Introduction

Pancreas adenocarcinoma remains one of the most lethal malignancies with an expected 5-year survival of <5%. There are close to 40,000 new diagnoses each year and a similar number of deaths making it the fourth most common cause of cancer related death in the United States[1]. Despite significant progress in breast cancer, colon cancer and melanoma, there has been no appreciable change in the mortality from pancreas cancer in the past four decades. This is due in large part to its tendency to metastasize early, its resistance to chemotherapy and its ability to evade immune detection. While the exact mechanism of evasion is incompletely understood, there appears to be a relative increase in the number of immunosuppressive elements both in the tumor microenvironment and systemically. While previous studies investigated the role of regulatory T-cells (Treg) [2-4] more recent focus has been on the accumulation of myeloid derived suppressor cells (MDSC). What drives the accumulation of these cells is a subject of active investigation.

Exosomes are 30-120nm vesicles originally discovered as a mechanism by which reticulocyte shed unwanted cell surface proteins and unneeded organelles during maturation to erythrocytes[5,6]. They form as a result of programmed invagination of the cell surface lipo-proteinmembrane during the endocytic pathway. The limiting membrane of the endosome undergoes endoluminal budding creating multiple vesicles within the endosome. These multivesicular bodies (MVB) fuse with the cell surface releasing the lipid vesicles which are termed exosomes into the surrounding stroma and circulation. Due to their unique genesis, exosomes contain trapped samples of cytosolic elements such as mRNA and microRNA as well as cell surface lipids and proteins from their parental cell. Both cytosolic and cell surface components of exosomes are enriched with specific proteins, lipids and nucleic acids, which aids in their identification and purification. The most abundant surface proteins on exosomes are a family of adhesions molecules called tetraspanins (CD9, CD63 and CD81)[7].

Exosome release has been identified from many normal cells including immune cells (T-cells, B-cells, dendritic cells), epithelial cells, neurons and muscle cells. In recent years, research has shifted from the physiologic role of microvesicles to identification and characterization of tumor-derived exosomes. In the past decade, exosomes have been isolated from various cancer cell lines including glioma, breast, ovarian, mesothelioma, melanoma and colon cancers[7]. They have been implicated in tumor progression and metastases by multiple mechanisms including, promotion of angiogenesis, elimination of chemotherapeutic agents, education of bone marrow derived stem cells and most notably, immune suppression.

We hypothesize that pancreas cancer derived exosomes are responsible for the expansion of MDSCs both in the local tumor microenvironment and systemic circulation. This occurs by direct contact with exosome bound proteins or via autocrine or paracrine exosome mediated cytokine release. We will use human pancreatic cancer cell lines and peripheral blood mononuclear cells (PBMC) to investigate the role of tumor-derived exosomes in MDSC expansion and function.

Methods

Exosome isolation: Following 48 hours of culture in RPMI supplemented with 10% exosome depleted FBS, 75mL of media was harvested and subjected to serial centrifugation as previously described[8]. Briefly, supernatant was centrifuged at 2000g x 10 min to clear cells and 10,000g x 30 min to remove cellular debris. The resultant supernatant was centrifuged at 100,000g x 70 min with the pellet containing exosomes. Exosome-free supernatant was saved for use as control. The exosomes were washed of

contaminating proteins with PBS and after a second ultracentrifugation, resuspended in PBS. Protein concentration was measured by Bradford assay and purity of isolation confirmed by dynamic light scatter and nanoparticle tracking analysis and by western blot for the exosome specific markers.

Immune cell culture:
PBMC were harvested from the buffy coat of various healthy donors by Ficoll gradient. After red blood cell lysis, PBMC were washed and placed in to RPMI media prepared with exosome free FBS. Cells were cultured in the presence or absence of exosomes and collected at various time points to determine phenotype. To determine effect on differentiation, bone marrow derived cells with or without tumor derived exosomes were cultured in low or high dose GM-CSF to force maturation to dendritic cells or macrophages, respectively. Cells were phenotyped after 4 days in culture.

Flow cytometry: Cells were harvested, washed and suspended in FACS buffer. Surface staining was performed to phenotype cells using antibodies to CD45, CD3, CD11b, CD11c, CD14 and CD 33. For cytokine staining, cells were activated with PMA/ionomycin in the presence of golgi stop and plug for 4 hours. After washing, fixing and permeabilizing, cells were stained for intracellular cytokines.
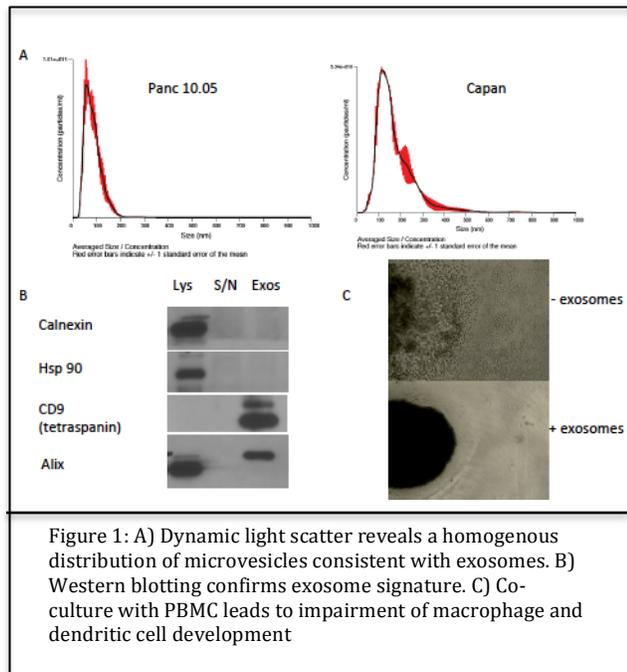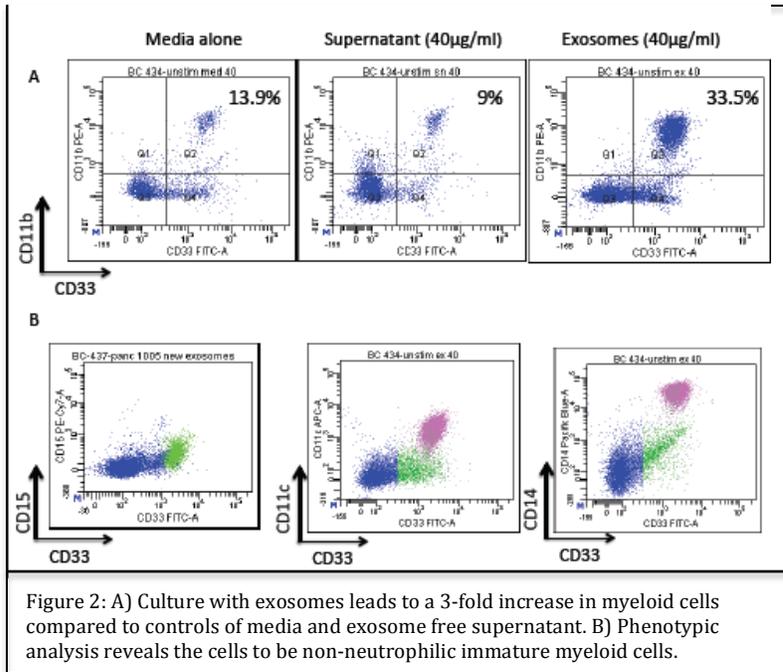

Figure 1: A) Dynamic light scatter reveals a homogenous distribution of microvesicles consistent with exosomes. B) Western blotting confirms exosome signature. C) Co-culture with PBMC leads to impairment of macrophage and dendritic cell development

Results:

Highly pure exosomes can be harvested from pancreatic cancer cell lines using ultracentrifugation. After 4 days in culture, the supernatant from pancreatic cancer cell lines was subjected to serial centrifugation. The resultant material was diluted in PBS and subjected to analysis with dynamic light scatter and nanoparticle tracking analysis using the Nanosight LM10 (Malvern, Worcestershire, UK). Because exosomes are 30-120nm in size, they are not visible with standard microscopy. By focusing a laser onto the colloidal suspension, scattered light can be collected from individual microvesicles and by measuring Brownian motion, size determined. Assessment of various cell lines determined the concentration to be 6.10e+012 +/- 2.92e+011 particles/75ml media and 6.84e+012 +/- 1.09e+011 particles/75ml media for the pancreatic cancer cell lines Panc 10.05 and Capan, respectively. Analysis of the size distribution determined the particle population to be very pure with mean sizes of 84nm and 103nm (Figure 1a). To confirm the identity of the microvesicles, western blotting was performed and confirmed presence of the exosome specific markers CD9 and Alix, but not the cytoplasmic proteins calnexin and HSP90 (Figure 1b).

Culture with pancreatic cancer derived exosomes leads to an immature monocytic phenotype. PBMC were harvested from multiple donors and cultured with or without exosomes. After 4 days, cells were harvested for phenotypic analysis. Visual inspection

of culture plates revealed very few adherent cells in PBMC cultured with exosomes compared to control (Figure 1c). FACS analysis revealed an expansion of myeloid cells as shown by a 3-fold increase in CD11b$^+$CD33$^+$ staining (Figure 2a). Further phenotypic analysis confirmed that the cells were not neutrophils (CD15$^-$) but represented undifferentiated monocytes

Figure 2: A) Culture with exosomes leads to a 3-fold increase in myeloid cells compared to controls of media and exosome free supernatant. B) Phenotypic analysis reveals the cells to be non-neutrophilic immature myeloid cells.

(CD33$^+$CD11b$^+$CD11c$^+$CD14$^+$) (Figure 2b). CFSE stained activated lymphocytes were then cultured in the presence of these immature myeloid cells and demonstrated impaired activation and proliferation.

Pancreas cancer derived exosomes arrest myeloid differentiation. To determine if the increased monocytic population represented failure of cells to differentiate, monocytes were sorted from PBMC and cultured in the presence of low and high dose GM-CSF to drive differentiation into dendritic cells or macrophages, respectively. In the presence of exosomes, fewer monocytes were driven towards a

Figure 3: Exosomes have little effect on the cytokine profile of sorted lymphocytes, but appear to force CD3+ cells to a CD4+ phenotype.

macrophage differentiation compared to control (18 vs 56%, respectively). The same was not seen for differentiation of dendritic cells in low dose GM-CSF where the phenotype remained essentially unchanged (35% and 37%, respectively).
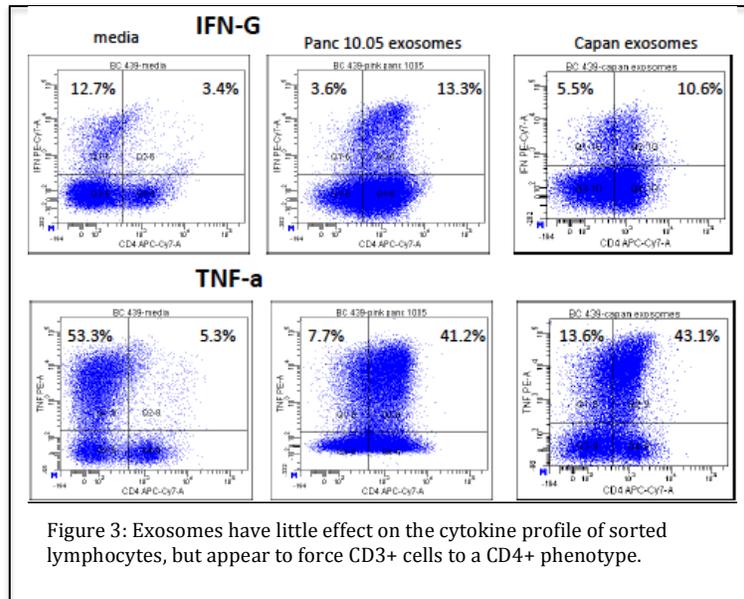
Exosomes do not alter proliferation or apoptosis of myeloid cells. Myeloid cells were separated from human PBMC and stained with the proliferation marker CFSE. After culture with various concentrations or exosomes, no increased cellular proliferation was

observed. After similar culture conditions, myeloid cells were assayed for apoptosis using the marker Annexin V. There was no observed difference in myeloid apoptosis after exosome culture.

Lymphocytes cultured with pancreas cancer derived exosomes were driven to a CD4+ phenotype with no change in cytokine secretion. To determine the effect of pancreas cancer derived exosomes on lymphocytes, CD3+ cells were sorted from PBMC by negative selection. After incubation for 24 hours in exosomes derived from Panc 10.05 and Capan cell cultures, lymphocytes were activated with PMA/Ionomycin for 4 hours and cytokines measured using flow cytometry. While exosome cultured cells were driven to a CD4 phenotype, cytokine secretion remained the same as demonstrated by FACS for IL-2, IL-6, IFN-G and TNF-alpha (Figure 3).

Discussion
Despite significant progress in breast cancer, colon cancer and melanoma, there has been no appreciable change in pancreas cancer mortality in the past four decade. Key factors contributing to poor cancer specific outcomes include its relative resistance to modern chemotherapy and radiation and its proclivity towards metastasis early in tumor development. Along with these factors, the pancreas microenvironment is composed of suppressive immune elements which render immunotherapeutics ineffective. There appears to be an abundance of immature myeloid cells which function to suppress cytotoxic lymphocytic activity.

Gabitass et al. measured circulating MDSCs and Tregs from 131 patients with pancreas, esophageal and gastric cancers[9]. Authors identified a significantly greater proportion of MDSCs in patients with pancreas cancer compared to non-cancer controls as well as higher levels of circulating Arginase 1. Of note, patients with a larger population of MDSCs had a worse overall survival compared to those with normal levels. A similar study by Khaled et al. found higher levels of MDSCs in both the circulation and tumors of patients with pancreatic cancer compared to non-cancer controls[10]. There are multiple possible factors which may contribute to this population expansion including: 1) stimulation of proliferation, 2) resistance to apoptosis, 3) increased bone marrow production of myeloid progenitors and 4) impairment of myeloid differentiation into mature dendritic cells and macrophages. While cytokines remain the most widely studied mediator of expansion, recent studies have looked at the role of tumor-associated microvesicles in MDSC function and expansion including tumor-derived exosomes[6,7].

Exosomes are 30-100nm vesicles originally discovered as a mechanism by which reticulocytes shed unwanted cell surface proteins and unneeded organelles during maturation to erythrocytes[5]. Recent studies have focused on the ability of tumor-derived exosomes to promote the expansion of immunosuppressive regulatory T-cells and MDSCs. Xiang et al.[11] found a 3-fold increase in splenic MDSCs in mice after repeated intravenous injections of breast cancer derived exosomes. The proposed mechanism for this expansion was exosomal prostaglandin (PGE2) and TGF-B as demonstrated by abrogation of MDSC population changes upon administration of blocking antibodies. A similar study by Chalmin et al.[12] found that interactions with colon cancer derived exosomes resulted in activation of MDSCs as determined by heighted ability to suppress antigen specific T-cell function.

In the current report, we investigated the impact of pancreas cancer derived exosomes on immune cells to assess their ability to promote immunosuppression. Using serial centrifugation, exosomes could be reliably harvested from various pancreas cancer cell lines. Using dynamic light scatter and nanoparticle tracking we were able to determine

that we had achieved a pure population of exosomes devoid of other microvesicles including cellular blebs and apoptotic bodies. Immunoblotting confirmed their identity as exosomes.

While untreated PBMC in culture tend to create an adherent cell population on the bottom of culture wells, addition of exosomes abrogated this phenomenon. To determine the cause of this, cells were subjected to phenotyping using cell surface markers for lymphocytes (CD3), NK cells (CD56), myeloid cells (CD33 and CD11b), neutrophils (CD15), macrophages (CD14) and dendritic cells (CD11c). We were able to determine that culture with exosomes resulted in expansion of an immature monocytic population which expressed all myeloid lineage markers with the exception of CD15 and was not adherent. There was a proportional decrease in the adherent mature macrophages and dendritic cells.To determine if these cells were immunosuppressive, they were cultured in the presence of activated lymphocytes and found to impair lymphocytic expansion, a feature characteristic of myeloid derived suppressor cells.

Increased immature monocytes can be due to three potential causes: 1) expansion, 2) resistance to apoptosis, 3) failure of progression to mature myeloid cells such as macrophages and dendritic cells. Simple tests for proliferation and apoptosis failed to reveal any effect of exosomes on myeloid survival or death. To determine the latter, monocytes were forced down differentiation paths using varying doses of GM-CSF. Exosomes were able to effectively impair macrophage differentiation, although they had little effect on dendritic cell formation.

An interesting observation was found with respect to the impact of exosomes on lymphocytes. After co-culture, there was no increase in cytokine production, but cells shifted to a CD4 phenotype. This will be the source of further investigation in the future.

In conclusion, it has been well established that the immune microenvironment of pancreas cancer if rife with suppressive cells including MDSCs. We have demonstrated that pancreatic cancer derived exosomes can contribute to this suppressive phenotype by arresting myeloid differentiation leading to accumulation of immunosuppressive immature monocytes. Future efforts to derail tumor exosome production and/or signaling could result in reduced peritumoral immunosuppression and improved efficacy of immunotherapeutics.

Reference:

1   Hidalgo, M. Pancreatic cancer. *The New England journal of medicine* **362**, 1605-1617, doi:10.1056/NEJMra0901557 (2010).
2   Liyanage, U. K. *et al.* Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *Journal of immunology* **169**, 2756-2761 (2002).
3   Moo-Young, T. A. *et al.* Tumor-derived TGF-beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer. *Journal of immunotherapy* **32**, 12-21, doi:10.1097/CJI.0b013e318189f13c (2009).
4   Viehl, C. T. *et al.* Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Annals of surgical oncology* **13**, 1252-1258, doi:10.1245/s10434-006-9015-y (2006).
5   Harding, C., Heuser, J. & Stahl, P. Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *European journal of cell biology* **35**, 256-263 (1984).

6   Pan, B. T. & Johnstone, R. M. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**, 967-978 (1983).

7   Thery, C. Exosomes: secreted vesicles and intercellular communications. *F1000 biology reports* **3**, 15, doi:10.3410/B3-15 (2011).

8   Thery, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]* **Chapter 3**, Unit 3 22, doi:10.1002/0471143030.cb0322s30 (2006).

9   Gabitass, R. F., Annels, N. E., Stocken, D. D., Pandha, H. A. & Middleton, G. W. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. *Cancer immunology, immunotherapy : CII* **60**, 1419-1430, doi:10.1007/s00262-011-1028-0 (2011).

10   Khaled, Y. S., Ammori, B. J. & Elkord, E. Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients. *Journal of immunology research* **2014**, 879897, doi:10.1155/2014/879897 (2014).

11   Xiang, X. *et al.* Induction of myeloid-derived suppressor cells by tumor exosomes. *International journal of cancer. Journal international du cancer* **124**, 2621-2633, doi:10.1002/ijc.24249 (2009).

12   Chalmin, F. *et al.* Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *The Journal of clinical investigation* **120**, 457-471, doi:10.1172/JCI40483 (2010).