Published in final edited form as:

Cancer Immunol Immunother. 2012 September ; 61(9): 1373–1385. doi:10.1007/s00262-011-1178-0.

Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid derived suppressor cells which promote primary tumor growth

Matthew R. Porembka¹, Jonathan B. Mitchem¹, Brian A. Belt¹, Chyi-Song Hsieh², Hyang-Mi Lee², John Herndon¹, William E. Gillanders^{1,3}, David C. Linehan^{1,3}, and Peter Goedegebuure^{1,3}

¹Department of Surgery, Washington University School of Medicine, Saint Louis, MO 63110

²Department of Rheumatology, Washington University School of Medicine, Saint Louis, MO 63110

³Alvin J. Siteman Cancer Center, Washington University School of Medicine, Saint Louis, MO 63110

Abstract

Purpose—Myeloid derived suppressor cells (MDSC) are a heterogeneous population of immunosuppressive cells that are upregulated in cancer. Little is known about the prevalence and importance of MDSC in pancreas adenocarcinoma (PA).

Experimental Design—Peripheral blood, bone marrow, and tumor samples were collected from pancreatic cancer patients, analyzed for MDSC (CD15⁺CD11b⁺) by flow cytometry, and compared to cancer free controls. The suppressive capacity of MDSC (CD11b⁺Gr-1⁺) and the effectiveness of MDSC depletion were assessed in C57BL/6 mice inoculated with Pan02, a murine PA, and treated with placebo or zoledronic acid, a potent aminobisphosphonate previously shown to target MDSC. The tumor microenvironment was analyzed for MDSC (Gr1⁺CD11b⁺), effector T cells, and tumor cytokine levels.

Results—Patients with PA demonstrated increased frequency of MDSC in the bone marrow and peripheral circulation which correlated with disease stage. Normal pancreas tissue showed no MDSC infiltrate, while human tumors avidly recruited MDSC. Murine tumors similarly recruited MDSC that suppressed CD8⁺ T cells *in vitro* and accelerated tumor growth *in vivo*. Treatment with zoledronic acid impaired intratumoral MDSC accumulation resulting in delayed tumor growth rate, prolonged median survival, and increased recruitment of T cells to the tumor. This was associated with a more robust type 1 response with increased levels of IFN- γ and decreased levels of IL-10.

Conclusions—MDSC are important mediators of tumor-induced immunosuppression in pancreatic cancer. Inhibiting MDSC accumulation with zoledronic acid improves the host anti-tumor response in animal studies suggesting that efforts to block MDSC may represent a novel treatment strategy for pancreatic cancer.

Keywords

myeloid derived suppressor cells; pancreatic cancer; zoledronic acid

Introduction

Tumor-induced alterations in bone marrow myelopoiesis are driven by growth factors and cytokines secreted by the primary tumor resulting in myeloid derived suppressor cell (MDSC) expansion and mobilization [1–3]. MDSC are a heterogeneous population of immature and undifferentiated myeloid cells found in increased numbers in numerous cancers including breast, prostate, renal cell, melanoma, and lung [4–9]. The prevalence of these cells in cancer patients correlates with disease stage, and normalization in MDSC numbers occurs after successful treatment [10]. Once mobilized to the tumor stroma, MDSC promote primary tumor growth and invasion through a variety of mechanisms: arginase production, iNOS and ROS upregulation, L-selectin down regulation, cysteine depletion, promotion of neoangiogenesis, and regulatory T cell recruitment [11–22,6,23–26,7]. MDSC also promote tumor-induced immunosuppression and host immune evasion by inhibiting lymphocyte activation and antigen recognition [27,28].

The identification and classification of MDSC in human cancers remains a difficult problem due to the considerable heterogeneity of this group of myelomonocytic cells. Although MDSC in murine models of cancers reliably express Gr-1⁺CD11b⁺, there is no direct analogous cell surface marker for Gr-1 in humans. The heterogeneity of MDSC in patients with malignant disease has made characterization of these cells difficult clinically due to the lack of a singular marker. Multiple reports including several reviews have been published that established a clear link between CD15⁺ MDSC in cancer patients and Gr-1⁺ MDSC in mice [1, 8–11]. Recent studies demonstrate that induction of various subpopulation of MDSC is directly related to the specific cytokine profile of the inducing tumor [29]. Numerous subpopulations have been described in various tumors and include CD15⁺CD11b⁺, CD33⁺CD11b⁺, and CD14⁺CD11b⁺, as well as others [30–32,20,6,12,33,34]. In the course of our prior studies, we observed that human pancreatic adenocarcinoma avidly recruits CD11b⁺CD15⁺ myeloid cells to the primary tumor.

Pancreatic adenocarcinoma, henceforth called pancreatic cancer, is a treatment refractory malignancy that represents the fourth leading cause of cancer death in the United States [35]. We have previously observed that pancreatic cancer creates a highly immunosuppressive tumor microenvironment through the production of inhibitory cytokines and the recruitment of immunosuppressive cells [36–38,33], including T regulatory cells. While several reports have implicated MDSC in growth of pancreatic cancer in mouse models [39,40], few reports have suggested that MDSC play an important role in patients with pancreatic adenocarcinoma [7].

Treatments targeting MDSC have demonstrated impaired tumor growth, increased survival, and improved response to vaccine therapies in murine models of breast cancer, glioma, and melanoma [41–45]. Zoledronic acid, a potent aminobisphosphonate and osteoclast inhibitor initially developed to treat osteoporotic bone disease, has demonstrated efficacy as an anticancer agent used to treat bony metastasis and prolong disease-free survival in patients with breast cancer [46,47]. The anti-neoplastic mechanism of zoledronic acid remains poorly understood. Direct apoptotic effects have been reported [48,49] as well as indirect mechanisms including inhibition of neoangiogenesis [50–52], modulation of T cell immune response [53], and prevention of myeloid cell differentiation into MDSC [54,45].

Few studies have targeted MDSC in pancreatic cancer [55]. In this study, we demonstrate that MDSC represent a clinically relevant immunosuppressive cell subset in human pancreatic cancer and that targeting MDSC in a murine model of pancreatic cancer using zoledronic acid reduces MDSC prevalence and tumor growth.

Material and Methods

Immunofluorescence

Paraffin-embedded tissue sections of archived human pancreatic ductal adenocarcinoma were used for immunofluorescence following standard procedures. Confocal images were scanned at 400X with an axiovert 100M microscope equipped with a LSM 510 confocal system and software (Zeiss). For co-localization of CD15, Foxp3, Arginase-1, and CD11b, rat anti-human Foxp3 (eBioscience, 10ug/ml), goat anti-human CD11b (Abcam, Cambridge, MA, 1:150), rabbit anti-human arginase 1 (Sigma), and polyclonal mouse anti-human CD15 (Abcam, Cambridge, MA, 1:50) were used, visualized respectively with Alexafluor 488 Donkey anti-rat, Alexafluor 555 Donkey anti-goat, Alexafluor 488 goat anti-rabbit and Alexafluor 647 donkey anti-rabbit secondary antibodies diluted 1:200 (Invitrogen, Carlsbad, CA).

Flow cytometric analysis of human blood, bone marrow, and tumor in patients with pancreatic adenocarcinoma

Informed consent was prospectively obtained from all patients prior to obtaining human tissue or blood according to the institutionally approved Human Studies Committee Protocol. Peripheral blood samples were collected in vacuum tubes containing EDTA (BD Biosciences; San Jose, CA) and processed immediately. Cell surface staining was performed on whole blood (100µL aliquots) and PBMC isolated by Ficoll-density centrifugation using monoclonal antibodies for CD15 (BD Biosciences), CD33 (BioLegend; San Diego, CA), and CD11b (BD Biosciences) according to the manufacturer's instructions. For whole blood samples, red cells were lysed prior to analysis.

Bone marrow aspirates were obtained from the iliac wing and transferred to vacuum tubes containing EDTA (BD Biosciences). Cells were isolated by Ficoll-density centrifugation and frozen in DMSO with 10% FBS. Cells were then thawed using CTL wash (Cellular Technology Ltd, Shaker Heights, OH), washed, and stained with CD45, CD11b, CD15 and CD33 (BioLegend). Analysis was immediately performed.

Samples of fresh human pancreatic tumor tissue were obtained from patients immediately after tumor extirpation and placed into cold RPMI. Tumor tissue was minced into 2–3 mm size pieces, mechanically dissociated using the Miltenyi GentleMACS tissue dissociator, and digested in a buffer containing 1mg/ml collagenase, 2.5 U/ml hyaluronidase and 0.1mg/ml DNase for 15 minutes. The cell suspension was then separated from tissue debris by serial filtration (70µm, 40µm cell strainer). Cell surface staining was performed using monoclonal antibodies for CD15, CD45, CD33, and CD11b (BioLegend) according to the manufacturer's instructions after treatment with Fc-block. Corresponding isotope controls yielded no significant staining. All results are expressed as a percentage of CD45⁺ cells.

Mice and tumor cell line

C57BL/6 and OT-1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a specific pathogen-free animal facility at Washington University and were used according to protocols approved by the Institutional Animal Care and Use Committee. The murine pancreatic adenocarcinoma cell line Pan02 is a non-metastatic tumor line, syngeneic to C57BL/6 [56], and was obtained from the DCT Tumor Repository (NCI-Frederick Cancer Research and Development Center, Bethesda, MD).

Mouse model of pancreatic cancer

Six-week-old C57BL/6 mice were injected subcutaneously on the right hind leg with 0.1×10^6 Pan02 cells, as previously described [37]. Treatment with zoledronic acid (Novartis

Pharma) (30 μ g/kg/100 μ L subcutaneously thrice weekly) or placebo (normal saline) was initiated 10 days after inoculation when the tumor became palpable. Tumor volume was determined by caliper measurement and recorded weekly. Animals were followed until death or until any single tumor measured greater than 2cm. Survival was recorded. All animal experiments were performed in triplicate and compared a minimum of 10 mice in each arm (placebo versus treatment).

Tumor immune cell infiltrate was analyzed at multiple time points after inoculation. Bone marrow was flushed from non-tumor bearing fibulae and femurs. Spleens and tumors were resected, and processed into single cell suspensions. Tumor tissue was minced into 2–3 mm size pieces, mechanically dissociated using the Miltenyi GentleMACS tissue dissociator, and digested in a buffer containing 1mg/ml collagenase, 2.5 U/ml hyaluronidase and 0.1mg/ml DNase for 15 minutes. The cell suspension was then separated from tissue debris by serial filtration (70 μ m, 40 μ m cell strainer).

Immunofluorescence labeling and flow cytometry

Monoclonal antibodies were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA) and BD Biosciences (San Jose, CA). Intracellular Foxp3 staining was performed according to the manufacturer's intracellular staining protocol (eBioscience). All samples were treated with Fc-block. Corresponding isotype controls yielded no significant staining. Prevalence of tumor-infiltrating leukocytes (CD45⁺) and immune cell subsets including MDSC (Gr-1⁺CD11b⁺), T cells (CD4, CD8), and T regulatory cells (Treg, CD4⁺Foxp3⁺) were measured using four-color flow cytometry. Results are expressed as a percentage of CD45⁺ cells.

***In vivo* suppression assay**

Gr-1⁺CD11b⁺ MDSC were isolated from mature Pan02 tumors (diameter >1cm) using fluorescence activated cell sorting. Purity of MDSC was >99%, confirmed by flow cytometry. Six week old C57BL/6 mice were inoculated with Pan02, MDSC, or a 1:1 combination of Pan02 and MDSC (10 mice per experimental arm). Caliper measurement was used to calculate tumor volume.

***In vitro* suppression assays**

CFSE was purchased from Invitrogen (Carlsbad, CA). 100,000 CFSE-labeled splenocytes obtained from OT-1 mice were co-cultured in 96-well plates with 1 μ m SIINFEKL peptide and varying concentrations of Gr-1⁺CD11b⁺ MDSC isolated from mature tumors, spleen, or bone marrow after successful CD11b⁺ magnetic bead separation (Miltenyi Biotec), according to the manufacturer's instructions. Purity was confirmed to be >97% by flow cytometry. After 72 hours, cells were subsequently harvested and the CD8⁺ TcR⁺ cell fraction was analyzed by flow cytometry for CFSE dilution.

Analysis of tumor cytokine concentrations using the BioPlex assay

Mature tumors from zoledronic acid treated and control mice were harvested, flash frozen using liquid nitrogen, and homogenized in a PBS solution containing a protease inhibitor cocktail (Roche Laboratories, Boulder, CO). Supernatant was reserved after centrifugation (10,000 RPM for 5 minutes at 4°C) and analyzed for IFN- γ , IL-2 and IL-10 according to the manufacturer's directions (BioPlex, BioRad Laboratories, Hercules, CA). The cytokine panel was analyzed using the Luminex 100 (Luminex, Inc., Austin, TX) plate reader and data processed using the accompanying proprietary software. Cytokine concentrations were normalized according to the mass of tumor homogenized.

Statistical analysis

Data were analyzed using Graph Pad Prism version 5.01 (GraphPad Software Inc., La Jolla, CA). Survival differences between treatment and control groups were compared using Kaplan-Meier analysis and differences were calculated using the log-rank test. Comparison of difference between groups was calculated using student's t-test with $p < 0.05$ considered to be statistically significant.

Results

Patients with pancreatic adenocarcinoma have increased numbers of CD11b⁺CD15⁺ myeloid cells in the bone marrow and the peripheral blood which are avidly recruited to the primary tumor

Peripheral blood, PBMC, and bone marrow aspirates were collected from patients with pancreatic adenocarcinoma and cancer free controls. Human samples were analyzed for the frequency of known markers of MDSC including CD33, CD15 and CD11b. Patients with pancreatic cancer had a significant increase in circulating CD11b⁺CD15⁺ myeloid cells both in whole blood samples (Figure 1A; controls $n = 5$, patients $n = 20$) and isolated PBMC when compared to controls (Figure 1B; controls $n = 12$, patients $n = 16$, $p < 0.0001$). Myeloid cell mobilization was most notable in patients with metastatic disease ($n = 11$; $68.2\% \pm 3.6\%$ of CD45⁺ cells), when compared to both normal controls ($n = 10$; $37.6\% \pm 3.6\%$; $p < 0.0001$) and patients with resectable pancreatic cancer ($n = 9$; $57.3\% \pm 3.5\%$; $p < 0.05$). A similar expansion of myeloid cells was observed in the bone marrow of pancreatic cancer patients ($47.7 \pm 3.9\%$ of CD45⁺ cells, $n = 8$) compared to controls ($22.3 \pm 1.4\%$, $p < 0.001$, $n = 16$; Figure 1C). A representative flow cytometric analysis is depicted in Figure 1C.

Normal human pancreas was devoid of infiltrating CD15 and CD11b expressing cells by immunofluorescent analysis (Figure 2A). In contrast, pancreatic cancers avidly recruited cells which coexpressed CD15 and CD11b (Figure 2B), which also coexpressed Arginase-1 (Figure 2C). Flow cytometric analysis of human pancreatic tumors allowed further phenotypic discrimination of infiltrating leukocytes (Figure 2C–D). The stroma of human pancreatic cancers was noted to have a dense CD45⁺ infiltrate which was predominantly composed of CD15⁺CD11b⁺ myeloid cells ($66.6\% \pm 3.2\%$); these cells also coexpressed CD33. By comparison, the percentage of tumor infiltrating CD4 and CD8 T cells was considerably smaller ($16.7\% \pm 2.1\%$ and $9.5\% \pm 1.6\%$, respectively).

Pan02 inoculated into C57BL/6 mice induces the mobilization of immunosuppressive MDSC and their recruitment to the primary tumor

To further study tumor-induced recruitment of MDSC, we used the non-metastatic murine pancreatic adenocarcinoma cell line, Pan02 (aka Panc02) [56]. After inoculation with Pan02, a progressive increase in prevalence of CD11b⁺Gr-1⁺ myeloid cells was observed in the spleen (7.8% vs. 18.8% at 30 days; $p < 0.01$) which was associated with decreased percentages of circulating CD4 (17.6% vs. 8.1% ; $p < 0.01$) and CD8 (13.7% vs. 6.9% ; $p < 0.01$) T cells, and an increased percentage of Foxp3⁺ Treg (Figure 3A). Mobilization and accumulation of CD11b⁺Gr-1⁺ cells in the bone marrow, spleen, and primary tumor microenvironment reliably occurred (Figure 3B). Flow cytometric analysis of the Pan02 tumors demonstrated that the murine tumor microenvironment closely recapitulates that of human pancreatic cancer. Just as in human tumors, the majority of infiltrating CD45⁺ leukocytes in Pan02 tumors was comprised of myeloid cells (human: 68% , Pan02: 78%). Conversely, the percentage of immune effector cells was minimal.

The suppressive potential of the CD11b⁺Gr-1⁺ cells isolated from various compartments (bone marrow, spleen, and tumor) was tested using assessment of CFSE dilution in OT-1 splenocytes stimulated with SIINFEKL peptide (Figure 3C). Bone marrow derived CD11b⁺Gr-1⁺ cells were minimally suppressive compared to vehicle control. Tumor-derived CD11b⁺Gr-1⁺ cells possessed the greatest ability to suppress proliferation of OT-1 splenocytes. The suppressive ability of spleen-derived CD11b⁺Gr-1⁺ cells was intermediate.

Tumor-derived CD11b⁺Gr-1⁺ cells suppress host antitumor activity, promote tumor engraftment, and progression of Pan02 tumors

The ability of CD11b⁺Gr-1⁺ cells to promote Pan02 tumor growth was tested *in vivo*. CD11b⁺Gr-1⁺ cells were isolated from mature Pan02 tumors grown in Ly5.1 congenic mice using fluorescence-activated cell sorting and coinjected with Pan02 tumor cells into wild type mice. When compared to mice injected with either tumor or isolated CD11b⁺Gr-1⁺ cells alone, mice injected with a mixture of the myeloid cells and Pan02 tumor cells demonstrated significantly larger tumors compared to mice inoculated solely with Pan02 (Figure 4, $p < 0.0001$). The addition of CD11b⁺Gr-1⁺ cells to the tumor appeared to accelerate initial growth. However, after approximately 14 days, the rate of growth of both the admixture and Pan02 alone was equal. Subsequent flow cytometric examination for Ly5.1⁺ cells in Pan02 tumors at 27 days revealed the absence of donor CD11b⁺Gr-1⁺ cells (data not shown).

Treatment with zoledronic acid results in smaller tumors and improved survival

Mice were inoculated with Pan02 and zoledronic acid treatment was administered thrice weekly by subcutaneous injections. There were no adverse side effects noted in non-tumor bearing mice treated with zoledronic acid (data not shown). Mice treated with zoledronic acid demonstrated significantly delayed tumor growth rate and smaller tumors when compared to animals treated with placebo ($p < 0.0001$; Figure 5A). Importantly, treatment resulted in prolonged median survival (zoledronic acid: 73 days, placebo: 59 days, $p = 0.02$; Figure 5B).

Zoledronic acid impairs the production and recruitment of MDSCs into the periphery, as well as into the tumor microenvironment resulting in an immune activated host tumor microenvironment

The antitumor effect of zoledronic acid occurred rapidly with evidence of response after 6 doses. Treatment with zoledronic acid resulted in a statistically significant decrease in intratumoral CD11b⁺Gr-1⁺ cells (78% in placebo vs. 51% in zoledronic acid at Day 34, $p < 0.05$; Figure 6A). Additionally, the composition of the tumor immune infiltrate was altered by treatment with zoledronic acid (Figure 6A–C), with increases in CD4⁺ (CD4: $4.4\% \pm 1.1\%$ vs. $12.2\% \pm 2.0\%$, $p = 0.02$) and CD8⁺ T cell tumor infiltrate ($3.9\% \pm 1.3\%$ vs. $10.6\% \pm 2.2\%$, $p = 0.04$), and a decrease in the percentage of CD4 cells expressing Foxp3 (Foxp3 as %CD4: $42.2\% \pm 5.0\%$ vs. $22.3\% \pm 6.5\%$, $p = 0.02$).

The functional consequence of altering the immune cell balance within the tumor microenvironment was investigated by comparing the cytokine profiles of zoledronic acid treated Pan02 tumors with placebo-treated controls (Figure 7). Various cytokine levels (IFN- γ , GM-CSF, and IL-10) were measured in homogenates created from fresh, mature tumors. Tumor-bearing mice exhibited an immunosuppressive cytokine phenotype as evidenced by elevated levels of IL-10 and lower concentrations of IFN- γ . Treatment with zoledronic acid functionally altered the tumor microenvironment towards an immunostimulatory phenotype with statistically significant increased levels of IFN- γ ($p < 0.05$) and decreased levels of IL-10 ($p < 0.05$). Despite a significant alteration in the cytokine profiles between zoledronic

acid treated animals and placebo controls, no difference was observed in tumor concentrations of GM-CSF ($p > 0.05$).

Discussion

Pancreatic cancer remains one of the most aggressive malignancies, with a mortality rate which approaches its incidence [35]. Despite many innovative treatments, including immunomodulatory therapies, pancreatic cancer remains highly resistant to treatment [57–59]. One manner in which pancreatic cancer evades therapeutic response is by the induction of an immunosuppressive microenvironment. We, and others, have previously described the role of Treg in pancreatic cancer as an efficient means of preventing adequate host antitumor responses [60,36,37]. Recently, myeloid derived suppressor cells (MDSC) have been accepted as important mediators in tumor-induced immunosuppression.

MDSC are now recognized as a key immunosuppressive cell type in the development and progression of malignancy [33]. The prevalence of these cells and their immunosuppressive capacity has been reported in multiple different malignancies including head and neck, prostate, renal cell, and breast [4–9]; however, relatively little research about these cells has been carried out in pancreatic cancer. In this publication, we quantify the frequency of MDSC in human pancreatic cancer, and investigate the role of MDSC depletion in a mouse model of pancreatic adenocarcinoma as a therapeutic modality.

Early research by Schmielau and Finn suggested an increased number of “low density granulocytes” present in patients with pancreatic cancer that possessed an immunosuppressive phenotype [7]. This study focused exclusively on patients with metastatic disease and demonstrated that circulating CD15⁺ cells isolated from peripheral blood were able to block T cell function. Although this study laid essential groundwork for the study of MDSC in human cancer patients, it did not thoroughly characterize the cells of interest. We have expanded on this research and demonstrated that the CD15⁺ cells previously observed represent CD15⁺CD11b⁺ MDSC. Other authors have demonstrated a similar increase of granulocytic MDSC in human cancers including bladder cancer, renal cell cancer, colon cancer, and lung cancer [30,12,6,20]. In the current study, we demonstrate that CD15⁺CD11b⁺ cells are not only upregulated in the peripheral blood of patients with pancreatic cancer, but they are also observed in increased prevalence in the bone marrow; most importantly, these cells infiltrated tumors in large numbers. In addition, increasing prevalence of the CD11b⁺CD15⁺ cells in peripheral blood correlates with more advanced disease state. Patients with biopsy proven pancreatic adenocarcinoma have increased numbers of phenotypic MDSC when compared to normal, age-matched controls, and patients with metastatic disease had significantly increased numbers over patients with local disease (Figure 1). In addition, these cells were found to coexpress Arginase-1, a key method of MDSC immunosuppression that has repeatedly been demonstrated throughout the literature [30,12,6,20,33,76]. These findings are similar to other studies which correlate MDSC prevalence with clinical disease stage in patients with advanced cancer including pancreatic cancer [10].

Through soluble mediators, the primary tumor has been implicated in the generation of MDSC in the bone marrow, mobilization into the peripheral circulation, homing to the primary tumor, and imprinting of the immunosuppressive phenotype [2,3,27,45,32,29]. This is the first publication demonstrating increased numbers of MDSC in the bone marrow of patients with solid tumors. Numerous factors are associated with the mobilization of these cells from the bone marrow including IL-6, IL-10, GM-CSF, G-CSF, SCF, and CCL2 [61,2,62,63,42,27,64,65,32,29,66]. The variation in MDSC phenotype between different

cancer types is likely related to differential production of chemokines by the primary tumor which results in a heterogeneous myeloid cell population [67,32,29].

The above mentioned heterogeneity has made the study of these cells difficult in human patient populations. We observed that human pancreatic cancer tumors avidly recruit CD15⁺CD33⁺CD11b⁺ MDSC into the peripheral circulation and to the primary tumor. In human pancreatic adenocarcinoma, these cells represent the majority of tumor infiltrating leukocytes and greatly outnumber tumor infiltrating lymphocytes, including Treg (Figure 2). We recently observed that a subset of CD11b⁺ cells with a monocytic phenotype (CD14⁺CD33⁺) also infiltrate human pancreatic cancers, and ongoing efforts focus on functional and phenotypic characterization of this subset, as monocytic MDSC have been described in a variety of human cancers as well as animal models [33].

Interestingly, comparison of the circulating MDSC to those found within the tumor microenvironment demonstrates notable differences in cell surface marker phenotype. CD15 expression was variable between tumor-associated and circulating MDSC. In addition, CD33 expression on tumor-derived MDSC segregated into two populations: CD33^{hi} and CD33^{lo}. Although phenotypic analyses were unable to be carried out on the MDSC subgroups because of small quantities of human specimen, it can be postulated that the differential expression of cell surface markers was the result of imprinting on the MDSC mobilized from the bone marrow by tumor-derived factors. Recently Rodrigues *et al* described that monocytes from normal donors exposed to malignant glioma cells adopted an immunosuppressive phenotype and exhibited increased CD33 expression [68]. Importantly, it was also noted that direct cell contact was necessary to “educate” normal donor monocytes to develop maximal immunosuppressive phenotype, a finding which is supported by our data as we observed maximal suppression induced in tumor-confined MDSC. Additionally, a recent published report suggests that peripheral MDSC are skewed towards suppressive function but require activation in the tumor microenvironment to develop full suppressive potential [69], also supported by our data.

Variable “imprinting” or “education” of MDSC was observed in our Pan02 mouse model of pancreatic cancer. In tumor bearing mice, Gr-1⁺CD11b⁺ cells were isolated from various compartments and their individual suppressive potential was measured on T cell proliferation, with tumor associated MDSC having the highest suppressive potential. Although all MDSC shared the same cell surface markers, their ability to suppress antigen mediated T cell response varied. MDSC isolated from the bone marrow were essentially inert; splenocyte-derived MDSC were immunosuppressive but by a much smaller degree than MDSC isolated from tumor. The immunosuppressive variability suggests that MDSC mobilization from the bone marrow and the development of the immunosuppressive phenotype may be isolated processes, separated both temporally and anatomically. Current cell surface markers used to identify MDSC in both animal models and humans are not sufficient given the phenotypic and functional variability of these heterogeneous cells. This is especially true in humans where cell surface markers are less specific than in murine model and the actual MDSC cell population is likely a subset of CD15⁺CD11b⁺ cells.

Given the abundance of MDSC in the primary tumor and circulation of patients with pancreatic cancer, we hypothesized that these cells play an instrumental role in the engraftment and support of the primary tumor. We selectively targeted MDSC using zoledronic acid, a potent and bone avid aminobisphosphonate that has previously been shown to target MDSC in breast and cervical cancer [44,70,45].

In mice inoculated with Pan02, zoledronic acid effectively decreased the rate of accumulation of MDSC in the tumor microenvironment resulting in impaired tumor growth

and prolonged survival; however, the exact mechanism of impaired MDSC accumulation by zoledronic acid is unclear [44,50,70,48,51,52,49,71]. Regardless of the mechanism, zoledronic acid effectively decreased accumulation of MDSC to the tumor and phenotypically altered the tumor microenvironment. This allowed for the host to mount a more robust T cell response, as evidenced by a shift in the cytokine profile from immunosuppressive to immunostimulatory and increased prevalence of effector CD4 and CD8 T cells. The resulting decreased tumor growth and improved survival suggests that MDSC play an important role in the progression of pancreatic cancer.

It is possible that zoledronic acid has a direct effect on the primary tumor, as *in vitro* studies have shown anti-tumor activity [48,49]. However, bioavailability studies looking at soft tissue and bone concentrations of zoledronic acid demonstrate that even with supratherapeutic daily dosing greater than used in this study (150 µg/kg/day), zoledronic acid did not accumulate at clinically appreciable levels in the pancreas or soft tissues [72]. Concentrations of zoledronic acid detected in bony tissues were up to 1500-fold greater than the non-mineralized tissues and at least 4000-fold higher than in plasma [72]. Therefore, it is unlikely that zoledronic acid would have direct anti-tumor effects *in vivo* [48,49].

Although we observed a significant reduction in rate of tumor growth and an improvement in survival in mice treated with zoledronic acid, all animals eventually died from progressive disease. There are several possible reasons why zoledronic acid was not able to cure mice inoculated with Pan02. First, tumors promote immunosuppression and immune evasion through multiple redundant mechanisms, including Treg. Zhang *et al* elegantly demonstrated that although elimination of these cells is sufficient to abrogate tumor growth, it is unable to effect complete tumor eradication [73]. This suggests that local tumor-induced immunosuppression is multifactorial and single arm therapies are unlikely to be sufficient.

Second, Treg also play an important role in immune invasion in pancreatic cancer [60,36,37,74]. Treatment with zoledronic acid resulted in a decreased percentage of CD4+Foxp3+ cells. While the concomitant increase in tumor-associated CD4 T cells resulted in an unchanged absolute number of Treg, the increased prevalence of CD4+Foxp3+ cells altered the ratio of immune effectors to immune suppressors in favor of immune effectors. However, the continued presence of Treg likely plays an important role in immune evasion in the setting of decreased MDSC.

Our above data indicates that MDSC are an important immunosuppressive cell active in the immune evasion elicited by pancreatic adenocarcinoma, and that depletion of these cells may provide a novel therapeutic modality for the treatment of this aggressive malignancy. Zoledronic acid is effective in depleting tumor-associated MDSC; however its mechanism of action is unknown and leaves room for improvement, as all mice in our studies eventually progressed to death from disease. Combination therapy with chemotherapeutic modalities may provide improved outcomes [75]. In this vein, we are currently conducting a clinical trial to investigate the immune effects and clinical efficacy of zoledronic acid in patients with resectable pancreatic cancer (ClinicalTrials.gov identifier: NCT00892242). Information gained from such trials will be useful to dissect the complex immunologic role of MDSC and therapeutic benefit of MDSC targeting strategies. Novel therapeutic modalities targeting tumor-induced immunosuppression with immunostimulatory treatments and chemotherapy are urgently needed to overcome this aggressive malignancy.

References

1. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009; 9(3):162–174. nri2506 [pii]. 10.1038/nri2506 [PubMed: 19197294]

2. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, Ugel S, Sonda N, Biciato S, Falisi E, Calabrese F, Basso G, Zanovello P, Cozzi E, Mandruzzato S, Bronte V. Tumor-Induced Tolerance and Immune Suppression Depend on the C/EBPbeta Transcription Factor. *Immunity*. 2010 S1074-7613(10)00202-5 [pii]. 10.1016/j.immuni.2010.05.010
3. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. *Immunol Rev*. 2008; 222:162–179. IMR602 [pii]. 10.1111/j.1600-065X.2008.00602.x [PubMed: 18364001]
4. Liu CY, Wang YM, Wang CL, Feng PH, Ko HW, Liu YH, Wu YC, Chu Y, Chung FT, Kuo CH, Lee KY, Lin SM, Lin HC, Wang CH, Yu CT, Kuo HP. Population alterations of L: -arginase- and inducible nitric oxide synthase-expressed CD11b(+)/CD14 (-)/CD15 (+)/CD33 (+) myeloid-derived suppressor cells and CD8 (+) T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J Cancer Res Clin Oncol*. 200910.1007/s00432-009-0634-0
5. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, Castelli C, Mariani L, Parmiani G, Rivoltini L. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol*. 2007; 25(18):2546–2553. 25/18/2546 [pii]. 10.1200/JCO.2006.08.5829 [PubMed: 17577033]
6. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J, Ochoa AC. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J Exp Med*. 2005; 202(7):931–939. jem.20050715 [pii]. 10.1084/jem.20050715 [PubMed: 16186186]
7. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res*. 2001; 61 (12):4756–4760. [PubMed: 11406548]
8. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol*. 2001; 166 (1):678–689. [PubMed: 11123553]
9. Almand B, Resser JR, Lindman B, Nadaf S, Clark JI, Kwon ED, Carbone DP, Gabrilovich DI. Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res*. 2000; 6 (5):1755–1766. [PubMed: 10815894]
10. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother*. 200810.1007/s00262-008-0523-4
11. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res*. 2010; 70(1):68–77. 0008-5472.CAN-09-2587 [pii]. 10.1158/0008-5472.CAN-09-2587 [PubMed: 20028852]
12. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, Ochoa AC. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res*. 2009; 69(4):1553–1560. 0008-5472.CAN-08-1921 [pii]. 10.1158/0008-5472.CAN-08-1921 [PubMed: 19201693]
13. Li H, Han Y, Guo Q, Zhang M, Cao X. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol*. 2009; 182(1):240–249. 182/1/240 [pii]. [PubMed: 19109155]
14. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. *J Immunol*. 2009; 183(2):937–944. jimmunol.0804253 [pii]. 10.4049/jimmunol.0804253 [PubMed: 19553533]
15. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Sotomayor E, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol*. 2009; 182(9):5693–5701. 182/9/5693 [pii]. 10.4049/jimmunol.0900092 [PubMed: 19380816]
16. Movahedi K, Guillems M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschijn A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. 2008; 111(8):4233–4244. blood-2007-07-099226 [pii]. 10.1182/blood-2007-07-099226 [PubMed: 18272812]

17. Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kubler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. *Clin Cancer Res.* 2008; 14(24):8270–8278. 14/24/8270 [pii]. 10.1158/1078-0432.CCR-08-0165 [PubMed: 19088044]
18. Kusmartsev S, Eruslanov E, Kubler H, Tseng T, Sakai Y, Su Z, Kaliberov S, Heiser A, Rosser C, Dahm P, Siemann D, Vieweg J. Oxidative stress regulates expression of VEGFR1 in myeloid cells: link to tumor-induced immune suppression in renal cell carcinoma. *J Immunol.* 2008; 181(1):346–353. 181/1/346 [pii]. [PubMed: 18566400]
19. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006; 66(2):1123–1131. 66/2/1123 [pii]. 10.1158/0008-5472.CAN-05-1299 [PubMed: 16424049]
20. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, McDermott D, Quiceno D, Youmans A, O'Neill A, Mier J, Ochoa AC. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* 2005; 65(8):3044–3048. 65/8/3044 [pii]. 10.1158/0008-5472.CAN-04-4505 [PubMed: 15833831]
21. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol.* 2005; 174(2):636–645. 174/2/636 [pii]. [PubMed: 15634881]
22. Sinha P, Clements VK, Ostrand-Rosenberg S. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. *Cancer Res.* 2005; 65(24):11743–11751. 65/24/11743 [pii]. 10.1158/0008-5472.CAN-05-0045 [PubMed: 16357187]
23. Serafini P, De Santo C, Marigo I, Cingarlini S, Dolcetti L, Gallina G, Zanovello P, Bronte V. Derangement of immune responses by myeloid suppressor cells. *Cancer Immunol Immunother.* 2004; 53(2):64–72. 10.1007/s00262-003-0443-2 [PubMed: 14593498]
24. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 2004; 64(16):5839–5849. [pii]. 10.1158/0008-5472.CAN-04-046564/16/5839 [PubMed: 15313928]
25. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol.* 2002; 168 (2):689–695. [PubMed: 11777962]
26. Schmielau J, Nalesnik MA, Finn OJ. Suppressed T-cell receptor zeta chain expression and cytokine production in pancreatic cancer patients. *Clin Cancer Res.* 2001; 7 (3 Suppl):933s–939s. [PubMed: 11300494]
27. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol.* 2006; 16(1):53–65. S1044-579X(05)00058-1 [pii]. 10.1016/j.semcancer.2005.07.005 [PubMed: 16168663]
28. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol.* 2010; 184(6):3106–3116. jimmunol.0902661 [pii]. 10.4049/jimmunol.0902661 [PubMed: 20142361]
29. Lechner MG, Megiel C, Russell SM, Bingham B, Arger N, Woo T, Epstein AL. Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. *J Transl Med.* 2011; 9:90. 1479-5876-9-90 [pii]. 10.1186/1479-5876-9-90 [PubMed: 21658270]
30. Eruslanov E, Neuberger M, Daurkin I, Perrin GQ, Algood C, Dahm P, Rosser C, Vieweg J, Gilbert SM, Kusmartsev S. Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. *Int J Cancer.* 10.1002/ijc.26123
31. Brandau S, Trellakis S, Bruderek K, Schmaltz D, Steller G, Elian M, Suttman H, Schenck M, Welling J, Zabel P, Lang S. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *J Leukoc Biol.* 2011; 89(2):311–317. jlb.0310162 [pii]. 10.1189/jlb.0310162 [PubMed: 21106641]

32. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J Immunol.* 185(4): 2273–2284. jimmunol.1000901 [pii]. 10.4049/jimmunol.1000901 [PubMed: 20644162]
33. Goedegebuure P, Mitchem JB, Porembka MR, Tan MC, Belt BA, Wang-Gillam A, Gillanders WE, Hawkins WG, Linehan DC. Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer. *Curr Cancer Drug Targets.* 2011; 11(6): 734–751. EPub-Abstract-CCDT-138 [pii]. [PubMed: 21599634]
34. Chioda M, Peranzoni E, Desantis G, Papalini F, Falisi E, Solito S, Mandruzzato S, Bronte V. Myeloid cell diversification and complexity: an old concept with new turns in oncology. *Cancer Metastasis Rev.* 30(1):27–43.10.1007/s10555-011-9268-1 [PubMed: 21267772]
35. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60(5):277–300. caac.20073 [pii]. 10.3322/caac.20073 [PubMed: 20610543]
36. Liyanage UK, Goedegebuure PS, Moore TT, Viehl CT, Moo-Young TA, Larson JW, Frey DM, Ehlers JP, Eberlein TJ, Linehan DC. Increased prevalence of regulatory T cells (Treg) is induced by pancreas adenocarcinoma. *J Immunother.* 2006; 29(4):416–424. 00002371-200607000-00008 [pii]. 10.1097/01.cji.0000205644.43735.4e [PubMed: 16799337]
37. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol.* 2002; 169 (5):2756–2761. [PubMed: 12193750]
38. Tan MC, Goedegebuure PS, Belt BA, Flaherty B, Sankpal N, Gillanders WE, Eberlein TJ, Hsieh CS, Linehan DC. Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer. *J Immunol.* 2009; 182(3):1746–1755. 182/3/1746 [pii]. [PubMed: 19155524]
39. Zhao F, Obermann S, von Wasielewski R, Haile L, Manns MP, Korangy F, Greten TF. Increase in frequency of myeloid-derived suppressor cells in mice with spontaneous pancreatic carcinoma. *Immunology.* 2009; 128(1):141–149. IMM3105 [pii]. 10.1111/j.1365-2567.2009.03105.x [PubMed: 19689743]
40. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res.* 2007; 67(19):9518–9527. 67/19/9518 [pii]. 10.1158/0008-5472.CAN-07-0175 [PubMed: 17909062]
41. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res.* 2009; 15(6):2148–2157. 1078-0432.CCR-08-1332 [pii]. 10.1158/1078-0432.CCR-08-1332 [PubMed: 19276286]
42. Pan PY, Wang GX, Yin B, Ozao J, Ku T, Divino CM, Chen SH. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. *Blood.* 2008; 111(1):219–228. blood-2007-04-086835 [pii]. 10.1182/blood-2007-04-086835 [PubMed: 17885078]
43. Jia W, Jackson-Cook C, Graf MR. Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma+vaccination model. *J Neuroimmunol.* 2010; 223(1–2):20–30. S0165-5728(10)00121-9 [pii]. 10.1016/j.jneuroim.2010.03.011 [PubMed: 20452681]
44. Coscia M, Quaglino E, Iezzi M, Curcio C, Pantaleoni F, Riganti C, Holen I, Monkkinen H, Boccadoro M, Forni G, Musiani P, Bosia A, Cavallo F, Massaia M. Zoledronic acid repolarizes tumor-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway. *J Cell Mol Med.* 2009 JCM926 [pii]. 10.1111/j.1582-4934.2009.00926.x
45. Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. *Cancer Res.* 2007; 67(23):11438–11446. 67/23/11438 [pii]. 10.1158/0008-5472.CAN-07-1882 [PubMed: 18056472]
46. Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S, Menzel C, Jakesz R, Seifert M, Hubalek M, Bjelic-Radisic V, Samonigg H, Tausch C, Eidtmann H, Steger G, Kwasny W, Dubsy P, Fridrik M, Fitzal F, Stierer M, Rucklinger E, Greil R, Marth C. Endocrine

- therapy plus zoledronic acid in premenopausal breast cancer. *N Engl J Med*. 2009; 360(7):679–691. 360/7/679 [pii]. 10.1056/NEJMoa0806285 [PubMed: 19213681]
47. Diel IJ, Solomayer EF, Bastert G. Bisphosphonates and the prevention of metastasis: first evidences from preclinical and clinical studies. *Cancer*. 2000; 88(12 Suppl):3080–3088. [pii]. 10.1002/1097-0142(20000615)88:12+<3080::AID-CNCR27>3.0.CO;2-W [PubMed: 10898355]
 48. Marten A, Lilienfeld-Toal M, Buchler MW, Schmidt J. Zoledronic acid has direct antiproliferative and antimetastatic effect on pancreatic carcinoma cells and acts as an antigen for delta2 gamma/delta T cells. *J Immunother*. 2007; 30(4):370–377. [pii]. 10.1097/CJI.0b013e31802bff1600002371-200705000-00002 [PubMed: 17457212]
 49. Tassone P, Tagliaferri P, Viscomi C, Palmieri C, Caraglia M, D'Alessandro A, Galea E, Goel A, Abbruzzese A, Boland CR, Venuta S. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. *Br J Cancer*. 2003; 88(12):1971–1978. [pii]. 10.1038/sj.bjc.6600986600986 [PubMed: 12799645]
 50. Ferretti G, Fabi A, Carlini P, Papaldo P, Cordiali Fei P, Di Cosimo S, Salesi N, Giannarelli D, Alimonti A, Di Cocco B, D'Agosto G, Bordignon V, Trento E, Cognetti F. Zoledronic-acid-induced circulating level modifications of angiogenic factors, metalloproteinases and proinflammatory cytokines in metastatic breast cancer patients. *Oncology*. 2005; 69(1):35–43. OCL2005069001035 [pii]. 10.1159/000087286 [PubMed: 16088233]
 51. Santini D, Vincenzi B, Dicuonzo G, Avvisati G, Massacesi C, Battistoni F, Gavasci M, Rocci L, Tirindelli MC, Altomare V, Tocchini M, Bonsignori M, Tonini G. Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res*. 2003; 9 (8):2893–2897. [PubMed: 12912933]
 52. Santini D, Vincenzi B, Galluzzo S, Battistoni F, Rocci L, Venditti O, Schiavon G, Angeletti S, Uzzalli F, Caraglia M, Dicuonzo G, Tonini G. Repeated intermittent low-dose therapy with zoledronic acid induces an early, sustained, and long-lasting decrease of peripheral vascular endothelial growth factor levels in cancer patients. *Clin Cancer Res*. 2007; 13(15 Pt 1):4482–4486. 13/15/4482 [pii]. 10.1158/1078-0432.CCR-07-0551 [PubMed: 17671133]
 53. Tsagozis P, Eriksson F, Pisa P. Zoledronic acid modulates antitumoral responses of prostate cancer-tumor associated macrophages. *Cancer Immunol Immunother*. 2008; 57(10):1451–1459. 10.1007/s00262-008-0482-9 [PubMed: 18297280]
 54. Wolf AM, Rumpold H, Tilg H, Gastl G, Gunsilius E, Wolf D. The effect of zoledronic acid on the function and differentiation of myeloid cells. *Haematologica*. 2006; 91 (9):1165–1171. [PubMed: 16956814]
 55. Nagaraj S, Youn JI, Weber H, Iclozan C, Lu L, Cotter MJ, Meyer C, Becerra CR, Fishman M, Antonia S, Sporn MB, Liby KT, Rawal B, Lee JH, Gabrilovich DI. Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. *Clin Cancer Res*. 2010; 16(6):1812–1823. 1078-0432.CCR-09-3272 [pii]. 10.1158/1078-0432.CCR-09-3272 [PubMed: 20215551]
 56. Corbett TH, Roberts BJ, Leopold WR, Peckham JC, Wilkoff LJ, Griswold DP Jr, Schabel FM Jr. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. *Cancer Res*. 1984; 44 (2):717–726. [PubMed: 6692374]
 57. Linehan DC, Tan MC, Strasberg SM, Drebin JA, Hawkins WG, Picus J, Myerson RJ, Malyapa RS, Hull M, Trinkaus K, Tan BR Jr. Adjuvant interferon-based chemoradiation followed by gemcitabine for resected pancreatic adenocarcinoma: a single-institution phase II study. *Ann Surg*. 2008; 248(2):145–151. [pii]. 10.1097/SLA.0b013e318181e4e900000658-200808000-00001 [PubMed: 18650621]
 58. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007; 25(15):1960–1966. JCO.2006.07.9525 [pii]. 10.1200/JCO.2006.07.9525 [PubMed: 17452677]
 59. Picozzi VJ, Kozarek RA, Traverso LW. Interferon-based adjuvant chemoradiation therapy after pancreaticoduodenectomy for pancreatic adenocarcinoma. *Am J Surg*. 2003; 185(5):476–480. S0002961003000515 [pii]. [PubMed: 12727570]

60. Linehan DC, Goedegebuure PS. CD25+ CD4+ regulatory T-cells in cancer. *Immunol Res.* 2005; 32(1–3):155–168. IR:32:1-3:155 [pii]. 10.1385/IR:32:1-3:155 [PubMed: 16106066]
61. Dolcetti L, Peranzoni E, Ugel S, Marigo I, Fernandez Gomez A, Mesa C, Geilich M, Winkels G, Traggiai E, Casati A, Grassi F, Bronte V. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *Eur J Immunol.* 2010; 40(1): 22–35.10.1002/eji.200939903 [PubMed: 19941314]
62. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, Liu Y, Li D, Yuan Y, Zhang GM, Feng ZH. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. *Cancer Lett.* 2007; 252(1):86–92. S0304-3835(06)00684-7 [pii]. 10.1016/j.canlet.2006.12.012 [PubMed: 17257744]
63. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine Growth Factor Rev.* 2010; 21(1):41–48. S1359-6101(09)00116-6 [pii]. 10.1016/j.cytogfr.2009.11.009 [PubMed: 20005149]
64. Christopher MJ, Rao M, Liu F, Woloszynek JR, Link DC. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *J Exp Med.* 2011; 208(2):251–260. jem.20101700 [pii]. 10.1084/jem.20101700 [PubMed: 21282380]
65. Greenbaum AM, Link DC. Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. *Leukemia.* 2011; 25(2):211–217. leu2010248 [pii]. 10.1038/leu.2010.248 [PubMed: 21079612]
66. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest.* 2010; 120(7):2423–2431. 41649 [pii]. 10.1172/JCI41649 [PubMed: 20516641]
67. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol.* 2010; 22(2):238–244. S0952-7915(10)00022-1 [pii]. 10.1016/j.coi.2010.01.021 [PubMed: 20171075]
68. Rodrigues JC, Gonzalez GC, Zhang L, Ibrahim G, Kelly JJ, Gustafson MP, Lin Y, Dietz AB, Forsyth PA, Yong VW, Parney IF. Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. *Neuro Oncol.* 2010; 12(4):351–365. nop023 [pii]. 10.1093/neuonc/nop023 [PubMed: 20308313]
69. Haverkamp JM, Crist SA, Elzey BD, Cimen C, Ratliff TL. In vivo suppressive function of myeloid-derived suppressor cells is limited to the inflammatory site. *Eur J Immunol.* 2011; 41(3): 749–759.10.1002/eji.201041069 [PubMed: 21287554]
70. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest.* 2004; 114(5):623–633.10.1172/JCI22087 [PubMed: 15343380]
71. Aft R. Bisphosphonates in breast cancer: antitumor effects. *Clin Adv Hematol Oncol.* 2011; 9 (4): 292–299. [PubMed: 21558988]
72. Weiss HM, Pfaar U, Schweitzer A, Wiegand H, Skerjanec A, Schran H. Biodistribution and plasma protein binding of zoledronic acid. *Drug Metab Dispos.* 2008; 36(10):2043–2049. dmd. 108.021071 [pii]. 10.1124/dmd.108.021071 [PubMed: 18625688]
73. Zhang B, Zhang Y, Bowerman NA, Schietinger A, Fu YX, Kranz DM, Rowley DA, Schreiber H. Equilibrium between host and cancer caused by effector T cells killing tumor stroma. *Cancer Res.* 2008; 68(5):1563–1571. 68/5/1563 [pii]. 10.1158/0008-5472.CAN-07-5324 [PubMed: 18316622]
74. Viehl CT, Moore TT, Liyanage UK, Frey DM, Ehlers JP, Eberlein TJ, Goedegebuure PS, Linehan DC. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Ann Surg Oncol.* 2006; 13(9):1252–1258.10.1245/s10434-006-9015-y [PubMed: 16952047]
75. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* 1997; 15 (6):2403–2413. [PubMed: 9196156]

76. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol.* 2008; 181(8):5791–5802. 181/8/5791 [pii]. [PubMed: 18832739]

Figure 1. CD11b⁺CD15⁺ myeloid cells are upregulated in the peripheral blood and bone marrow of patients with pancreatic cancers when compared to normal controls

Multicolor flow cytometric analysis of peripheral blood samples of patients with confirmed pancreatic cancer was obtained prior to surgery. Patients with pancreatic cancer (n = 16) had a significant increase in the prevalence of CD11b⁺CD15⁺ PBMC compared to cancer free controls (n = 12) (***p < 0.0001). Those patients with unresectable, metastatic disease (n = 11) had the greatest burden of CD11b⁺CD15⁺ in whole blood compared to those with resectable disease (n = 9) or controls (n = 10) (B: *p < 0.01, **p < 0.05, ***p < 0.0001). Analysis of bone marrow cells from patients with pancreatic adenocarcinoma (n = 16) also revealed increased populations of CD11b⁺CD15⁺ compared to cancer free controls (n = 8; C: *p < 0.01). A representative flow cytometric analysis from PBMC is shown (D).

Figure 2. Immune subset analysis of human pancreatic cancer tumors

Normal human pancreas (A) was essentially devoid of any CD15⁺ or CD11b⁺ cells as determined by immunohistochemistry; however, patients with pancreas adenocarcinoma were noted to have a dense immune infiltrate composed primarily of cells which coexpressed CD15 and CD11b (B: CD11b blue, CD15 red, CD15 and CD11b coexpression magenta). Treg (Foxp3) were noted at a much lower prevalence (green). These cells were also found to highly coexpress Arginase-1 (C: CD15, red, Arginase-1, green, CD15 and Arginase-1 coexpression, orange/yellow, and nuclear stain, blue). Quantification of immune cell subsets in human pancreatic cancer samples was performed (CD15⁺CD11b⁺, CD33⁺CD11b⁺, CD8⁺, and CD4⁺) (D). Primary, resected tumors from patients with pathologically-confirmed pancreas adenocarcinoma were subjected to multicolor flow cytometric analysis (E). Gating of CD45⁺ cells was initially performed to distinguish immune infiltrate from pancreatic tumor cells.

Figure 3. Inoculation of C57Bl/6 mice with Pan02 induces systemic immunosuppressive changes
 In mice with mature tumors, immunosuppressive cells including MDSC and Foxp3⁺CD4⁺ Treg were significantly elevated in circulating immune cells isolated from the spleen (A: **p < 0.01 and ***p < 0.0001, respectively). A reciprocal effect was observed on CD8 and CD4 effector T cells (A: *p < 0.05 and **p < 0.01, respectively). The prevalence of Gr-1⁺CD11b⁺ cells varied between individual compartments in tumor bearing mice (B). Gr-1⁺CD11b⁺ cells isolated from each compartment were tested for their suppressive potential on T cell proliferation as measured by CSFE dilution. Tumor-derived Gr-1⁺CD11b⁺ cells exhibited the greatest suppression, while splenocyte- and bone marrow-isolated Gr-1⁺CD11b⁺ cells were able to suppress an antigen-mediated T cell response to a lesser degree. The shaded graphs represent proliferation of OT-1 T cells in the absence of MDSC (C).

Figure 4. Gr-1⁺CD11b⁺ cells coinjected with Pan02 tumor cells facilitate more rapid tumor engraftment

Tumor-derived Gr-1⁺CD11b⁺ cells from Ly5.1 congenic mice (indicated in the figure as “MDSC”) were isolated by FACS and coinjected with Pan02 tumor cells into wild type mice. Mice injected with a mixture of Gr-1⁺CD11b⁺ cells and Pan02 tumor cells demonstrated significantly larger tumors compared to mice inoculated with Pan02 alone ($p < 0.0001$). The addition of Gr-1⁺CD11b⁺ cells to tumor facilitated transient accelerated initial growth.

Figure 5. Treatment with zoledronic acid resulted in delayed tumor growth and prolonged survival in mice inoculated with Pan02

Six week old C57Bl/6 mice were inoculated with 100,000 Pan02 tumor cells and treated with zoledronic acid or placebo. Mice treated with zoledronic acid demonstrated significantly delayed tumor growth (A, * $p < 0.0001$) and prolonged median survival (B, $p < 0.05$) when compared to control animals treated with saline.

Figure 6. Treatment with zoledronic acid decreases the accumulation of MDSC in the tumor microenvironment

Serial treatment with zoledronic acid reduces the number of tumor infiltrating Gr-1⁺CD11b⁺ MDSC and Foxp3⁺CD4⁺ Treg in mice inoculated with Pan02 tumors over time (A, **p* < 0.05). A reciprocal increase in immune effector CD4 and CD8 cells is observed (B, **p* < 0.05). A representative flow cytometric analysis on day 34 obtained from a tumor sample is shown (C).

Figure 7. MDSC blockade functionally altered the tumor microenvironment

In addition to increased recruitment of effector T cells, a more robust type 1 response with increased levels of IFN- γ (* $p < 0.05$) and decreased levels of IL-10 (* $p < 0.05$) was observed. There was no difference in tumor levels of GM-CSF with zoledronic acid treatment.