Tumor-educated Macrophages Promote Tumor Growth and Peritoneal Metastasis in an Orthotopic Nude Mouse Model of Human Pancreatic Cancer

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Abstract. *Background: Macrophages promote tumor growth by stimulating tumor-associated angiogenesis, cancer-cell invasion, migration, intravasation, and suppression of antitumor immune responses. Materials and Methods: Ten transgenic nude mice, ubiquitously expressing green fluorescent protein (GFP), were injected subcutaneously with the human pancreatic cancer cell line, BXPC3, stably expressing red fluorescent protein (RFP). GFP-expressing macrophages from the GFP mice with the subcutaneous BxPC3-RFP tumor were harvested and defined as "tumor-educated macrophages". Macrophages were also harvested from transgenic GFP mice (n=10) without tumors and identified as "naïve macrophages." The tumor-educated and naïve macrophages were then implanted into BxPC-3-RFP tumor-bearing non-transgenic nude mice and compared for their ability to enhance tumor progression. Results: In the control group, without macrophage injection, the average primary tumor weighed 668 mg and only three mice (30%) developed peritoneal metastases, which averaged 72 mg. The naïve-macrophage group had an average tumor weight of 823 mg (p=0.51) and 50% developed peritoneal metastases, whose weight averaged 975 mg (p=0.029). The group treated with tumor-educated macrophages had an average primary tumor weight of 2095 mg (p=0.001) and 75% of mice developed peritoneal metastases, whose weight averaged 2135 mg (p=0.008). Conclusion: These results suggest that macrophages influence tumors, and tumors influence macrophages, and tumor-educated promote tumor progression. Tumor-educated macrophages may be a target for therapy of metastatic cancer.*

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The tumor microenvironment (TME) is increasingly being shown to influence tumor behavior. A striking example is orthotopic implantation in mouse models of intact tumor tissue, including the entire tumor stroma, complete with fibroblasts, macrophages, and other hematopoetic cells, which results in a clinically relevant model, especially with regard to metastasis, in contrast to orthotopic injection of cancer cells alone, where metastasis is rare (1). The present report focuses on the role of macrophages in tumor progression.

In pathologic tumor specimens, a high density of tumor-associated macrophages correlates with poor prognosis in several studies (2, 3). There is an increasing body of evidence linking cancer with chronic inflammation, a process that depends on macrophage production of inflammatory cytokines (3). For instance, Balkwill *et al*. showed reduction of cancer risk by treatment with anti-inflammatory drugs (4). Tumors are able to recruit macrophages to create a microenvironment that causes suppression of immune functions (4). Macrophages may promote tumor progression by chronic inflammation, matrix remodeling, promotion of tumor-cell invasion, intravasation, angiogenesis, and seeding at distant sites (4). One recently proposed mechanism suggests that vascular cell adhesion molecule-1 (VCAM-1)
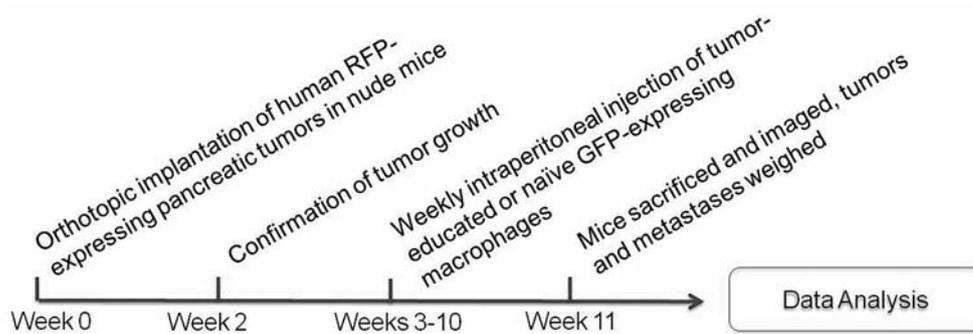
Figure 1. *Timeline of experiments. Mice underwent orthotopic implantation of BxPC3-RFP human pancreatic tumor at week 0. The presence of tumor was confirmed in each mouse at week 2 using a fluorescent light source. Green fluorescent protein (GFP) expressing macrophages from either naïve or tumor-educated mice were harvested and added via intraperitoneal injection to the appropriate study group once weekly for weeks 3-10. At week 11, mice in the tumor-educated group became pre-morbid and the experiment was ended. Please see Materials and Methods for details.*

promotes lung metastasis in breast cancer by tethering cancer cells to lung metastasis-associated macrophages (5).

There have been several experiments that focus on blocking single cytokines or co-factors associated with macrophages in an attempt to determine how tumor-associated macrophages lead to enhanced tumor progression. For example, the macrophage growth factor, colony-stimulating factor (CSF-1), has been studied by several groups. A knock-out of CSF-1 function leads to a reduced rate of tumor progression and almost complete suppression of tumor metastasis in a mouse model of breast cancer. In contrast, overexpression of CSF-1 accelerated tumor progression and metastasis in the mouse model (6). In other experiments, elimination of signaling factors involved in inflammation, such as nuclear factor kappa-B (NF-κB), showed that this signaling pathway is important for tumor initiation and growth (7).

In the present study, we compare primary and metastatic tumor growth following the addition of either unexposed, *i.e.* tumor-naïve macrophages, or macrophages previously exposed to pancreatic cancer in a mouse model, *i.e.* tumor-educated macrophages. We utilize multicolored fluorescent proteins to color code macrophages and tumors using imaging technology pioneered by our laboratories (8-10). The goal of this study was determine whether increasing the inflammatory potential within the TME, particularly with tumor-educated macrophages, would lead to increased tumor growth and metastasis.

Materials and Methods

Cell culture. The human pancreatic cancer cell line BxPC-3, stably expressing red fluorescent protein (RFP), was maintained in RPMI (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA). All cell lines were cultured at 37°C with 5% CO_2.

Animal care. Athymic nu/nu mice were maintained in a barrier facility on high-efficiency particulate air (HEPA)-filtered racks at AntiCancer Inc. The animals were fed with autoclaved laboratory rodent diet (Teckland LM-485; Western Research Products, Orange, CA, USA). All surgical procedures and intravital imaging were performed with the animals anesthetized by intramuscular injection of 0.02 ml of a solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals under PHS Assurance Number A3873-1

Orthotopic tumor implantation. Models of human metastatic pancreatic cancer were established in nude mice by transplantation of tumor fragments of the BxPC-3 tumor, stably expressing RFP, harvested from subcutaneous tumors previously established in the flank of nude mice. A small incision was made in the left pararectal line through the skin and peritoneum. The tail of the pancreas was exposed and a 1 mm tumor fragment was sutured into the tail of the pancreas using 8-0 monocryl sutures (11-13).

Macrophage harvest. Two groups of mice were established for macrophage harvest: i) a naïve-macrophage donor group which consisted of transgenic nude mice engineered to express green fluorescent protein (GFP) in all cells (n=10), and ii) a tumor-educated macrophage donor group which consisted of the GFP-expressing nude mice with established subcutaneous BxPC3-RFP tumors (n=10). Each week the GFP-expressing nude mice from either the naïve- or tumor-educated donor groups were anesthetized using intramuscular injection of 0.02 ml of a solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. RPMI-1640 medium (6 ml, room temperature) was then injected into the intraperitoneal (*i.p.*) space of each mouse using a 10 ml syringe and the mice were gently massaged for 5 minutes. The syringe was then reinserted into the *i.p.* space and all possible RPMI-1640 medium was removed and placed into a plastic petri dish. The dishes were then incubated at 37°C for 4 hours. RPMI-1640 medium was then removed and each plate was washed 10-15 times with 10 ml PBS, or until all red blood cells, fibroblasts and other cellular debris was removed. Plates were visualized under a fluorescence microscope
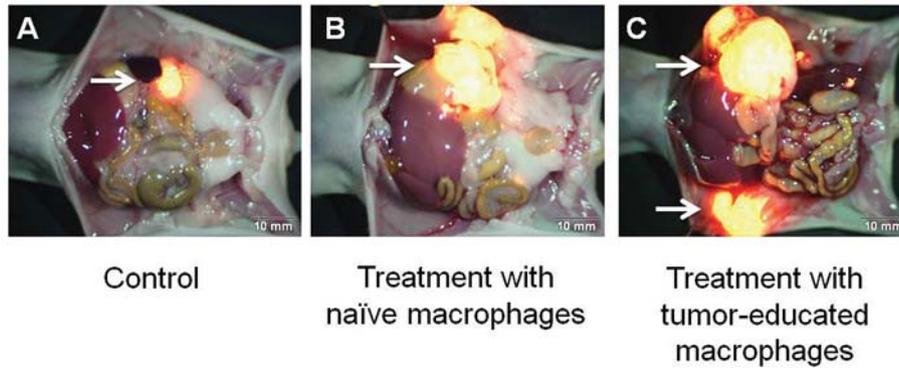
Figure 2. *Red fluorescent protein (RFP) tumor growth in control compared with naïve and tumor-educated macrophage-treated mice after eight weeks of macrophage treatment (representative mice). a: Control mouse with small primary tumor foci and no evidence of metastasis. b: mouse treated with naïve-macrophages with large primary tumor and local peritoneal metastasis (arrow). c: mouse treated with tumor-educated macrophages with large primary tumor and distant peritoneal metastases (arrows).*
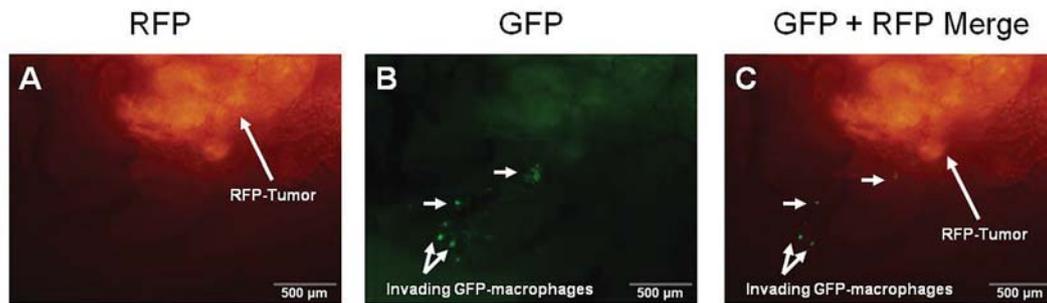

Figure 3. *Interaction of GPF-expressing macrophages with RFP-expressing BxPC3 human pancreatic tumor in a live animal imaged with the Olympus OV-100, 24 hours after intraperitoneal injection of GFP-expressing macrophages. a: Orthotopic pancreatic tumor imaged under an RFP filter. b: A GFP filter was used to image the same region as shown in panel a in order to capture invading macrophages. c: Overlay images of panels a and b, showing invading macrophages approaching the pancreatic tumor.*

in order to confirm the presence and approximate number of GFP-expressing macrophages which had adhered to the dish. Approximately 10^6 macrophages were harvested per mouse. Using a rubber spatula, macrophages were then scraped from the petri dish, collected in 10 ml PBS, and placed in a 50 ml plastic tube. Samples from each group were pooled into the same collecting tube and pelleted using standard centrifugation techniques for 10 minutes. PBS was then removed, macrophages were resuspended in 100 μl PBS per mouse and injected *i.p.* into the appropriate mice in each study arm.

Study arms. Orthotopic tumors were allowed to grow for 14 days, at which time the presence of tumor was confirmed by non-invasive fluorescence imaging. The mice were then separated into the following three groups: i) a control group whose tumors were allowed to grow without addition of macrophages (n=10); ii) a 'naïve', which received weekly *i.p.* injection of 10^6 GFP-expressing macrophages, from non-tumor-bearing mice, per mouse (n=10); and iii) a tumor-educated group, which received weekly *i.p.* injection of 10^6 GFP-expressing macrophages, from tumor-bearing mice, per mouse (n=10).

Imaging of animals and of macrophage-tumor interactions. Mice were imaged using the OV-100 Small Animal Imaging System (Olympus Corp, Tokyo, Japan). To image the interaction of GFP macrophages with RFP BxPC3 human pancreatic tumors, mice underwent laparotomy 24 hours after *i.p.* injection of GFP-expressing macrophages. The orthotopic pancreatic tumor was imaged in the live animal with the OV-100 under an RFP filter. A GFP filter was used to image the same region in order to capture invading macrophages. Overlay images of the GFP and RFP panels were utilized to demonstrate invading macrophages approaching the pancreatic tumor.

Study endpoint. Mice were monitored for signs of a pre-morbid state, at which time the mice were sacrificed by CO_2 asphixiation. At termination, animals were weighed, imaged, and both primary tumors and metastases were weighed and recorded. A timeline of the study is illustrated in Figure 1. The study ended with termination of the group treated with tumor-educated macrophages after approximately 11 weeks due to a pre-morbid state identified in all mice in this group. This included cachexia, lethargy, visibly large external tumors, and decreased appetite. All mice in each of the

three study arms were sacrificed at that time, imaging was performed, and total tumor weight was obtained.

Data analysis. Primary tumor and metastases weights were analyzed using Microsoft Excel and groups were compared using the Student's *t*-test.

Results and Discussion

After eight weeks of weekly macrophage treatments, the tumor-educated macrophage-treated group were cachectic, lethargic, had restricted movement due to large-volume ascites and tumor size, and had poor nutritional intake. Two mice from this group died before tumor weights could be obtained. Conversely, mice in both the control and naïve-macrophage-treated groups were active, eating well, with no signs of illness, although tumors were externally visible in the majority of mice in the latter group. All remaining mice were sacrificed on week 11 post-tumor implantation, following eight rounds of weekly *i.p.* macrophage injections.

All animals were imaged using the OV-100, and total body weight, primary tumor weight, and metastatic tumor weight were recorded for each specimen. A representative image of a single mouse from each group is illustrated in Figure 2. Fluorescence-imaging confirmed that all mice had visible tumors two weeks following orthotopic implantation.

In the control group, all ten mice survived the experiment. Two mice had only sub-millimeter primary or metastatic tumors at necropsy that were too small to be weighed. The average primary tumor in the control group weighed 668 mg and only three mice (30%) developed peritoneal metastases, with an average weight of 72 mg.

In the naïve-macrophage-treated group, there were nine surviving mice. One mouse was found dead at week four post-implantation and although tumor was present, it did not appear to be the cause of death. The average tumor weight in the surviving animals in this group was 823 mg versus 668 mg in the control mice (p=0.51). Five out of nine mice developed peritoneal metastases, with an average weight of 975 mg compared to three of 10 mice in the control group, with an average weight of 72 mg (p=0.029).

There were eight surviving mice in the group treated with tumor-educated macrophages. Two succumbed due to tumor burden, but data could not be obtained on these animals. The surviving animals had an average primary tumor weight of 2085 mg compared to 668 mg in the control group (p=0.001). Six out of eight mice (75%) developed peritoneal metastases, which averaged 2135 mg compared to three of ten mice in the control group with an average weight of 72 mg (p=0.008). It was possible to see GFP-expressing macrophages interacting with established RFP-expressing pancreatic tumors as early as 24 hours after the injection of macrophages (Figure 3).

When comparing groups treated with naïve or tumor-educated macrophages, the primary tumor weight was significantly greater in the tumor-educated macrophage-treated group (p=0.003). The average weight of metastases was 2.2 times greater. However, this did not reach statistical significance (p=0.17).

It has been repeatedly shown that there is a strong clinical association between poor survival and increased macrophage density in thyroid, lung, and hepatocellular cancer (14-16). In this study, we developed a nude mouse model of human pancreatic cancer, which demonstrates that the addition of macrophages previously exposed to pancreatic cancers, tumor-educated macrophages, specifically promotes tumor growth and peritoneal metastasis, compared to naïve-macrophages. The results of the present study may explain the relationship between macrophage density in tumors and poor prognosis in clinical cancer.

Future experiments will explore the mechanism of tumor education of macrophages and their subsequent ability to promote tumor growth and metastasis.

Acknowledgements

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