UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inhibition of myeloid cell PI3Kγ is a potential therapeutic approach to treat pancreatic cancer

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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The Thesis of Chanae Rhea Hardamon is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

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DEDICATION

This thesis is dedicated to my mom, who has been my greatest source of support, comfort, reassurance, and love.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
PI3K	Phosphatidylinositol 3-kinase
PI3Kγ	Phosphatidylinositol 3-kinase gamma
GPCR	G protein-coupled receptor
RTK	Receptor tyrosine kinase
TLR/ILR	Toll-like receptor/Interleukin receptor
VCAM	Vascular cell adhesion protein
TAM	Tumor-associated macrophage
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
VEGF-A	Vascular endothelial growth factor
Arg-1	Arginase-1
WT	Wild-type

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understanding. Whenever I have been unsure about certain things, Dr. Russell has provided clarity, support, and reassurance. For all of that, I am truly grateful.

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Last, but not least, I would like to thank my mom for being the most wonderful person and friend that I have ever known. With her constant love, support, willingness to listen, and ability to provide the best motherly advice, she has helped me become who I am today.

ABSTRACT OF THE THESIS

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This thesis relates to a working hypothesis that tumor inflammation plays a significant role in initiating and perpetuating tumor growth, angiogenesis and metastasis. While normal inflammatory responses aid in wound healing, fighting infections, and destroying pathogens, tumor inflammation is self-perpetuating and plays a role in facilitating the disease, in part by inducing immunosuppression.

Gr1+CD11b+ myeloid cells are the most prevalent inflammatory cells found in tumors, where they directly promote tumor angiogenesis and immunosuppression. Recent studies in our laboratories have shown that the phosphatidylinositol 3- kinase (PI3K) catalytic subunit isoform p110γ, which is mainly expressed by Gr1+CD11b+ myeloid cells, directly promotes myeloid cell invasion and consequently, tumor immunosuppression. Genetic and pharmacological suppression of PI3Kγ activity substantially reduces myeloid cell trafficking to orthotopic Pdx1Cre;Kras+; p53+/- and other pancreatic ductal carcinomas, reduces expression of immunosuppressive factors, and inhibits pancreatic tumor growth and metastasis. These studies indicate that PI3Kγ inhibitors may be useful therapeutics for pancreatic ductal carcinoma.

Chapter 1- Introduction

In 2012, an estimated 43,920 people in the United States will be diagnosed with pancreatic cancer and an estimated 37,390 people will die from this disease.[23] One reason for a high mortality rate is due to late diagnosis. Normally, patients with pancreatic cancer experience little to no symptoms early on, which puts the patient at a severe disadvantage when the cancer is finally detected. Once the cancer has progressed and metastasized, it becomes more difficult to treat. Mild symptoms of pancreatic cancer include fever, loss of appetite, itching, depression, weight loss, and abdominal pain which are not symptoms someone might initially associate with this disease. Because pancreatic cancer is able to develop and progress discreetly, it is sometimes referred to as a "silent killer." Usually by the time the cancer is detected, it has spread to other organs leaving no option for surgery, which is the only cure for cancer at this time. With limited therapies available and pancreatic cancer research being underfunded, treating this aggressive disease is a challenge.

Behavioral, environmental, and hereditary factors have been associated with the cause of pancreatic cancer.[2,3] When a cell becomes cancerous, the cellular DNA becomes damaged and acquires mutations that result in chromosomal instability. More than 90% of pancreatic tumors have mutations in two genes that regulate normal cellular processes within the cell. These two genes are *Ras* and *TP53* (*Trp53* in mice).[2,21] Ras proteins are small GTPases that alternate between active and inactive states. Ras guanosine triphosphate (Ras-GTP) is the active version of Ras which undergoes hydrolysis to become inactive Ras guanosine diphosphate (Ras-GDP). Ras

proteins control crucial signaling pathways that regulate cell fate, differentiation, survival, and angiogenesis.[1,15] Oncogenic *Ras* is the one of the most common gain-of-function mutations in human cancer, being active in 90% of pancreatic tumors.[2] In tumors, *Ras* permanently remains in the active GTP state, inducing abnormal cellular activity. [1] *TP53* is a tumor-suppressor gene that codes for the tumor-suppressor protein, p53. The p53 protein is a transcription factor that regulates pathways of the cell cycle, apoptosis, and DNA repair. Wild-type p53 also protects the cell under stress conditions and prevents normal cells from developing malignancies. Mutations in *TP53* can cause p53 to be inactive in cancer cells which would interfere with normal functioning of the cell cycle, apoptotic pathways, and DNA damage repair.[9] Impairing normal cellular processes with these mutations allow cancer cells to survive. Mutant *TP53* and *Ras* allow cells to evade DNA damage control checkpoints and apoptotic signals, creating cells that are invasive and immortal.[2,12,15,21] Although oncogenic *TP53* and *Ras* are not the only mutations exclusive to pancreatic cancer cells, mutations of both genes function to reinforce genetic instability as well as the aggressive and metastatic character of pancreatic cancer.

Inflammation is a key factor in tumor development and progression, as well as angiogenesis[8,11,17.] Tumor and stromal cells release chemokines, cytokines, and growth factors that recruit immune cells to the tumor microenvironment, thereby causing immunosuppression[5,18,22]. Studies show that immunomodulatory hematopoietic cells such as macrophages directly collaborate with tumor cells within close proximity, providing further ammunition for the cancer to progress[7,16]. Recent studies have

shown that the catalytic subunit of Phosphatidylinositol 3-Kinase (PI3K), p110γ, is mainly expressed by GR1+CD11B myeloid cells, playing a critical role in tumor inflammation and growth by promoting myeloid cell trafficking to tumors and by inhibiting anti-tumor immunity[13]. Myeloid cells can differentiate into tumor-associated macrophages (TAMs), which aid tumor growth by expressing immunosuppressive and pro-angiogenic factors[6,20].

Schmid and colleagues discovered that chemoattractants activate GPCRs, TRKs, and TLR/ILRs which in turn activate p110γ in myeloid cells. Active p110γ will then activate integrin α4β1, allowing myeloid cells to infiltrate tumors[13] (Figure 1). A subpopulation of bone marrow-derived GR1+CD11B+ myeloid cells have been shown to cause immunosuppression and aid in tumor progression and angiogenesis, enabling the tumor microenvironment.[10,13,14,19] GR1+CD11B+ cells easily incorporate into tumors, decreasing tumor cell necrosis and apoptosis. [19] When inflammatory cells, like myeloid cells, are recruited to the tumor microenvironment, inflammatory chemokines, like IL-1β, are produced and these chemokines stimulate tumor infiltration.[14] Myeloid cells will strongly adhere to the tumor cells and endothelium after being stimulated by chemoattractants and will then adhere to tumor cells via endothelial cell markers, like VCAM, and/or integrins (i.e. integrin α4β1).[14] TAMs are part of the myeloid lineage and contribute to a significant portion of the tumor. TAMs secrete chemoattractants, promote angiogenesis and tumor growth, and contribute to the invasive behavior of cancer cells.[16]

In this research project, the role of inhibiting myeloid cell PI3Kγ in the progression of pancreatic cancer is investigated using the LMP pancreatic cancer cell line. LMP was derived from a liver metastasis from an *LSL-KRas*$^{G12D/+}$*; LSL-Trp53*$^{R172H/+}$ *; Pdx-1Cre* (KPC) mouse. KPC mice develop pancreatic similar in nature to human pancreatic cancer in terms of behavior (invasive and metastatic), morphology (epithelial-like), and genetic abnormalities.[4] The efficacy of PI3Kγ inhibitor, TG100-115, to reduce myeloid cell infiltration of the LMP tumor is evaluated. The results of these studies are discussed in terms of how GR1+CD11B+ myeloid cells contribute to the progression of pancreatic cancer by promoting tumor growth, chemoattractant production, angiogenesis, and metastasis.

Chapter 2 – Materials and Methods

Immunocompetent mice

The Jackson Laboratory supplied eight week old B6129SF1/J female mice to perform all in vivo mouse studies. These hybrid mice are from the first filial generation and were bred from female C57BL/6J and male 129S1/SvImJ mice.

Cell culture

The LMP pancreatic tumor cell line was derived from a spontaneous liver metastasis from an *LSL-KRas*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx-1Cre* mouse. Cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin, sodium pyruvate, sodium bicarbonate, l-glutamine, and essential medium nonessential amino acids. The cells tested negative for mycoplasma using the Mycoplasma Plus PCR primer set from Stratagene.

The mCherry LMP cells were a gift from Dr. Andrew Lowy's lab. The LMP cells were labeled with mCherry fluorescent protein.

Orthotopic tumor implantation

On Day 0, one million murine LMP cells (or mCherry LMP) were orthotopically implanted into the pancreas of eight week old B6129SF1/J female mice. A small 6- to 10-mm transverse incision was made on the left flank of the mouse through the skin and peritoneum. The tail of the pancreas was exposed through this incision and 1X 10^6 cells, in a 10µL final volume, were injected into the pancreatic tail

using a Hamilton syringe (Hamilton Co). On completion, the pancreas was returned to the abdomen and the incision was closed in 2 layers using 6.0 Ethibond nonabsorbable sutures. Tumors were allowed to grow for one week before treatment with inhibitor or control substance.

In vivo PI3K γ inhibitor studies

On Day 8 and continuing for a total of 14 days, the mice were treated by intraperitoneal injection twice a day with the inert control or 2.5mg/kg of PI3Kγ inhibitor, TG100-115. All mice from the treatment (n=10 or 12) and control group (n=10 or 12) were terminated on Day 22. These in vivo studies were performed twice.

Quantification of myeloid cells in tumors by flow cytometry (FACS)

As previously described[14], tumors were excised, minced and digested to single cell suspensions for 2h at 37°C in 10ml of Hanks Balanced Salt Solution (HBSS, GIBCO) containing 1 mg/ml Collagenase type IV (Sigma), 10 μg/ml Hyaluronidase type V (Sigma) and 20 units/ml DNase type IV (Sigma). Red blood cells were solubilized with RBC Lysis Buffer (eBioscience) and cells were incubated in FC-block (BD Bioscience), followed by anti- CD11b and Gr1 antibodies. Staining with FC block ensures that any antibody staining that is detected is specific and a direct result of the antigen-binding portion of the antibody binding to the antigen on the cells surface. To exclude dead cells, 0.5μg/ml propidium iodide (PI) was added before data acquisition by FACS Calibur (BD Bioscience).

Animal Imaging and tissue preservation

After completing the two-week treatment regimen, the mice were sacrificed and intravital images were taken to evaluate primary pancreatic and metastatic tumor burden. Whole-body imaging of mice implanted with mCherry LMP tumors was performed with the OV-100 Small Animal Imaging System, containing an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus Corp). All images were analyzed with ImageJ v1.440 (National Institutes of Health) to determine total tumor area (primary tumor and metastatic burden). The OV-100 provided visualization of the LMP mCherry cells for tumor burden quantification with ImageJ as well as the recording of metastatic organs. Tumor and organ weights were recorded and cryopreserved in OCT for antigen quantification and RNA isolation.

Gene expression

Total RNA was isolated from cells and tissues using TRIZOL from Invitrogen. cDNA was prepared from 1μg RNA from each sample and qPCR was performed using gene specific primers for GAPDH, Il-1β, Arginase-1, and Il-6 from Qiagen (QuantiTect Primer Assay). qPCR for VEGF-A expression was performed with sense primers: 5‟GCTGTGCAGGCTGCTCTAAC3‟ and anti-sense primers: 5‟CGCATGATCTGCATGGTGAT3‟. Relative expression levels were normalized to Gapdh expression according to the formula <2^- (Ct gene of interest – Ct GAPDH)>. Fold increase in expression levels were calculated by comparative Ct method <2^- (ddCt)> 10.

Quantification of myeloid cells and blood vessels in tissues by immunohistochemistry

As previously described[14], LMP tumors were grown orthotopically in the pancreas for 21 days, and received the PI3Kγ inhibitor or control substance for a total of 14 days. The pancreata were cryopreserved in O.C.T., cryosectioned and immunostained for CD11b using clone M1/70 (BD Bioscience), for F4/80 using clone BM8 (eBioscience), and for CD31 using clone MEC13.3 (BD Bioscience). Slides were counterstained with DAPI (Invitrogen). Tissues were analyzed for CD11b, F4/80, and CD31 using Metamorph image capture and analysis software (Version 6.3r5, Molecular Devices). Haematoxylin and eosin staining was performed by the Moores UCSD Cancer Centre Histology Shared Resource to confirm the presence of cancer in the pancreas.

***The data was analyzed for statistical significance with an unpaired two-tailed Student's t-test. $P<0.05$ was considered to be significant.

Chapter 3 – Myeloid Cell PI3Kγ Inhibition Decreases Tumor Growth and Metastasis

To visualize the efficacy of PI3Kγ inhibition on tumor malignancy in vivo, we established an orthotopic mouse model of murine pancreatic cancer using mCherry LMP pancreatic cancer cells, which do not express PI3Kγ, in 24 mice. 1 X 10^6 LMP cells were implanted on Day 0 and randomized into two groups: control and treatment. On Day 8, the mice received 2.5 mg of PI3Kγ inhibitor or control solution via intraperitoneal injection every 12 hours for a total of 14 days. On Day 22, all mice were sacrificed and evaluated for tumor burden. Intravital images were taken with the Olympus OV-100 to evaluate primary pancreatic and metastatic tumor burden. Overall, there was a significant reduction in metastatic lesions and a decrease in tumor growth in the treatment group when compared to control mice ($P<0.01$ vs. control). Likewise, there was a significant decrease in metastasis to the colon, spleen, liver, and diaphragm (Figure 2, 3B). Inhibiting PI3Kγ led to a decrease in primary tumor weight (Figure 3A), which is supported by previous studies of other tumor types (Schmid et al, 2011). The control mice had an average primary tumor weight of 0.56591 grams whereas the mice treated with the PI3Kγ had an average tumor weight of 0.42332 grams ($P< 0.01$) (Figure 3A). Images obtained with the OV-100 show a 37% decrease in metastasis (Figure 3B) and 50% reduction in total mCherry LMP tumor burden (primary tumor and metastatic lesions) in the treatment group (Figure 3C). Quantification of images with ImageJ software show a mean mCherry LMP tumor

area of 77.26071 mm^2 was achieved in the treatment group as opposed to 153.5511 mm^2 ($P< 0.05$) in the control group.

Chapter 4 – Flow Cytometric Detection of CD11b+ and CD11b+Gr1+ Myeloid Cells in LMP Tumors

FACS analysis was done to evaluate pancreatic tumor inflammation by myeloid cells after PI3Kγ has been inhibited. Tumor-associated myeloid cells were isolated from LMP tumors from control and treatment groups and stained for CD11b+ and CD11b+Gr1+. The absolute number of CD11b+ and CD11b+GR1+ myeloid cells was quantified by flow cytometry (Figure 4). PI3Kγ inhibition decreases the number of tumor-derived CD11b+ and CD11b+Gr1+ myeloid cells in LMP tumors. These findings indicate that murine myeloid cells make up a substantial portion of the LMP tumor and this invasion is facilitated by PI3Kγ.

Chapter 5 – Blocking PI3Kγ Suppresses Angiogenesis and Macrophage Tumor Infiltration

Tumor-associated macrophages (TAMs) are part of the myeloid lineage and contribute to a significant portion of the tumor[18]. Because TAMs have a major role in perpetuating tumor inflammation, there was an interest to know to what degree macrophages were infiltrating the LMP tumors in control and treatment groups. To explore the presence of TAMs in LMP tumors, pancreatic tumor sections were stained by immunohistochemistry with F4/80 antibody to detect the extracellular surface antigen, F4/80, that is present on macrophages. Macrophage quantification shows a significant decrease (~50%) in macrophage infiltration in the PI3Kγ inhibitor treatment group, which suggests that PI3Kγ is a critical factor in macrophage infiltration in the tumor microenvironment (Figure 5).

Angiogenesis supplies tumors with the needed oxygen and nutrients to grow. Tumor angiogenesis is induced by growth factors such as vascular endothelial growth factor (VEGF) as well as TAMs[18]. CD31 is a surface marker on endothelial cells that enables the detection of blood vessels. Angiogenesis was measured in LMP tumors from the control and treatment groups by immunostaining of CD31. These studies show that myeloid cell PI3Kγ inhibition reduces new blood vessel formation by almost ten-fold (Figure 5).

Chapter 6 – Blockade of PI3Kγ Inhibits Tumor Inflammation

Myeloid cells are recruited to the tumor microenvironment in response to chemoattractant secretion from the tumor. Myeloid cells can differentiate into TAMs or tumor associated neutrophils (TANs) and express factors that further establish the tumor microenvironment. The inflammatory factors expressed by both tumors and inflammatory cells contribute to the progression of the cancer disease by promoting tumor growth and angiogenesis. We looked at chemoattractant gene expression in the pancreas of wild-type mice with LMP tumors after having received the PI3Kγ or control treatment for a total of 14 days. In order to determine if myeloid cell invasion contributed to inflammatory factor elevation, we performed real-time quantitative PCR analysis of *IL-1β*, *Arg-1*, *IL-6*, and *VEGF-A* using cDNA that was reverse transcribed from RNA isolated from LMP tumors from both treatment and control groups. All inflammatory factor gene expression was reduced in the mice treated with the PI3Kγ inhibitor (Figure 6). A drastic reduction of *Arg-1* is seen in the treatment group when compared to the control group. Surprisingly, not only was *IL-6* expression lessened in the treatment group, the observed decrease was less than the inherent expression of *IL-6* seen in LMP cells. Overall, we found that specific inflammatory factor genes are upregulated when cancer is present, indicating an important role in the progression of pancreatic cancer (Arg-1 and VEGF-A: $P < 0.01$; IL-6: $P < 0.05$; IL-1β: not significant).

Chapter 7 – Discussion

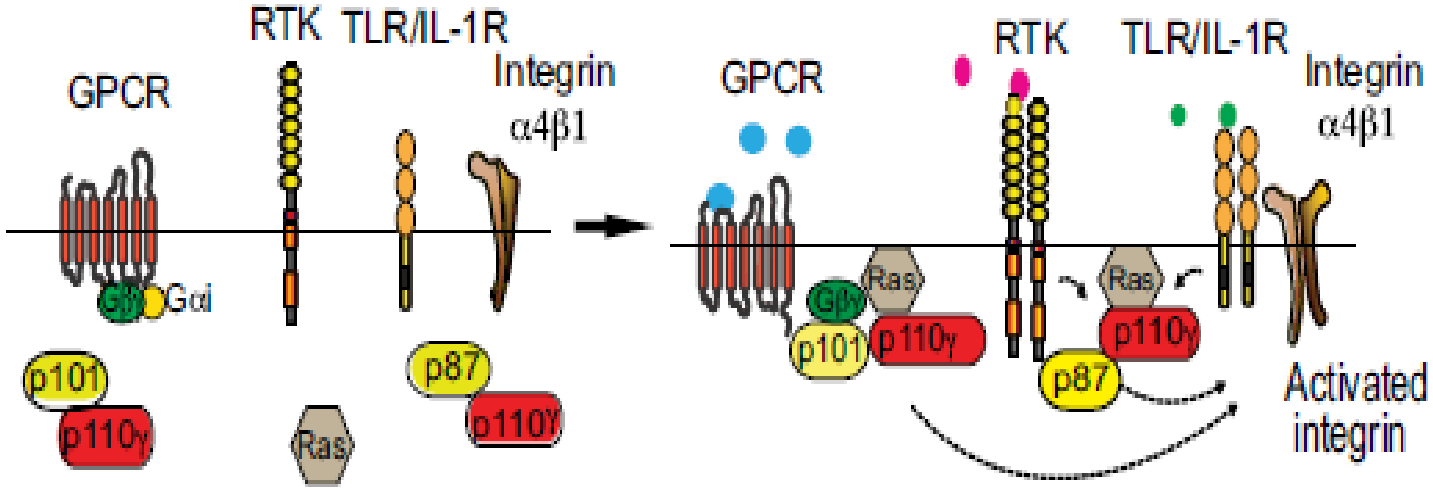
Tumor inflammation plays a significant role in perpetuating cancer progression and metastasis. Inflammatory responses are normal and necessary processes in wound healing, warding off infection, and destroying pathogens. When cancer invades a tissue, the tissue becomes damaged and inflammatory cells such as myeloid-derived suppressor cells, regulatory T cells, and lymphocytes are sent to the damaged tissue.[10] Once this recruitment happens, these immune cells secrete cytokines, chemokines, and other chemoattractants that contribute to tumor progression and stabilization of the tumor microenvironment.[10,14] Previous studies show that PI3Kγ is expressed almost exclusively by myeloid cells and that it plays an essential role in tumor inflammation and growth by promoting myeloid cell trafficking to tumors and by inhibiting anti-tumor immunity[13]. When WT mice with LMP tumors are treated with TG100-115 for 14 days, there is a reduction of metastasis to the colon, spleen, liver, and diaphragm as opposed to control mice treated with the inert control (Figure 2, 3B). Further, there is a decrease in LMP primary tumor weight (Figure 3A) and total tumor burden (Figure 3C), which suggests that inhibition of myeloid cell P13Kγ reduces the spread of pancreatic cancer.

Cancer cells have the ability to encourage the process of angiogenesis to take place as a way to ensure their survival by. Cell adhesion molecule (CD31), is a surface marker present on endothelial cells and LMP tumors were stained for CD31 as a way to detect blood vessel growth in the tumor in the control and treatment groups. The data shows a decrease in CD31 expression in the treatment group (Figure 5), which

indicates an important role that PI3Kγ has in encouraging new blood vessel formation during inflammation.

Cancerous cells release chemoattractants that recruit immune cells to the tumor microenvironment that perpetuate the spread of cancer and solidify its existence. Studies have shown that Arginase-1 production in the tumor microenvironment enhances tumor growth [24, 25]. Specifically, myeloid cells have been detected as a culprit in Arginase production in the tumor microenvironment[24]. IL-6 is known to promote tumor growth[10] and VEGF-A is a growth factor that is released by tumors to encourage adequate blood supply. These results show a decrease in inflammatory factors in mice treated with the inhibitor (Figure 6). Because inflammatory responses are mediated by myeloid cells, this reduction of inflammatory factors is supported by the decrease in myeloid cells in the PI3Kγ inhibitor treated LMP tumor (Figure 4).

Pancreatic cancer is aggressive in nature and there are limited options available to treat it. Tumor inflammation enables tumor growth along with angiogenesis. GR1+CD11B+ myeloid cells are part of inflammatory response that allows for tumor evasion by the immune system. Overall, PI3Kγ inhibitors might be a useful therapeutic agent to treat pancreatic cancer by suppressing tumor growth, metastasis, inflammatory, factor expression, and angiogenesis.



Schmid et al., 2011

Figure 1. Model of PI3Kγ control of myeloid cell invasion into tumors: GPCRs, RTKs, and TLR/ILRs activate PI3Kγ which promotes integrin α4β1 mediated myeloid cell adhesion to endothelium and transendothelial cell invasion.

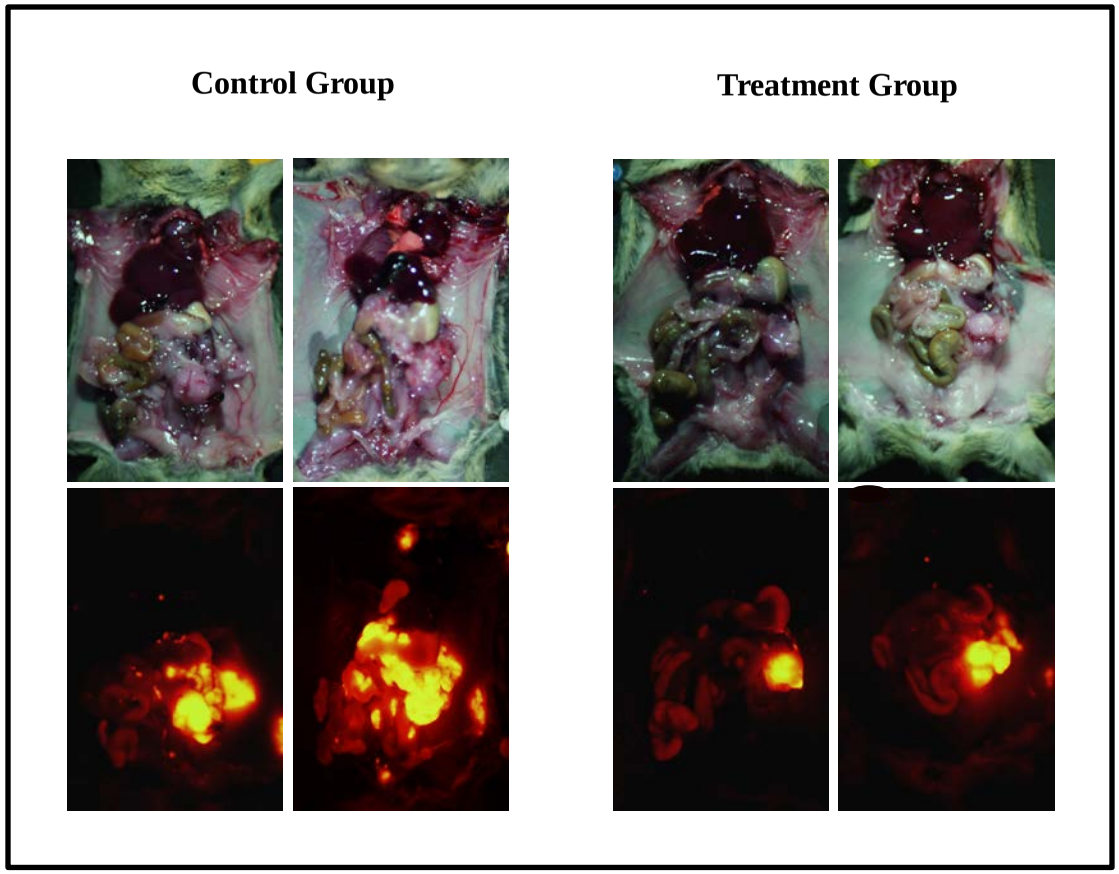


Figure 2. Fluorescent Images Show PI3Kγ Inhibition Reduces Metastasis. WT mice with LMP mCherry tumors that were treated with TG100-115 for 14 days showed a decrease in metastasis to the colon, spleen, liver, and diaphragm ($P < 0.05$) .

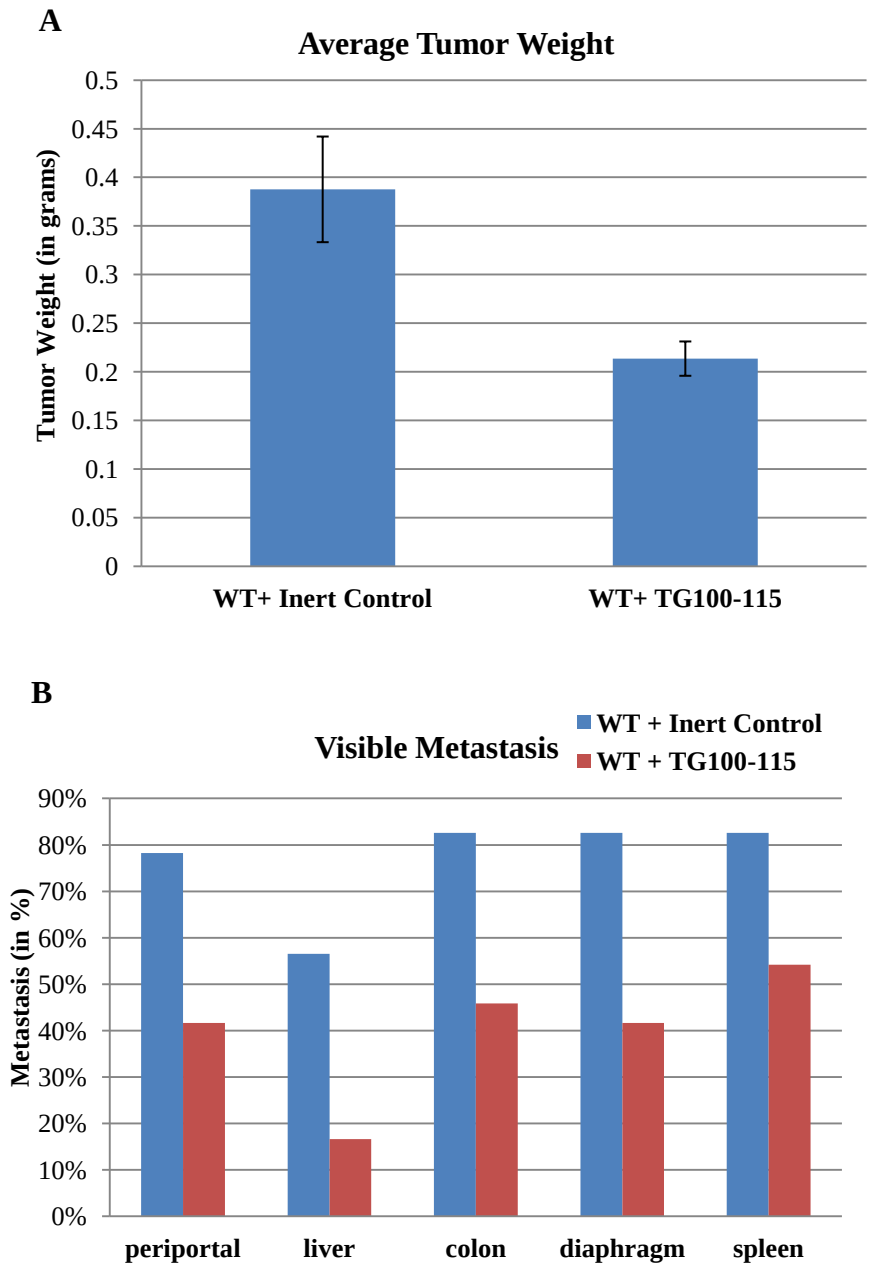


Figure 3. PI3Kγ Inhibition Decreases Tumor Growth and Metastasis.

(A) Primary tumor burden ($P<0.01$) (B) Number of metastases and (C) Total tumor area (primary tumor and metastatic lesions) were decreased in mice treated with TG100-115 ($P<0.05$).

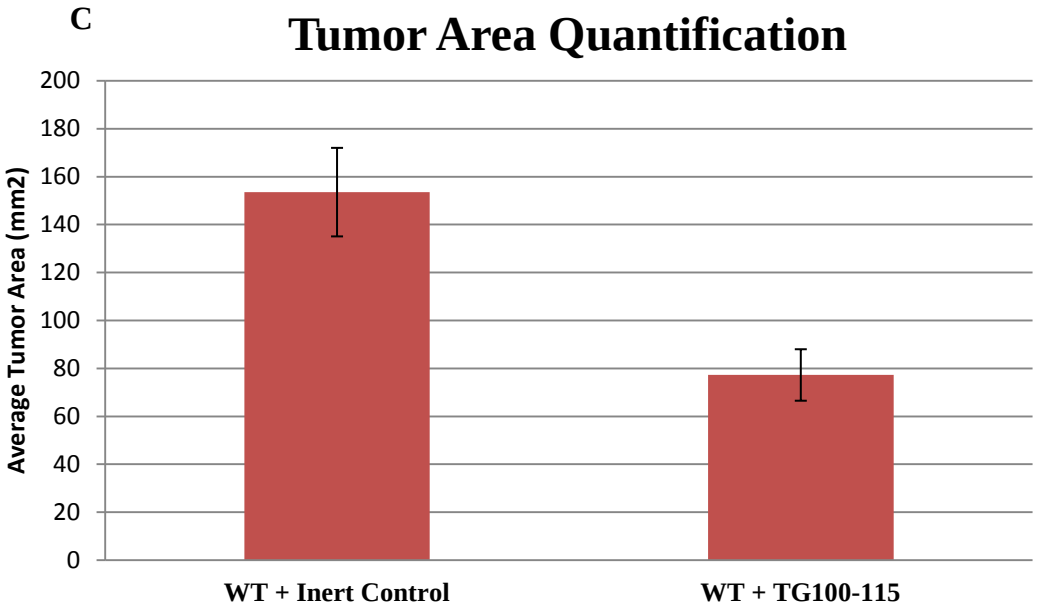


Figure 3. PI3Kγ Inhibition Decreases Tumor Growth and Metastasis (Continued)

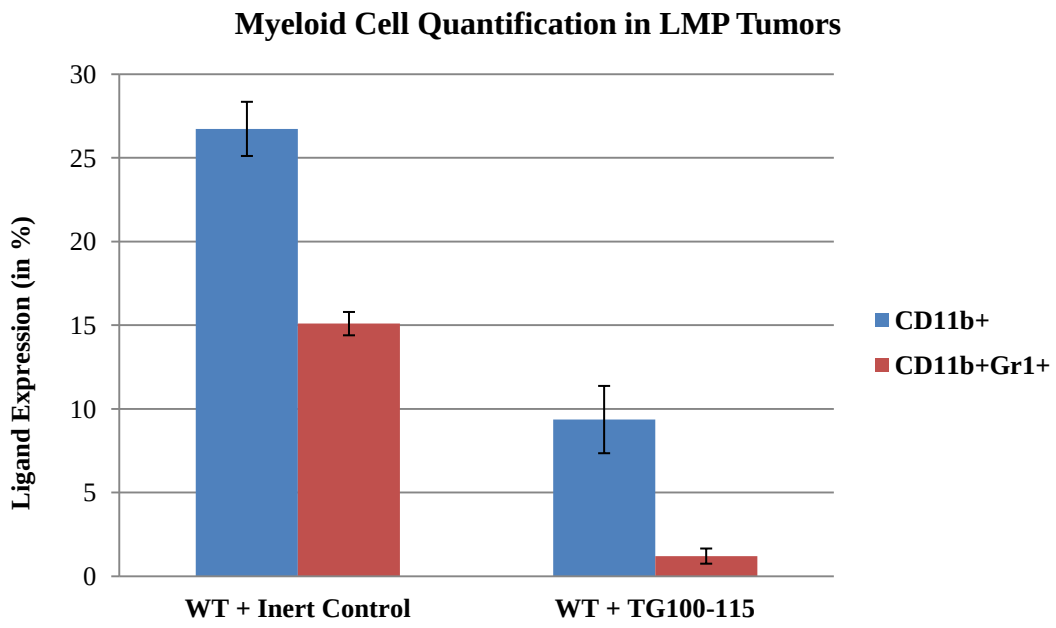


Figure 4. Flow Cytometric Detection of CD11b+ and CD11b+Gr1+ Myeloid Cells in LMP Tumors. Mononuclear cells were isolated from LMP tumors from control and treatment groups and stained for CD11b+ and CD11b+Gr1+ myeloid cells. PI3Kγ inhibition decreases the number of tumor-derived CD11b+ and CD11b+Gr1+ myeloid cells in LMP tumors. ($P<0.01$)

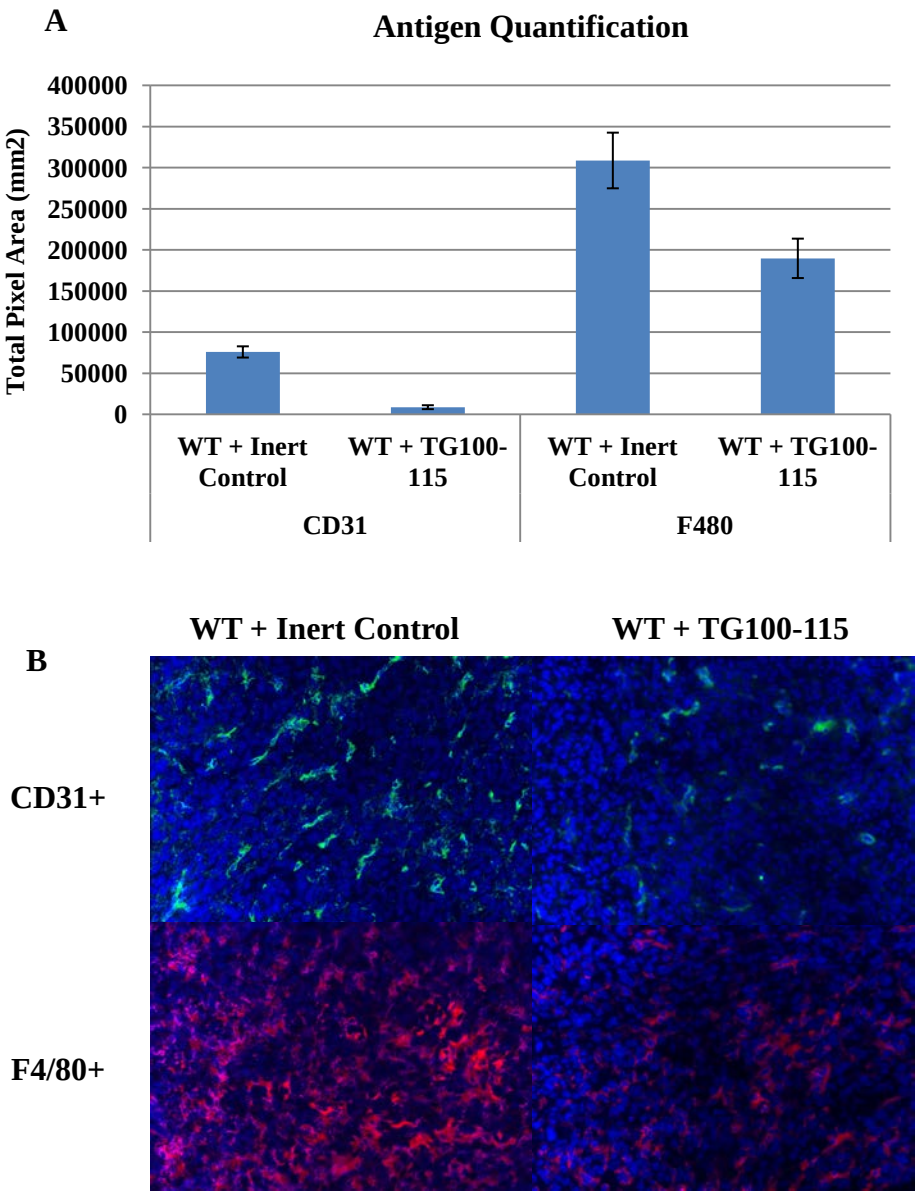


Figure 5. Blocking PI3Kγ Suppresses Angiogenesis and Macrophage Tumor Infiltration.

(A) Quantification of CD31+ or F4/80+ in treatment versus control mice ($P < 0.01$).

(B) Pancreas sections show CD31+ or F4/80+ expression in WT mice with LMP tumors after two week treatment with TG100-115 or control. Nuclei (blue, DAPI), endothelial cell adhesion molecule (green, CD31), macrophages (red, F4/80+).

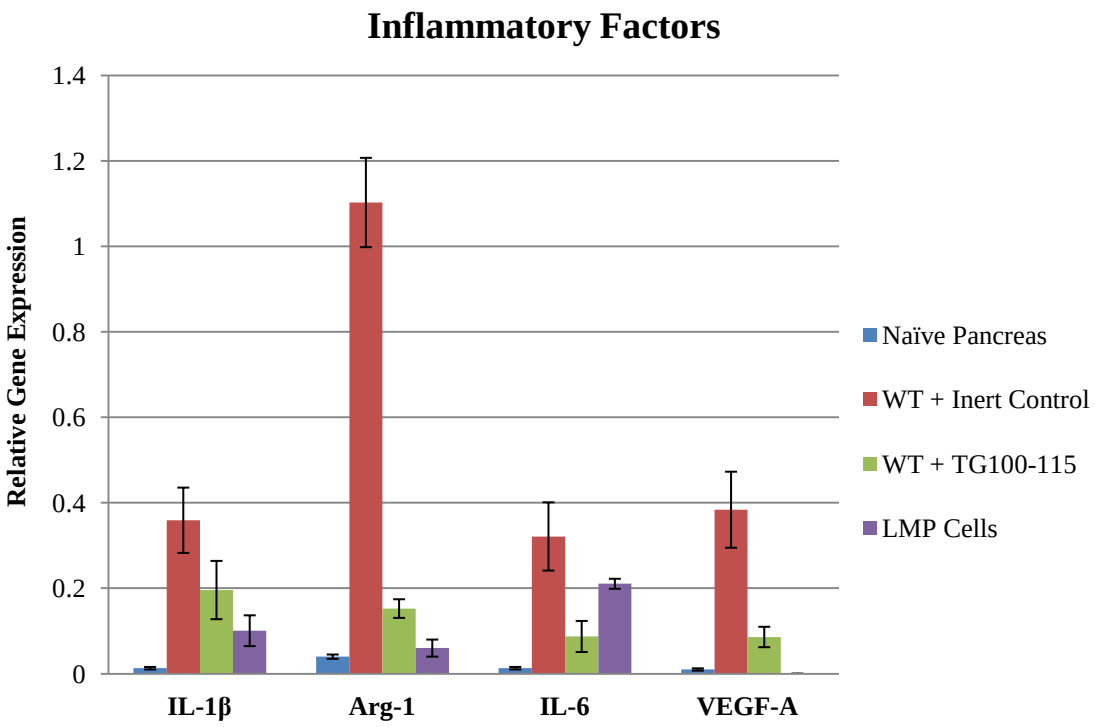


Figure 6. Blockade of PI3kγ Inhibits Tumor Inflammation. Chemoattractant gene expression in pancreas of WT mice with LMP tumors after receiving PI3Kγ inhibitor or control treatment.
Arg-1 and VEGF-A: $P < 0.01$
IL-6: $P<0.05$
IL-1β: $P>0.05$, not significant

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