MECHANISTIC STUDIES IN THE INFLAMMATORY RESPONSE OF PANCREATITIS AND PANCREATIC CANCER

- ROLE OF MYELOID DERIVED SUPPRESSOR CELLS -

By

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DEDICATION

To my amazing fiancé and future wife Suzette and to my beloved parents Humberto and Nora. Without your love and caring none of this would have happened. This humble work is a sign of my love to you.

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I. ABSTRACT

Tumor-infiltrating myeloid-derived suppressor cells (MDSCs), are important mediators of a tumor-permissive microenvironment that contributes to tumor growth and could account for the limited success of immunotherapeutic strategies. MDSCs suppress adaptive immunity by blocking T cell activation, inducing Treg accumulation, and inhibiting natural killer cell cytotoxicity against tumor cells. We investigated the roles of MDSCs in the regeneration of the exocrine pancreas associated with acute pancreatitis and the progression of acinar to ductal metaplasia.

Acute pancreatitis was induced in wild type and P48+/Cre;LSL-KRASG12D mice using caerulein and an early influx of MDSCs into the pancreas was observed flow cytometry and immunocytochemistry. Numbers of Gr1(+)CD11b(+) MDSCs increased over 20-fold in pancreata of mice with acute pancreatitis to account for nearly 15% of intrapancreatic leukocytes and have T cell suppressive properties. This marked accumulation of MDSCs returned to normal values within 24 hours of the insult in wild type mice; however, in the oncogenic KRAS mice, MDSCs levels remained elevated. When intrapancreatic MDSCs were depleted by administration of a CXCR2 antagonist (SB265610) in wild type mice the severity of acinar damage was increased. This was also accompanied by a delayed regeneration determined morphologically and with the mitotic immunomarker phospho-histone H3. Isolated intrapancreatic MDSCs from treated mice induce naïve acinar cells to undergo acinar ductal metaplasia when co-cultured in collagen 3D cultures. Purified splenic MDSCs failed to induce the phenotypic transdifferentiation.

We conclude that MDSCs are required for adequate pancreatic regeneration in wild type mice with acute pancreatitis and their persistent elevation in oncogenic KRAS mice is not only associated with immune-evasion, but may also function as direct enhancer of malignant proliferation.

II. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an inflammation-driven, highly aggressive malignancy with a dismal long term prognosis. Indeed, the disease exhibits a median survival of less than 6 months and a 5-year survival rate of 7%.[1] PDAC is the 4th leading cause of cancer-related death in both men and women in the Unites States. In 2015, it is estimated that more than 48,000 people in the United States will be diagnosed with pancreatic cancer and over 40,000 will die of this disease.[1,2] Even for patients with localized disease that is surgically resectable, the five-year prognosis is still poor.

PDAC evolves through a series of histopathological changes, referred to as pancreatic intraepithelial neoplasms (PanIN), accompanied by a recurrent pattern of genetic lesions the earliest and most ubiquitous of which is oncogenic activation of Kras.[3,4] Ninety-five percent of pancreatic cancers harbor activating mutations in Kras, representing the highest fraction of any tumor type.[5,6] The essential role of oncogenic Kras in the pathogenesis of PDAC is indicated by several genetically engineered mouse models where the conditional expression of the mutated allele of Kras in the pancreas is necessary and/or sufficient to drive disease progression from the early pre-invasive to a malignant stage.[7] Though the mechanisms by which oncogenic Kras contributes to the genesis and progression of PDAC have not been fully elucidated, the proliferative and survival advantages conferred on epithelial cells by expression of endogenous oncogenic Kras has been clearly implicated.[8,9]

Along with the known genetic alterations, there is a growing body of evidence for a strong relationship between inflammatory state and pancreatic cancer. Some of the inflammatory responses and pathways involved in inflammation have been linked to promotion and progression of pancreatic ductal adenocarcinomas.[10,11] Acute pancreatitis results from autodigestion of the pancreas by its own proteases. Enhanced pancreatic ductal permeability allows activated enzymes to leak from the duct and initiates pancreatic autodigestion. As the stimulus persists, the normal defense mechanisms of the

pancreas are overwhelmed by the released trypsin leading to pancreatic autodigestion and sets up a vicious cycle of active enzymes damaging cells, which then release more active enzymes. Inflammatory cytokines are overproduced in acute pancreatitis and mediate the systemic manifestations and pathophysiological changes of the disease. At the tissue level, the biochemical changes resulting from premature digestive enzyme activation damage the acinar cells, pancreatic interstitium, and vascular endothelium. Marked glandular invasion by macrophages and polymorphonuclear leukocytes in the early stages of animal and human pancreatitis has been shown in microscopic and radionuclide studies using Indium-111 tagged leukocytes. The activation of granulocytes and macrophages lead to the release of proteolytic and lipolytic enzymes, reactive oxygen metabolites, proinflammatory cytokines, and arachidonic acid metabolites. These local and systemic inflammatory substances overwhelm the scavenging capacity of endogenous antioxidant systems. The triad of activated pancreatic enzymes, microcirculatory impairment, and release of inflammatory mediators in its severe form can lead to a systemic insult and is associated with the development of systemic inflammatory response syndrome.[12]

Chronic pancreatitis, a well-established risk factor for pancreatic cancer, is believed to arise from repeated overt or silent episodes of acute pancreatitis.[13] As an example, one of the best characterized familiar pancreatic cancer syndromes is associated with development of chronic pancreatitis.[14,15] The pathogenesis of chronic pancreatitis and advanced pancreatic cancer are associated with inflammation and fibrosis. In fact, many of these inflammatory cytokines produced by pancreatic cancer act as autocrine and paracrine growth factors (IL-6, IL-1, TNF-α and TGF-β). Even though there is a strong link between chronic pancreatitis and pancreatic cancer; until the development of more sophisticated early detection measurements, screening is not recommended. [16,17]

Caerulein, a cholecystokinin (CCK)-analogue widely used in pancreatitis mouse models, rapidly accelerates cancer development in the context of Kras oncogenic activation.[11,18] Caerulein causes acinar cells reprogramming and pancreatitis might induce pancreatic cell regeneration and promote PDAC

development in oncogenic Kras mice. However, our understanding of these processes and ability to modify them is still very rudimentary. Our investigations of the mechanism will provide an increased understanding of the multifaceted roles of inflammation in regulating cell fate during PDAC carcinogenesis.

Among cells that negatively regulate the immune system in cancer, myeloid-derived suppressor cells (MDSCs) have a critical role in this process. MDSCs 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.[19] The prevalence of these cells in cancer patients correlates with disease stage, and normalization in MDSCs numbers occurs after successful treatment.[20,21] MDSCs in murine models of cancers reliably express Gr-1$^+$CD11b$^+$, whereas in humans, MDSCs are typically CD11b$^+$CD33$^+$HLA$^-$DR$^-$.[22] Tumor-induced alterations in bone marrow myelopoiesis are driven by growth factors and cytokines secreted by the primary tumor resulting in MDSCs expansion and mobilization. Once mobilized to the tumor stroma, MDSCs promote primary tumor growth and invasion through a variety of mechanisms such as arginase production, iNOS and ROS upregulation, L-selectin down regulation, cysteine depletion, promotion of neoangiogenesis, and regulatory T cell recruitment. MDSCs also promote tumor-induced immunosuppression and host immune evasion by inhibiting lymphocyte activation and antigen recognition. [19,23]

In both human PanIN tissue specimens and mouse models, GM-CSF production has been observed. The production of GM-CSF occurs by pancreatic ductal epithelial cells in a mutant Kras dependent manner and in vivo is required for the recruitment of Gr1$^+$CD11b$^+$ myeloid-derived suppressor cells. Pancreatic ductal cells that are driven by oncogenic Kras have been found to secret GM-CSF which promotes the proliferation and maturation of myeloid derived cell lineages. The evolution of pancreatic tumors has been shown to be critically dependent on the repression of T cell responses that would restrain tumor initiation.[9]

Pancreatic ductal adenocarcinoma is generally believed to arise predominantly from acinar cells through progression of pancreatic intraepithelial neoplasia (PanIN), ranging from low grade PanINs (termed PanIN1A, -1B) to high grade PanINs (termed PanIN-2, -3), to ductal adenocarcinoma.[3,4] Even surrounding early PanIN lesions there is a recruitment of immune cells. However, the role of PanIN associated immune infiltrates in PDAC is not well understood. It has been suggested that PanINs form an immune suppressive environment which allows them to escape immune surveillance and this is likely by recruitment of MDSCs. In patients with glioblastoma, colon, breast, the frequency of an immature MDSC population correlates with tumor burden and poor prognosis.[24]

In a physiological immune response to acute pancreatitis, pro-inflammatory signals are released by acinar cells undergoing necrosis or bystander cells to recruit effector T cells, immature dendritic cells (DCs), and macrophages. The immune response evolves by activating and expanding effector T cells through DC cross-presentation in the draining lymph nodes. Upon activation, T cells kill the damaged cells and secrete GM-CSF and other pro-inflammatory cytokines. When the pro-inflammatory cytokines reached a determinate concentration, MDSCs are recruited to turn off the immune response and promote tissue remodeling and repair.[12,25]

This project focuses on inflammation-driven pathogenesis of pancreatic cancer with the goal of examining the role of MDSCs in reprogramming pancreatic acinar cells as they undergo transdifferentiation in acinar-to-ductal metaplasia (ADM) in the development of pancreatic cancer. This process is important for regeneration and repair of normal pancreata and it can be altered in the presence of oncogenic Kras.

First, we will evaluate expansion and accumulation of MDSCs in a pancreatitis mediated acceleration of a pancreatic cancer mouse model. The $p48^{cre}/Kras^{G12D}$ mutant mouse is a transgenic model that generates features of human PDAC. By inducing pancreatitis in the Kras mice we expect to

see changes in the time course of disease progression and correlation with a sustained recruitment of MDSCs to the pancreas. Then, we will deplete MDSCs before induction of pancreatitis and evaluate the severity of inflammation. By removing the suppressor cells from the pancreas microenvironment, we expect a more severe response to the insult and a delay in the regeneration process. Finally, we hypothesized that co-localization of MDSCs will alter the acinar cell microenvironment to induce a phenotypic change into duct-like cells. To validate this theory, we will co-culture isolated pancreatic acinar cells with MDSCs from mice in which acute pancreatitis was induced. We will quantitate the ability of MDSCs to promote ADM and determine the gene expression of marker genes and key transcription factors in the reprogramming process.

Our research will address the gap in knowledge by defining the role of this heterogeneous group of cells as key mediators of tumor-induced immunosuppression, facilitators of acinar to ductal metaplasia and promoters of regeneration. Historically, chemotherapy or radiotherapy did not provide meaningful survival benefit in advanced pancreatic cancer. Current therapies have their limitations; thus, we are in dire need of newer treatment options. Contributions from this project will be significant because, by scrutinizing the role of MDSCs, new targets for immunotherapy can be developed.

III. METHODS AND MATERIALS

All animal experimental protocols were in accordance with institutional oversight committee requirements.

a. Animals

Male C57BL/6 (6-8 week old) and $p48^{+/Cre}$;LSL-$KRAS^{G12D}$ (4-8 week old) mice were obtained from The University of Arizona Cancer Center Experimental Mouse Shared Resource (EMSR). Mice were

genotyped using standard PCR procedure. Mice were housed in a pathogen-free environment and fed standard chow. Animal procedures were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC) .

b. Model of Acute Pancreatitis

Caerulein-induced pancreatitis is a well-studied model of the disease. The administration of supramaximal doses of this synthetic cholecystokinin analogue generates a distinct pancreatic response that includes diminished secretion, accumulation of secretory proteins within the pancreas and pancreatic injury.[18] Pancreatitis was induced using a regimen of 7 hourly intraperitoneal injections of caerulein (50 g/kg; Sigma-Aldrich) followed by a 48-hour rest period and a second regimen of 7 hourly intraperitoneal injections of caerulein before sacrifice at different time points (1, 12, 24, 48 and 72 hours after last caerulein injection).

c. Model of MDSCs depletion

C57BL/6 mice were treated with bi-daily intraperitoneal doses of CXCR2a (SB-265610) (4 mg/kg; Sigma-Aldrich, St Louis, MO). Treatment started one day before induction of pancreatitis with caerulein and continued until animal euthanasia.

d. Cellular Isolation and Flow Cytometry Analysis

To isolate mononuclear cells from the pancreas and spleen, organs were harvested via postmortem laparotomy and minced in small pieces using sterile scissors. The supernatants were passed through a 70 µm strainer and centrifuged. In order to isolate leukocytes, the resultant pellet was re-suspended in a Percoll gradient. Tubes were centrifuged at > 10 000 × g in an angle-head rotor for 25 mins and cells suspended in the middle interface were collected. Cell surface marker analysis was performed by flow cytometry using the BD FACSCANTO II (BD Biosciences; San Jose, CA) after incubating 5×10^5 cells with 1

µg anti-mouse CD16/CD32 purified antibody (Fc Block) (93) (eBioscience) and then labeling with 1 µg fluorescein isothiocyanate, phycoerythrin, or allophycocyanin-conjugated antibodies directed against Ly-6G/Ly-6C (Gr-1)(RB6-8C5) (Biolegend), CD45.2 (104), CD11b (M1/70), MHC Class II I-Ab (AF6-120.1) CD11c (N418), F4/80 (BM8) (all eBioscience).

e. Positive selection of gMDSC population

After preparation of a single-cell suspension from pancreas or spleen, as described previously, $CD11b^{+}Gr1^{+}$ cells were indirectly magnetically labeled with Anti-Ly-6G-Biotin and Anti-Biotin Microbeads (Miltenyi Biotec, Auburn, CA). After incubation, this suspension was loaded onto a column placed in a magnetic field. The magnetically labeled Ly6G+ cells, also known as positively selected cell fraction, were retained within the column and collected once the column is removed from the magnetic field. The cells not positively selected were analyzed by Flow Cytometry to confirm the efficacy of the isolation.

f. Co-culture of isolated activated gMDSCs with naïve acini in a collagen 3D gel

Pancreata from untreated wild type mice was minced in small pieces using sterile scissors. Pancreas was suspended in 5ml of cold HBSS containing 1mg of collagenase. Reaction was stopped after 16 minutes of incubaton by adding 5ml of cold HBSS with 5% FBS. The suspension was passed through a 70 µm strainer, centrifuged and washed. After removing the supernatant, the acinar units were resuspended in col 1X Waymouth media (containing 1% FBS, 0.5% gentamicin with 0.4 mg/ml of trypsin inhibitor and 1 ug-ml of dexamethasone). This suspension was then mixed with bovine collagen gel mixture (1:1) and 500 ul of the collagen gel/cell suspension was put into each of a 24-well plate and incubated. gMDSCs from spleen and pancreas of mice in which acute pancreatitis was induced were isolated as previously described and added to the collagen 3D gel. After 4 days of incubation, gels were stained with Cytokeratin 19 antibody (A53-B/A2) (Santa Cruz Biotechnology) and other gels were homogenized in

Trizol to isolate total RNA. Total RNA was converted to cDNA with random primers and analyzed with qPCR for relative gene expression of Hes-1 and CK-19.

g. qPCR analysis

Quantitative PCR was performed using a standardized preconfigured PCR array (SA Biosciences, Frederick, MD) on the Stratagene MX3000P (Stratagene, La Jolla, CA) according to the respective manufacturers' protocols. Alternatively, individual primers for BCL2 and ERK2 were obtained from Qiagen and PCR amplification was performed using the SYBR Green/ROX RT-PCR Master Mix (Qiagen).

h. Histology and Immunohistochemistry

For histological analysis, pancreatic specimens were fixed with 10% buffered formalin, dehydrated in ethanol, and then embedded with paraffin and stained with H&E. Acinar cell viability was calculated by examining 12 high-powered fields per slide and obtaining the mean percentage of viable acinar tissue. Immunohistochemistry was performed using antibodies directed against CD45, Ly6G-Ly6C (both BD Biosciences), Cytokeratin 19, Amylase (both Santa Cruz Biotechnology) and CD33 (PWS44) (Cell Marque). To evaluate for regeneration within the pancreatic tissue, we used Phospho-Histone 3 antibody (Cell Signaling, Danvers, MA) which highlights phosphorylation sites of mitosis. Photographs were taken with Axiovert 40 microscope (Carl Zeiss, Oberkochen, Germany). Images were captured on an Axiovert 200M fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The number of mitotic nuclei identified with Phospho-Histone 3 were counted using Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA). Photographs of 20X magnification were analyzed, and the total number of nuclei was obtained.

i. Statistics

Data are presented as mean ± standard error of mean. Statistical significance was determined by the Student's t test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). P values <.05 were considered significant.

RESULTS

a. Intrapancreatic MDSCs expand in Acute Pancreatitis

To assess the significance of MDSCs in acute pancreatitis described previously, we first tested whether the intrapancreatic $CD11b^{+}Gr1^{+}$ MDSCs population expands after initiation of pancreatitis from 2 treatments with 7-hourly caerulein injections separated by a 48-hour period of rest (Figure 1C). We found that while MDSCs were rare in the normal pancreas, the total number of intrapancreatic MDSCs increased markedly in acute pancreatitis, reaching a peak at 1 hour after the last dose of caerulein. Intrapancreatic MDSCs numbers returned to normal by 48 hours after the last caerulein injection. From previous experiments, we know that the total number of other leukocyte subgroups increases in acute pancreatitis; however, there was a disproportional increase in MDSCs.

In particular, the fraction of granulocytic intrapancreatic $CD11b^{+}Gr1^{high}$ MDSCs (gMDSCs) expanded from a baseline of 1%–3% to nearly 15% of all CD45+ intrapancreatic leukocytes (Figure 1D). Conversely, the number of splenic gMDSCs remained constant in acute pancreatitis, suggesting that MDSC expansion is a pancreas-specific phenomenon (Figure 1E). Histologic analysis with H&E of these pancreata demonstrates severe acinar injury and presence of inflammatory cells, especially in Days 0 and 1. The infiltration of these immune cells is much less evident by Day 3 and this is accompanied by decreased

acinar necrosis (Figure 1F). The origins of intrapancreatic MDSCs in acute pancreatitis are not entirely certain, given the experimental limitations of tracking MDSCs in situ.

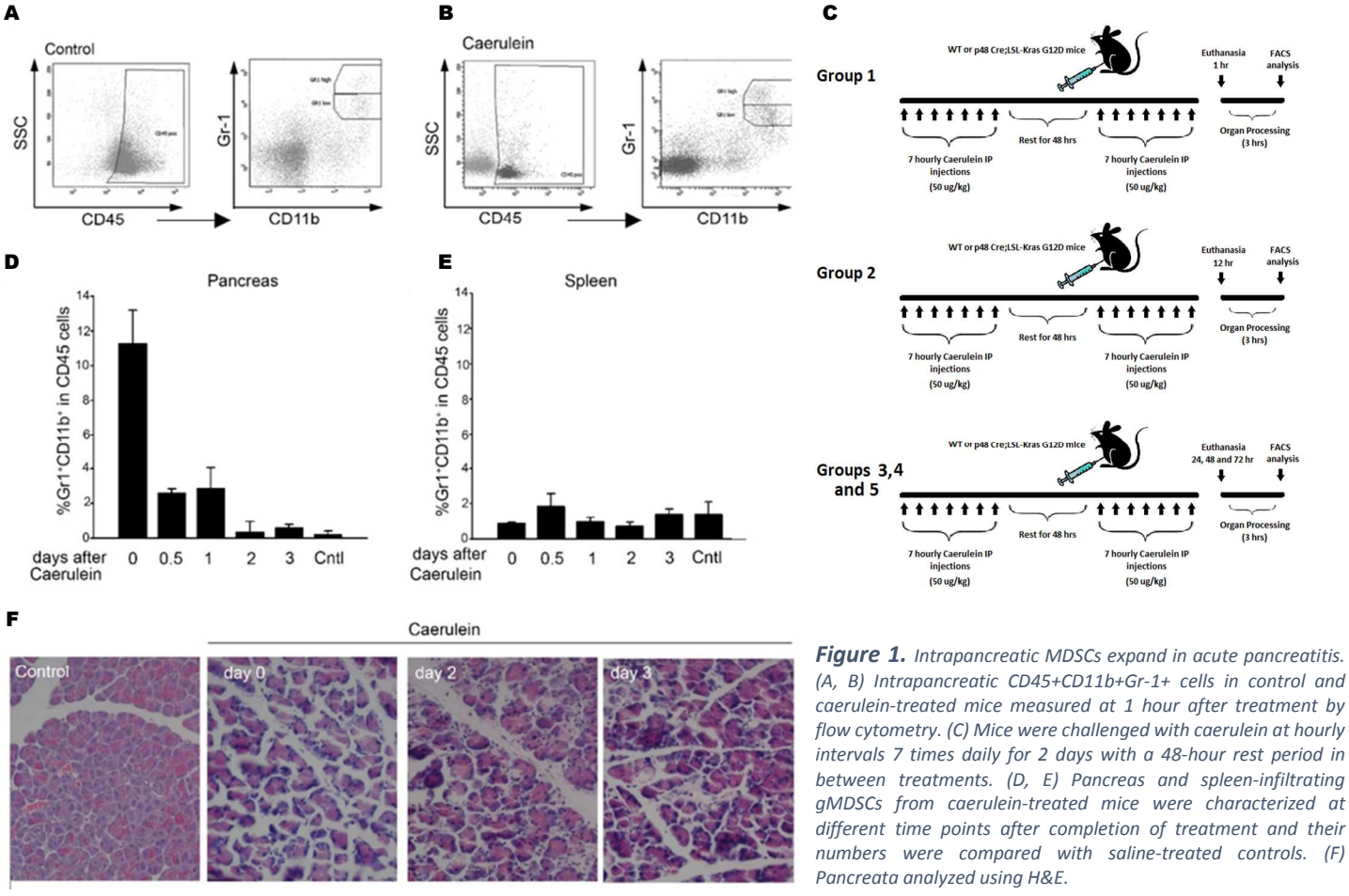

Figure 1. *Intrapancreatic MDSCs expand in acute pancreatitis. (A, B) Intrapancreatic CD45+CD11b+Gr-1+ cells in control and caerulein-treated mice measured at 1 hour after treatment by flow cytometry. (C) Mice were challenged with caerulein at hourly intervals 7 times daily for 2 days with a 48-hour rest period in between treatments. (D, E) Pancreas and spleen-infiltrating gMDSCs from caerulein-treated mice were characterized at different time points after completion of treatment and their numbers were compared with saline-treated controls. (F) Pancreata analyzed using H&E.*

b. Acute Pancreatitis in the Oncogenic Kras mouse model is characterized by a sustained accumulation of gMDSCs and a more severe acinar injury.

When pancreatitis was induced in p48$^{+/Cre}$;LSL-KRAS^{G12D} mice, the increment of gMDSCs in the pancreas was more abrupt and sustained compared to wild type mice (Figure 2A-B). Mice were euthanized at 12 hours and 1 day after the last caerulein injection (Figure 2C). Pancreata was analyzed using H&E and percentage of acinar non-viability was calculated (Figure 2D). While the fraction of nonviable acini was 25% in the wild type mice after caerulein challenge at time point "0.5 after caerulein"; this percentage increased to close to 50% of non-viability in the oncogenic Kras mice (Figure 2E).

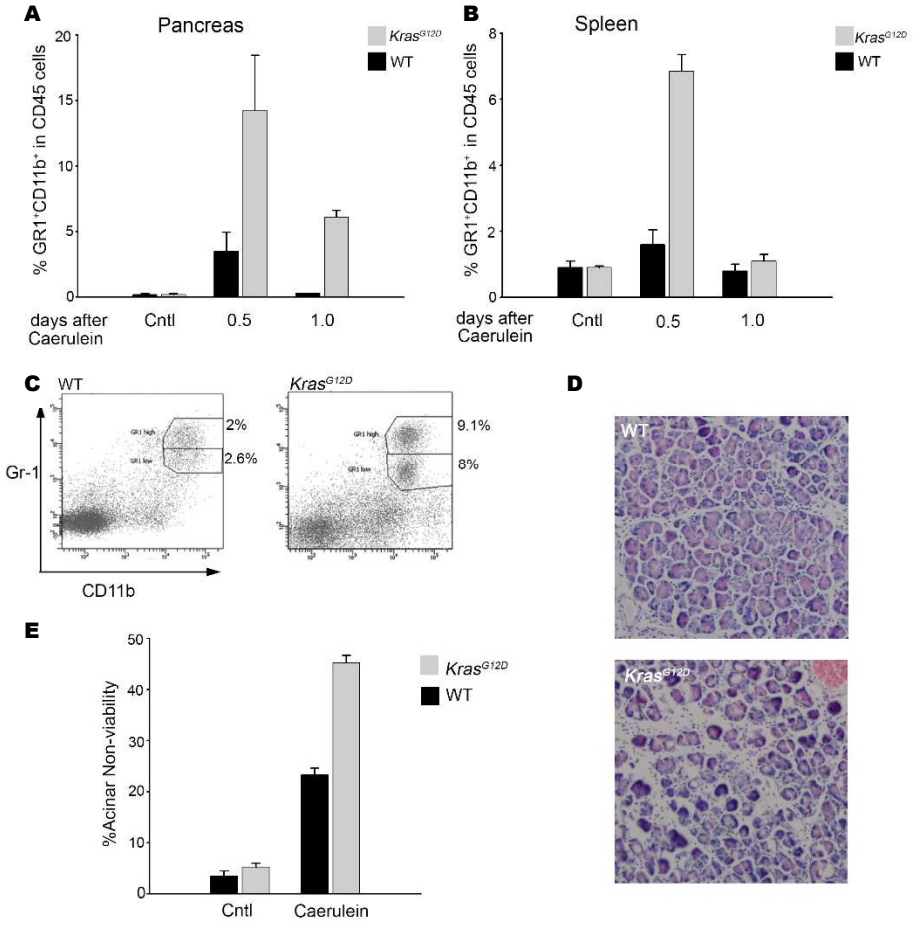

Figure 2. *Acute pancreatitis in the oncogenic Kras mouse model. (A-B) Pancreas and spleen-infiltrating gMDSCs from caerulein-treated mice were characterized at 2 time points after completion of treatment and their numbers were compared with saline-treated controls. (C) Representative flow cytometry analysis of Intrapancreatic CD45+CD11b+Gr-1+ cells in wild type and oncogenic Kras mice. (D) Pancreata analyzed using H&E. (E) Comparision of acinar non-viability percentage in the two models studied.*

c. Pancreatic gMDSCs are activated and have an immunosuppressive expression profile in Acute Pancreatitis

To determine whether intrapancreatic granulocytic MDSCs (gMDSCs) are in an activated immunosuppressive state after induction of acute pancreatitis, we compared the mRNA expression profile of pancreatic gMDSCs from caerulein-induced pancreatitis to splenic gMDSCs. As previously described, gMDSCs were isolated by positive selection using magnetic labeling (Figure 3B). To test efficacy of the selection, the negative selected cells were tested by flow cytometry, observing absence of gMDSCs (Figure 3C). Pancreatic activated gMDSCs mRNA were analyzed by qPCR; level expression of Arginase 1, iNOS and IL-10 were markedly increased compared to splenic gMDSCs controls (Figure 3A).

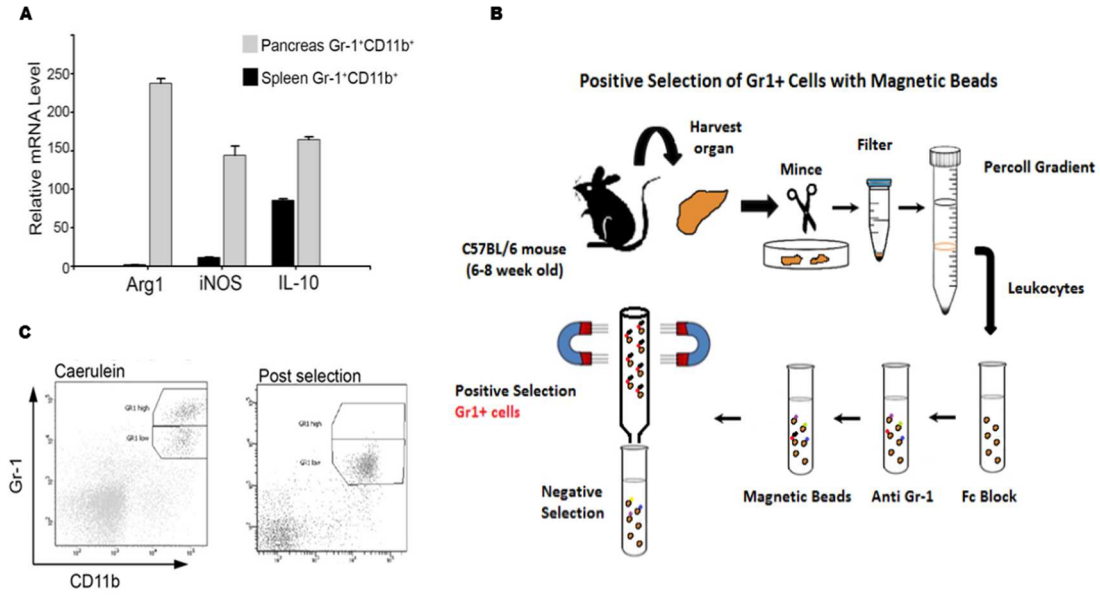

Figure 3. *Intrapancreatic activated gMDSCs have Immunossupressive profile. (A) Pancreatic and splenic gMDSCs of caerulein-treated mice were assayed for gene expression by qPCR. Relative mRNA level is indicated. (B) Positive selection of activated gMDSCs with magnetic labelling. (C) Efficacy of the selection was tested by flow cytometry.*

d. MDSCs recruitment is inhibited by a CXCR2 antagonist

Because MDSCs expand and have immunosuppressive properties in acute pancreatitis, we postulated that they contribute significantly to intrapancreatic modulation of inflammation. To test this hypothesis, we employed wild type mice, in which MDSC depletion was achieved with the use of a Chemokine ligand 2 (CXCL2) receptor antagonist (Figure 4A). CXCL2 is a small cytokine that belongs to the CXC chemokine family, secreted by monocytes, macrophages, and acinar cells. Its receptor, CXCR2, is found in MDSCs.

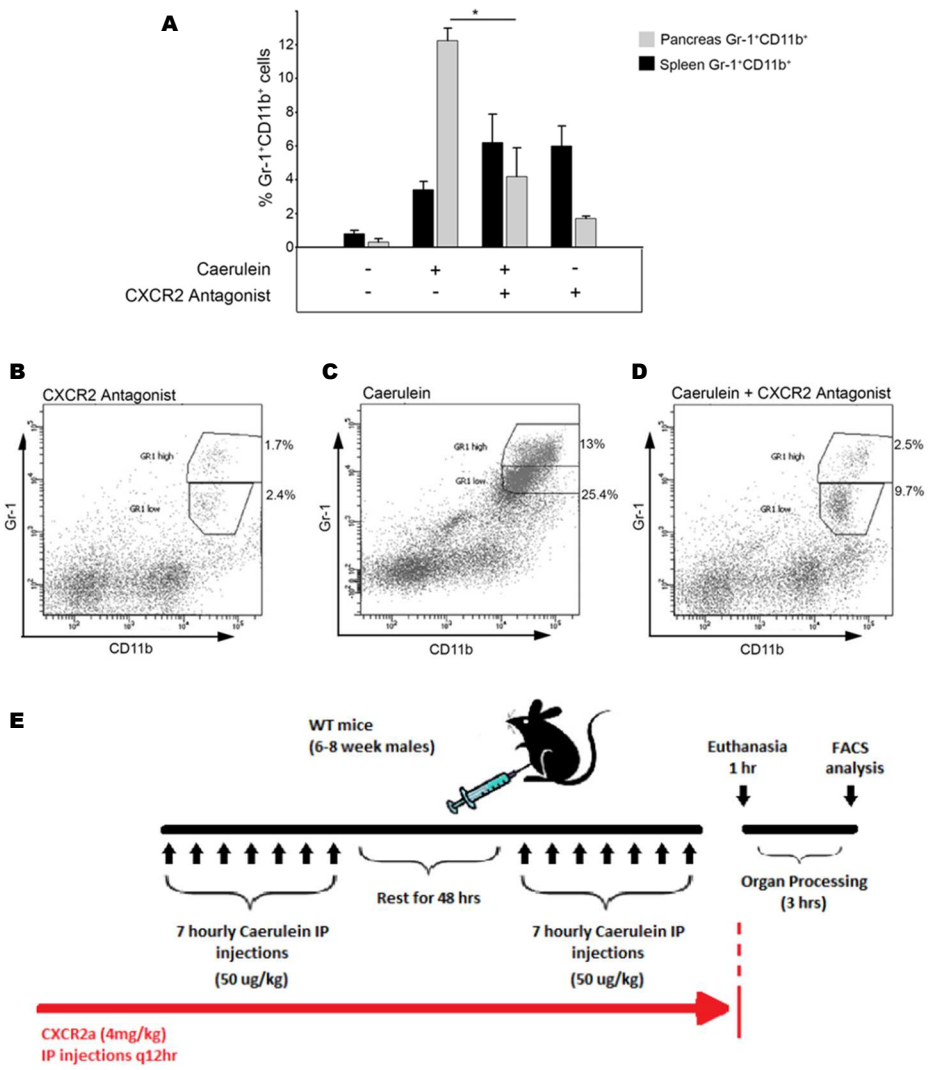

Figure 4. *MDSCs recruitment is inhibited by a CXCR2a. (A) Percentage of CD11b+Gr-1+ cells out of total CD45+ cells in wild type mice treated with caerulein only, caerulein + CXCR2a, CXCR2a only, and saline controls. (B-D) Representative flow cytometry of different groups. (E) CXCR2a (SB-205610) treatment began 24 hours before caerulein challenge and continued until sacrifice.*

CXCL2 has been shown to play a major role in recruiting MDSCs in inflammation. Different groups of researchers have attempted to block this receptor in different mouse models. The mb 1A8 anti-CXCR2 monoclonal antibody has been developed and used in a pancreatic cancer mouse model to block MDSCs. We chose to use a receptor antagonist (CXCR2 antagonist) SB-265610 to block this signaling axis. Wild type mice were treated or mock-treated with SB-265610 before caerulein challenge (Figure 4E). Pancreata was analyzed at 1 hour after last caerulein treatment. gMDSCs population which increases to nearly 15% of all CD45+ intrapancreatic leukocytes after induction of pancreatitis, are reduced to only 2.5% when depleted with SB-265610 (Figure 4B-D).

e. Intrapancreatic gMDSCs depletion delays acinar regeneration

Wild type pancreatic acini transiently de-differentiates and rapidly regenerates following injury. Acini responds to injury by adopting a ductal-like phenotype. This reversible phenomenon is called acinar to ductal metaplasia (ADM). On the other hand, acini possessing mutant Kras are sensitized to persistent dedifferentiation and ADM/PanIN formation. Loss of acinar cell differentiation also drives pancreatic cancer initiation, providing a mechanistic link between pancreatitis and cancer risk.

To investigate the role of MDSCs in acinar regeneration following acute pancreatitis we depleted the gMDSCs population and harvested pancreata 4 days after the last caerulein treatment (Figure 5A). Approximately 25% of acini were nonviable in wild type mice after caerulein only challenge. The fraction of nonviable acini increased to 60% in mice depleted of gMDSCs (Figure 5B). These results can be compared to the regeneration time course following acute pancreatitis (Figure 1F). Formalin-fixed, paraffin-embedded pancreata were examined by DAPI (4',6-diamidino-2-phenylindole) and stained with an antibody to mitosis-specific marker phospho-histone H3 (Figure 5C-D). We counted the number of mitotic nuclei as described previously.

Taken together, these data suggest that MDSCs may exert their protective effects by limiting inflammation and promoting regeneration following acute pancreatitis.

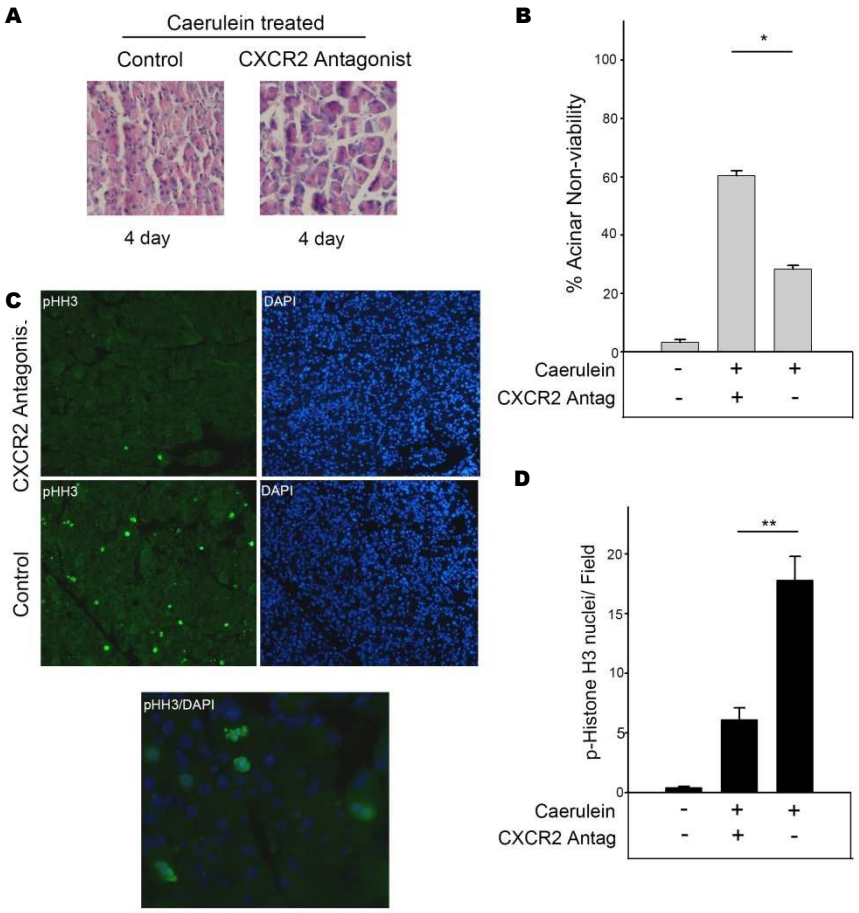

Figure 5. *Acinar regeneration is delayed following gMDSCs depletion. (A) Mice were sacrificed 4 days after the final dose of caerulein and pancreata examined by H&E staining. (B) Percentage of acinar non-viability was calculated in each group. (C) Formalin-fixed, paraffin-embedded pancreata were then examined by 4',6-diamidino-2-phenylindole and phosphor-histone 3 staining (*P .05; **P .01).*

f. Activated gMDSCs induce ductal-like transformation of the naïve acini

Since gMDSCs absence in the context of inflammation delays regeneration of the pancreatic acini we postulated that these cells not only modulate the inflammatory response but also may play a direct role in the acinar to ductal metaplasia. In order to test this hypothesis, acinar units were isolated from naïve

pancreata of wild type mice and plated in a 3D gel. Activated intrapancreatic gMDSCs (gMDSCs isolated from mice after induction of acute pancreatitis) were added to the gel. After a 4-day incubation period, the acinar units changed to a ductal phenotype (Figure 6A). The presence of ductal-like cells was confirmed with CK-19 staining (Figure 6B). This dramatic increase in ductal structures was calculated in over 10-fold compared to controls. TGFα, cytokine known to activate EGFR and stimulate acinar de-differentiation, was used as a positive control (Figure 6C). Supernatants were studied for gene expression. A significant increase in CK-19 and HES-1 expression was observed when compared to controls (Figure 6D).

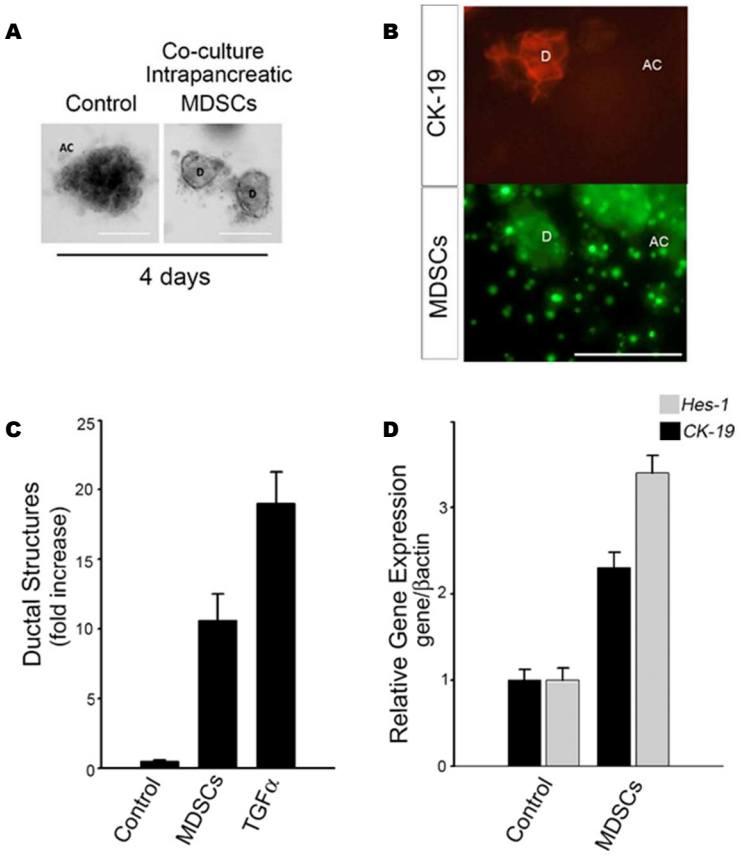

Figure 6. *Activated gMDSCs induce ductal-like transformation of the naïve acini. (A) 3D gel co-culture of acinar units with MDSCs and controls after 4 days of incubation. (B) 3D gel stained with CK-19 and gMDSCs labelled with cell tracker green. (C) Fold increase of ductal structures in controls vs gels with gMDSCs. TGFα used as a positive control. (D) Gene expression of HES-1 and CK-19 in co-cultured gels.*

VI. V. DISCUSSION

Despite decades of intense research efforts, pancreatic ductal adenocarcinoma remains a top cause for cancer related deaths in the United States. There is no reliable method for early detection and for the ~10-25% of pancreatic cancer patients who have a surgically resectable disease at diagnosis, the 5-year survival is still very low (~10%) due to recurrent disease. [26]

Acute pancreatitis is a disease with significant medical and economic impact, yet its specific immunological pathogenesis is uncertain. We have shown here that MDSCs, immunosuppressor cells by nature, play a key role in the modulation of inflammatory response of acute pancreatitis and the extend of organ-specific injury. Using a widely applied and well-studied animal model of acute pancreatitis that mimics human disease, we revealed an expansion of the granulocytic MDSC (gMDSCs) population specific to the pancreas. gMDSCs also expressed an immunosuppressive phenotype characterized by gene expression of Arginase 1, iNOS and IL-10. Again, these changes were noted to be pancreas-specific; relative mRNA levels of spleen MDSCs remained constant in pancreatitic mice. The presence of gMDSCs in this microenvironment is necessary to modulate the T cell response; otherwise, the unopposed T cell function will translate in a more severe pancreatitis.

When the model was applied to the oncogenic Kras mice, not only a more abrupt and sustained elevation of gMDSCs levels was observed; but also a more severe acinar injury. Other groups of investigators have demonstrated the role of the Kras mutated acinar cell in early recruitment of gMDSCs via secretion of GM-CSF. This early recruitment assists the neoplastic cells in escaping from the immune recognition, in invading the surrounding tissues, and in seeding to distal site.

The recognition of a discrete and immunogenic body of gMDSCs in the pancreas led us to investigate whether gMDSCs prevents a more destructive inflammatory response in acute pancreatitis. We found that depletion of gMDSCs using the CXCR2a SB-265610 in mice treated with caerulein resulted in

increase in the severity of pancreatitis and delayed acinar regeneration. The severe acinar injury observed in caerulein-treated, gMDSC-depleted mice could be attributable to a protective influence of endogenous MDSCs on the injured/inflamed pancreas.

Since CD11b+Gr-1+ gMDSCs is an important suppressive myeloid subset, preventing its trafficking to inflammation site has a deleterious effect in acute pancreatitis. On the other hand, previous studies have demonstrated an enhanced Kras-dependent, GM-CSF production in early PanINs. This upregulation of GM-CSF will recruit CD11b+Gr-1+ cells to the tumor microenvironment, assisting the neoplastic cells in escaping from the immune recognition, in invading the surrounding tissues, an in seeding to distal site. For these reasons, CXCR2 has been identified as a novel target for modulating tumor immune escape.

We hypothesized that gMDSCs will not only have an immunosuppressive protective effect in acute pancreatitis but also will directly affect acinar cells. We found that when acinar units were co-cultured with activated gMDSCs, a differentiation into a ductal-like phenotype will occur. De-differentiation is a transient physiologic response to stress; however, when tissues are exposed to repetitive stress, this phenomenon can become permanent.

Acute pancreatitis is characterized by invasion of multiple cell types into the pancreas. We have demonstrated that gMDSCs are present in high numbers during inflammation. Absence of gMDSCs will worsen acinar injury and prevent regeneration. It is known that mutated ductal epithelial cells recruit gMDSCs by secreting high levels of GM-CSF. This will turn the microenvironment into a perfect niche for tumor progression.

Further work to elucidate the regulatory function of gMDSCs in pancreatitis may be a key step in immune-directed therapy against acute pancreatitis and PDAC.

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