°C with an Alexa Fluor 647 rat anti-mouse F4/80 antibody (Biolegend) or a PE mouse anti-human CD163 antibody (BD Biosciences). After rigorous washing with 0.5% BSA and 2 mmol/L EDTA in PBS, cells were incubated with an anti-Alexa Fluor 647 Microbeads (Miltenyi Biotec) or anti-PE Microbeads (Miltenyi Biotec) for 15 minutes at 4°C. F4/80+ or CD163+ cells were sorted by MACS separation according to the manufacturer's instruction (Miltenyi Biotec). The isolated cells were labeled with DiD dye and were immediately used for microinjection. The PE rat anti-mouse PDL1 (eBioscience) antibody, the PE rat anti-mouse CD206 (Biolegend) antibody and anti-PE Microbeads were used for further sorting to obtain the M1 and M2 subpopulations from the total pool of the F4/80+ TAM population.

Statistical analysis
Statistical analysis was performed using the standard Student t test by a Microsoft Excel program. Data were presented as means of determinants (±SEM) and P values < 0.05 were considered as statistically significant.

Results
Development of a multicolor-based system to monitor interactions between tumor cells, macrophages, and microvessels
To recapitulate the tumor microenvironment of clinical situations, we aimed to investigate in zebrafish the interactions between tumor cells and macrophages in association with angiogenesis. In this system, Dil dye-labeled human and mouse tumor cells were implanted in the PVS (Supplementary Fig. S1A). Macrophages were labeled with DiD dye as a non-overlapping color. Dil-tumor cells and DiD-macrophages were co-implanted in PVS of the same zebrafish and interactions between these cell types could be kinetically monitored in the living zebrafish body (Supplementary Fig. S1A).

To validate this system as a functional model, we chose a murine T241 fibrosarcoma as an example although other tumor cells also produce similar results (Supplementary Fig. S1B). Implantation of T241 fibrosarcoma cells alone did not result in invasiveness and metastasis in this model at day 4 (Supplementary Fig. S1B). Dil-T241 tumor cells and DiD-labeled F4/80+CD206+ TAMs isolated from mouse LLC tumors were successfully coimplanted into the PVS of each zebrafish embryo. We chose a ratio of 1:1 tumor cell:TAMs as an optimal ratio for our study as this ratio gave rise to best detection of both cell types. At day 0 after tumor–TAM cell implantation, the growth of implanted in situ tumors was restricted in the injection site and no diffusion patterns were observed (Supplementary Fig. S1C). However, prolonged incubation of injected embryos for additional 4 days led to a broad metastatic pattern in the fish body (Supplementary Fig. S1C). Importantly, single metastatic tumor cells could be clearly visualized and their association with macrophages could be easily detected in overlapping colors in different parts of the zebrafish body (Supplementary Fig. S1C). Moreover, coinjection of color-labeled tumor cells and macrophages into the transgenic Fli1:EGFP zebrafish that labels vascular endothelial cells (11, 17) allowed us to study the complex interactions between three important cellular components in the tumor microenvironment, that is, tumor cells, macrophages, and vascular endothelial cells (Supplementary Fig. S1C). Thus, this multiple color system, in part, recapitulates the tumor microenvironment and provides a unique opportunity to investigate interactions between various cellular components in primary tumors in facilitating cancer metastasis.

TAMs isolated from IL6 and TNFα–positive tumors promote cancer metastasis
To study the role of TAMs in promoting cancer metastasis, TAMs were isolated from Lewis lung carcinomas (LLC) that were genetically propagated to overexpress IL6 and TNFα. Wild-type T241 fibrosarcoma tumor cells alone without TAMs was used a control. At day 4 after implantation of wt T241 tumor cells without TAMs, very few tumor cells were disseminated from the primary

Table 1. Correlation of TAMs with cancer invasion and metastases in human patients

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Clinical metastasis</th>
<th>TAM metastases in zebrafish</th>
</tr>
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<tbody>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>Metastatic spreading to uterus, peritoneal cavity, rectum, ovaries</td>
<td>Highly+</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>Lung metastasis</td>
<td>Highly+</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>Metastatic spreading to peritoneal cavity and local lymph nodes (6/27)</td>
<td>Highly+</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>Lymph nodes metastases (3/32)</td>
<td>Highly+</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Extensive metastatic spreading in peritoneal cavity</td>
<td>Highly+</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Invasion of cervical struma, both ovaries, omentum, metastatic spreading in two local lymph nodes</td>
<td>Highly+</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>No metastasis</td>
<td>Low+</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>No metastasis</td>
<td>Low+</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>No metastasis</td>
<td>Low+</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>No metastasis</td>
<td>Low+</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>No metastasis</td>
<td>Low+</td>
</tr>
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+High metastatic, spreading of tumor cells to different region of zebrafish body.
+Few invasive tumor cells restricted to the primary sites.
Coimplantation of T241 tumor cells with TAMs isolated from wt LLC tumors resulted in a significant albeit modest effect of tumor cell dissemination (Fig. 1B). Interestingly, most metastatic tumor cells were uncoupled with TAMs (Fig. 1B, F, and G), indicating that TAMs in wt type of tumors displayed a modest effect in facilitating metastasis. In contrast,
coimplantation of T241 tumor cells with TAMs isolated from IL6- or TNFα-expressing LLC tumors led to marked tumor cell dissemination and metastasis (Fig. 1C–E). In these coimplanted zebrafish embryos, metastatic tumors were detectable in most regions of the zebrafish body. Intriguingly, most metastatic tumor cells were coupled with injected TAMs (Fig. 1C, F, and G), which showed overlapping positive signals of TAMs and tumor cells. These results show that the interaction between IL6- or TNFα-stimulated TAMs and tumor cells is crucial for cancer metastasis. Thus, cancer metastasis occurs through a macrophage-dependent mechanism.

In the tumor microenvironment, isolated macrophages could be exposed to multiple factors and cytokines. To exclude the effects of other factors and cytokines, bone marrow–isolated primary monocytes were therefore stimulated with IL6 and TNFα in vitro and their abilities in facilitating metastasis were analyzed in zebrafish. Expectedly, coimplantation of primary monocytes with T241 tumor cells did not result in significant enhancement of tumor cell dissemination and metastasis (Fig. 2A). In contrast, coimplantation of IL6- and TNFα-activated monocytes significantly potentiated the capacity of tumor invasiveness and metastasis (Fig. 2). Again, most disseminated tumor cells were associated with IL6- or TNFα-stimulated macrophages (Fig. 2). These findings further validate our notion that both IL6 and TNFα significantly enhance metastatic potentials of tumor cells by activation of macrophages.

M2 TAMs facilitate metastasis

It is known that tumor tissues contain at least two distinct populations of macrophages, that is, M1 and M2 macrophages (10). To study the role of these two populations of macrophages in facilitating cancer metastasis in our zebrafish model, we isolated the F4/80+PDL1+M1 and F4/80+CD206+M2 TAMs from LLC tumors. Interestingly, coimplantation of F4/80+PDL1+M1-TAMs with T241 tumor cells did not result in metastasis (Fig. 3). In contrast, F4/80+CD206+M2-TAMs markedly promoted cancer metastasis, leading to wide spreading of tumor cells in the zebrafish body (Fig. 3). Notably, the disseminated tumor cells including distal metastatic tumor cells were associated with the implanted M2 macrophages, suggesting that the M2, but not M1, macrophages promote tumor cell dissemination by the mechanism of intravasation into the circulation.

Mouse and human M2, but not M1, macrophages promote tumor cells dissemination and metastasis

To further define macrophage subpopulations that were responsible for cancer metastasis, we employed an in vitro

**Figure 2.**

TNFα- and IL6-stimulated mouse bone marrow–derived monocytes promote metastasis. A, T241 tumor cells (TC; red) plus vehicle-stimulated bone marrow-derived monocytes (BMDM; green), tumor cells (red) plus IL6-stimulated BMDMs (green), or tumor cells (red) plus TNFα-stimulated BMDMs (green) were implanted in the PVS of each zebrafish embryo. Blood vasculatures are shown in blue. Dashed lines encircle primary tumor areas. Arrowheads, disseminated overlapping color (yellow) tumor cells (red) coupled with macrophages (green). Bar in top and middle panels, 500 μm. Bar in bottom panels, 100 μm. B, quantification of the total number of disseminated tumor foci, the number of disseminated tumor foci in the trunk region, the total number of BMDM-TC double-positive metastatic foci, and the percentage of the disseminated BMDM-TC double-positive foci in T241 tumor-bearing zebrafish embryos implanted with tumor cells together with vehicle-, IL6-, or TNFα-stimulated BMDMs (n = 11 embryos/group). Quantification was analyzed at day 4 after tumor implantation. NS, not significant.
approach to induce monocytes differentiation toward M1- or M2-specific phenotypes. In vehicle-stimulated primary bone marrow monocytes, expression levels of PDL1 and CD86 as M1-specific markers, and PDL2 and CD206 as M2-specific markers were very low (Fig. 4A), indicating that these primary monocytes remained in their undifferentiated stages. For differentiation of primary bone marrow monocytes towards M1, the well-defined agents consisting of a combination of IFNγ with LPS was used (18). As expected, stimulation of bone marrow monocytes with IFNγ and LPS remarkably increased expression levels of PDL1 and CD86 (Fig. 4A). In contrast, expression levels of PDL2 and CD206 in IFNγ-LPS-stimulated monocytes remained unchanged (Fig. 4A). These data show that stimulation with IFNγ and LPS induced a specific M1 macrophage phenotype in our model system. Conversely, stimulation of bone marrow primary monocytes with a combination of IL4, IL10, and TGFβ drifted monocyte differentiation towards a M2 phenotype (18) that expressed specific markers of PDL2 and CD206, but not PDL1 and CD86 (Fig. 4A).

The IFNγ-LPS-stimulated PDL1+ and CD86+ M1 macrophages were then coimplanted with wt T241 tumor cells in zebrafish. Interestingly, coimplantation of PDL1+ and CD86+ M1 with tumor cells did not alter the metastatic potential of T241 tumor cells (Fig. 4B and C). In sharp contrast, the IL4–IL10–TGFβ–stimulated M2 macrophages markedly augmented T241 tumor metastasis, leading to spread of tumor cells in the zebrafish body (Fig. 4B and C). Consistent with isolated TAMs from IL6 and TNFα tumor cells, most disseminated tumor cells were coupled with the IL4–IL10–TGFβ–stimulated M2 macrophages (Fig. 4B and C). These findings indicate that the M2 macrophages are essentially required for cancer metastasis through the mechanism of intravasation.

To further relate our findings to clinical relevance, we performed a similar M1 and M2 subpopulation experiments with human primary monocytes isolated from human peripheral blood. Similar to mouse primary monocytes, the primary human monocytes expressed M1- and M2-specific markers at very low levels (Fig. 5A). Stimulation with IFNγ plus LPS drifted human monocyte differentiation towards the M1 phenotype by expressing PDL1 and CD86, whereas the IL4–IL10–TGFβ–stimulated monocytes expressed the CD206 M2–specific marker (Fig. 5A). Again, coimplantation of the CD206 M2, but not PDL1–CD86+ macrophages, with human OVCAR8 ovarian tumor cells resulted in massive tumor cell dissemination in different parts of the zebrafish body (Fig. 5B and C). Similar to mouse M2 macrophages, the disseminated tumor cells were frequently associated with the human M2 macrophages, suggesting that human M2 macrophages facilitate metastasis through the intravasation process. Inversely, implantation of human OVCAR8 ovarian tumor cells alone caused very few metastases in this model.

Clinical correlation of TAMs isolated from metastatic cancers with invasion and metastasis

These preclinical findings promoted us to study whether TAMs isolated from tumors of human patients correlated with the invasiveness and metastasis in zebrafish. For this reason, human CD163+ macrophages were isolated from fresh tumor tissues of human patients with various types of cancers. The patient data were summarized in Supplementary Table S1. The isolated human tumor CD163+ macrophages were coimplanted with human OVCAR8 ovarian tumor cells into zebrafish to assess their invasive features. Figure 6 showed examples of TAM-mediated invasiveness and metastasis in zebrafish in correlation in patients...
with endometrial cancer. In a nonmetastatic human endometrial cancer, the isolated TAMs did not significantly enhance metastatic capacity of OVCAR8 ovarian tumors (Fig. 6). Interestingly, coimplantation of TAMs isolated from a metastatic endometrial cancer resulted in wide spreading of tumor cells in the zebrafish body (Fig. 6). Similar positive correlations also observed in other cancer types including colorectal cancer, ovarian cancer, breast cancer, and non–small cell lung cancer (Fig. 6; Table 1). These findings not only provide compelling evidence that invasive cancers metastasize through a macrophage-dependent mechanism, but also validate the fact that our zebrafish model is a reliable system to predict metastatic potentials of human cancers.

Discussion

The formation of malignant cells is not sufficient to develop clinically manifested cancer disease and interactions of tumor cells with host cells are crucial for tumor growth, invasion, and distal metastasis. In the tumor microenvironment, the host structural and cellular components including vasculatures, inflammatory cells and stromal fibroblasts are key determinants for tumor growth and metastasis. Drugs targeting these host components have been proven to be effectively therapeutic approaches for cancer therapy in human patients. For example, drugs that block tumor angiogenesis are successfully used for treatment of various human cancers (19–21). Similarly, inhibition of inflammation-related signaling pathways is also effective for treatment of cancers in animal models and human patients (22, 23). Despite the abundant knowledge of these host molecular and cellular components in facilitating tumor growth and metastasis, current prognosis of cancer disease progression is mainly based on pathologic changes of malignant cells. Consequently, the role of tumor inflammation and angiogenesis in predicting cancer invasion and survival has been overlooked in human patients.

The primary aim of our present study is to reconstitute the tumor microenvironment that permits to investigate the role of macrophages in promoting cancer metastasis during the early stage of cancer development. Macrophages are probably one of the initial host cell types that interact with tumor cells at the early stage of cancer development. Tumor cells often cause an early inflammatory response at the site where they originated (24). Although infiltration of inflammatory macrophages is believed to combat tumor cells, these same cells could potentially be manipulated by malignant cells for promoting tumor growth and invasion. To date, no optimal animal models are available to

Figure 4. M2 but not M1 macrophages promote cancer metastasis. A, FACS analysis of PDL1 and CD86 as M1 macrophage markers, and PDL2 and CD206 as M2 macrophage markers in vehicle-, LPS-IFNγ-, or TGFβ-IL4-IL10-stimulated BMDMs. MFI, median fluorescence intensity. B, T241 tumor cells (TC; red) plus vehicle-stimulated bone marrow-derived monocytes (BMDM; green), tumor cells (red) plus LPS–IFNγ-stimulated BMDMs (green), or tumor cells (red) plus TGFβ–IL4–IL10-stimulated BMDMs (green) were implanted in the PVS of each zebrafish embryo. Blood vasculatures are shown in blue. Dashed lines encircle primary tumor areas. Arrowheads, disseminated overlapping color (yellow) tumor cells (red) coupled with macrophages (green). Bar in top and middle panels, 500 μm. Bar in bottom panels, 100 μm. C, quantification of the total number of disseminated tumor foci, the number of disseminated tumor foci in the trunk region, the total number of BMDM-TC double-positive metastatic foci, and the percentage of the disseminated BMDM-TC double-positive foci in T241 tumor-bearing zebrafish embryos implanted with tumor cells together with vehicle-, LPS-IFNγ-, or TGFβ–IL4–IL10-stimulated BMDMs (n = 11 embryos/group). Quantification was analyzed at day 4 after tumor implantation; NS, not significant.
study the role of TAMs in promoting tumor invasion and metastasis at the early stage of the tumor formation. In the current study, we have developed a multicolor detection system in zebrafish, which allows us to kinetically study the role of macrophages in promoting cancer invasion and metastasis at the single-cell level. One of the most significant and novel findings is that TAMs hijack tumor cells to distal regions when primary tumors are microscopic sizes. In human and mouse tumor models, we have shown that the TAMs are the key determinant for cancer metastasis. Without TAMs, tumor invasion and dissemination are largely impaired. Thus, our findings reveal a novel macrophage-dependent mechanism through which tumor cells metastasize to distal tissues and organs. Our findings also support some data reported in mouse tumor models that TAMs facilitate intravasation of tumor cells into the circulation (25, 26).

Although the exact detailed mechanism that underlies TAM-mediated tumor cell dissemination and metastasis remains unidentified at this writing, our current findings demonstrate that macrophages physically interact with tumor cells. In theory, physical interactions between TAMs and tumor cells would make it more difficult for tumor cell migration and intravasation. However, tumor cell–carrying macrophages might be able to further interact with and manipulate endothelial cells that permit intravasation of the tumor cell–macrophage complex into the circulation. Perhaps, macrophages produce proteases such as matrix metalloproteinases (MMP) that break down the basement membrane around the endothelium that becomes more susceptible for tumor cell invasion. The fact that the most disseminated tumor cells are coupled with macrophages supports this view. Also, it is known that the activated macrophages produce high levels of MMPs including MMP2 and MMP9 (27), which are known to facilitate cancer invasion in mouse tumor models. Another important notion of our current work is that the M2, but not M1, macrophages are primarily responsible for cancer cell dissemination. In addition to M2 macrophages isolated from tumor tissues, the IL4–IL10–TGFβ–induced M2 macrophages could also increase tumor invasion and metastasis. Therefore, inhibition of these cytokine-triggered signaling pathways could potentially provide new therapeutic options for treatment and prevention of cancer invasion and metastasis. These possibilities warrant clinical validation. Another interesting notion from the published work shows that NADPH oxidases regulate polarization of macrophages (28–30) and thus interference of NADPH oxidases would potentially inhibit the M2 macrophage transition.
In a limited number of human cancer patients, we are able to perform a proof-of-concept pilot study using freshly isolated TAMs from human tumors. In this double-blind pilot study, we have found a positive correlation of cancer metastasis in human patients and TAM-mediated metastasis in our zebrafish model. These findings not only support the fact that our zebrafish metastasis model is a reliable system, but also provide a possible new functional platform to predict cancer metastasis in human patients. In addition, this platform could be potentially be used for screening new therapeutic agents that block cancer invasion and metastasis. Taken together, our present works provides mechanistic insights on the role of TAMs in facilitating cancer metastasis and a new functional tool to predict cancer metastasis by investigating the behavior of TAMs. On the basis of this reliable model, new therapeutic drugs that block cancer invasion and metastasis could potentially be developed by targeting inflammatory cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X.-M. Zhang, R.A. Harris, Y. Sun, Y. Cao
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Wang, Z. Cao, X.-M. Zhang, M. Nakamura, M. Sun, J. Hartman, R.A. Harris, Y. Cao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Wang, X.-M. Zhang, J. Hartman, Y. Cao
Writing, review, and/or revision of the manuscript: J. Wang, X.-M. Zhang, J. Hartman, R.A. Harris, Y. Cao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Wang, Y. Cao
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