Metabolic reprogramming of tumor-infiltrating myeloid cells conveys protection against pancreatic ductal adenocarcinoma

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**NANYANG
TECHNOLOGICAL
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SINGAPORE

**METABOLIC REPROGRAMMING OF TUMOR-
INFILTRATING MYELOID CELLS CONVEYS PROTECTION
AGAINST PANCREATIC DUCTAL ADENOCARCINOMA**

SIEOW JE LIN

SCHOOL OF BIOLOGICAL SCIENCES

2019

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AGAINST PANCREATIC DUCTAL ADENOCARCINOMA**

SIEOW JE LIN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfillment of the requirement for the
degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from [1] paper(s) published in the following peer-reviewed journal(s) where I was the second co-author.

Chapter 3.1 is published as “Hweixian Leong Penny, Je Lin Sieow, Giulia Adriani, Wei Hseun Yeap, Peter See Chi Ee, Boris San Luis, Bernett Lee, Terence Lee, Shi Ya Mak, Ying Swan Ho, Kong Peng Lam, Choon Kiat Ong, Ruby Y. J. Huang, Florent Ginhoux, Olaf Rotzschke, Roger D. Kamm & Siew Cheng Wong: Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma. *Oncolmunology*, 5, <http://dx.doi.org/10.1080/2162402X.2016.1191731>. (2016).”

The contributions of the co-authors are as follows:

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- Je Lin Sieow, Wei Hseun Yeap, Peter See Chi Ee, Boris San Luis assisted with experimental work and analyzed data.
- Giulia Adriani designed, performed, and interpreted all the extravasation experiments, analyzed data, and wrote the parts in the manuscript relevant to extravasation.
- Bernett Lee and Terence Lee analyzed data.
- Shi Ya Mak and Ying Swan Ho performed the metabolomics experiments.
- Choon Kiat Ong provided material support.
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- Kong Peng Lam, Olaf Rotzschke, Roger D. Kamm and Florent Ginhoux reviewed the manuscript.
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Table of Contents

STATEMENT OF ORIGINALITY	III
SUPERVISOR DECLARATION STATEMENT.....	IV
AUTHORSHIP ATTRIBUTION STATEMENT	V
ACKNOWLEDGEMENTS.....	VII
TABLE OF CONTENTS.....	VIII
LIST OF FIGURES.....	XI
LIST OF TABLES	XIV
ABBREVIATIONS.....	XV
ABSTRACT.....	XVII
CHAPTER 1 INTRODUCTION.....	1
PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)	1
INFLAMMATION-ASSOCIATED CANCER DEVELOPMENT	4
MYELOID CELLS INFLUENCE ANTI-TUMORAL IMMUNITY	6
IMMUNOMETABOLISM	13
METABOLIC SIGNATURE OF MACROPHAGES	16
METABOLIC ALTERATIONS OF TUMOR-INFILTRATING NEUTROPHILS.....	17
PROJECT OUTLINE.....	20
HYPOTHESIS #1	21
HYPOTHESIS #2	22
HYPOTHESIS #3	23
CHAPTER 2 MATERIALS AND METHODS.....	24
GENERATION OF <i>IN VITRO</i> CONDITIONED MEDIA	24
DEVELOPMENT OF <i>IN VITRO</i> -GENERATED TAMs	24
FLOW CYTOMETRY	25
ENDOCYTOSIS ASSAY	25
RT-QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR) – <i>IN VITRO</i>	25
EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT) ASSAY.....	28

EXTRAVASATION ASSAY	28
LIVE METABOLIC FLUX ASSAY	29
CELL LINE AUTHENTICATION	30
MICE	31
GENERATION OF STABLE LUCIFERASE EXPRESSING 1242 MOUSE PDAC CELL LINE	31
<i>IN VIVO</i> BIOLUMINESCENT IMAGING (BLI).....	32
TISSUE DISSOCIATION.....	32
FLOW CYTOMETRY	33
MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) PRODUCTION.....	34
CELL CYTOTOXICITY ASSAY.....	34
IMMUNOFLUORESCENCE (IF).....	35
WESTERN BLOT.....	36
TRANSCRIPTOMICS.....	36
RT-QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) – <i>IN VIVO</i>	37
MULTIPLEX IMMUNOFLUORESCENCE (IF) STAINING AND ANALYSIS.....	40
STATISTICS	43
STUDY APPROVAL	43
CHAPTER 3 RESULTS.....	44
3.1 WARBURG METABOLISM IN TUMOR-CONDITIONED MACROPHAGES PROMOTES METASTASIS IN HUMAN PANCREATIC DUCTAL ADENOCARCINOMA	44
PDAC TUMOR-CONDITIONED MACROPHAGES EXHIBIT A MIXED M1/M2 PHENOTYPE	44
GLYCOLYTIC SIGNATURE OF TAMs	44
PANC1 AND MiaPaCa2 TAMs PROMOTE HIGHER RATES OF ANGIOGENESIS, INDUCED HIGHER RATES OF EPITHELIAL-TO-MESENCHYMAL TRANSITIONS AND AUGMENTED EXTRAVASATION AS COMPARED TO CONTROL HPNE MACROPHAGES	47
INHIBITION OF GLYCOLYSIS IN PANC1 AND MiaPaCa2 MACROPHAGES USING 2-DG INHIBITED THEIR PRO-METASTATIC PHENOTYPE.....	53
3.2 A METABOLIC REPROGRAMMING IN TUMOR MACROPHAGES VIA CONDITIONAL KNOCKOUT OF GLUT1 ATTENUATES TUMOR BURDEN IN PANCREATIC DUCTAL ADENOCARCINOMA.....	58
MACROPHAGES FROM TUMOR-BEARING MICE ARE HIGHLY GLYCOLYTIC.....	58
MACROPHAGE-SPECIFIC DELETION OF GLUT1 SIGNIFICANTLY REDUCES TUMOR BURDEN	61
THE PRO-INFLAMMATORY IL-1 β PATHWAY IS SUPPRESSED IN GLUT1 $\Delta^{\text{M}\Phi}$ MACROPHAGES	62
NK CELLS AND CTLs MEDIATE ANTI-TUMOR IMMUNITY IN GLUT1 $\Delta^{\text{M}\Phi}$ MICE	64
THE GLUT1 INHIBITOR WZB117 ATTENUATES TUMOR BURDEN <i>IN VIVO</i>	67

THE MACROPHAGE GLYCOLYTIC SIGNATURE PREDICTS POOR SURVIVAL IN HUMAN PDAC	72
3.3 CONDITIONAL KNOCKOUT OF HIF-1A IN TUMOR-INFILTRATING NEUTROPHILS CONVEYS PROTECTION AGAINST PANCREATIC DUCTAL ADENOCARCINOMA	75
PRO-TUMORAL NEUTROPHILS INFILTRATE THE PANCREAS OF TUMOR-BEARING MICE AND THEIR TARGETED DEPLETION IMPROVES TUMOR OUTCOME	75
PRO-TUMORAL NEUTROPHILS EXHIBIT A HIGHLY GLYCOLYTIC SIGNATURE AND ARE ADAPTED TO A HYPOXIC TUMOR MICROENVIRONMENT	79
NO REDUCTION IN GLYCOLYSIS AND CHANGE IN TUMOR GROWTH IN NEUTROPHIL-SPECIFIC DELETION OF GLUT1	83
NEUTROPHIL-SPECIFIC DELETION OF TRANSCRIPTIONAL FACTOR HIF-1A SIGNIFICANTLY ATTENUATES TUMOR BURDEN	84
HIF-1A ^{ΔNΦ} NEUTROPHILS EXERT DIRECT ANTI-TUMORAL EFFECTS	88
HIF-1A ^{ΔNΦ} NEUTROPHILS ACTIVATE THE ADAPTIVE IMMUNE RESPONSE	89
ANTI-TUMORAL IMMUNITY IN HIF-1A ^{ΔNΦ} OT MICE IS MEDIATED BY CD8+ T CELLS AND NK CELLS	91
HIF-1A ^{HI} , GLYCOLYTIC MARKER ^{HI} NEUTROPHILS FOUND IN PDAC PATIENTS MIRROR RESULTS FROM THE ORTHOTOPIC MOUSE MODEL	94
CHAPTER 4 DISCUSSION	98
CONCLUSION	112
APPENDIX	113
I. CHAPTER 3.1 – PUBLISHED	113
II. CHAPTER 3.2 – IN REVISION	129
III. CHAPTER 3.3 – IN REVISION	129
IV. REVIEW ARTICLE – IN REVISION	129
OTHER PUBLICATIONS	130
POSTER PRESENTATIONS	132
AWARDS	134
REFERENCES	135

List of Figures

Introduction:

Figure 1.1. Targeting endogenous *Kras*^{G12D} and *Trp53*^{R172H} expression to the mouse pancreas in a pancreatic cancer mouse model

Figure 1.2. Inflammation and cancer

Figure 1.3. Pro-tumoral macrophage mechanisms and their effects on effector cells

Figure 1.4. Effects of tumor-infiltrating neutrophil-derived products on the tumor microenvironment

Figure 1.5. HIF-1 promotes the expression of glycolytic enzymes and transporters during glycolysis

Results:

Chapter 3.1

Figure 1: PDAC tumor-conditioned macrophages exhibit a mixed M1/M2 phenotype

Figure 2: Glycolytic signature of TAMs

Figure 3: Panc1 and MiaPaCa2 macrophages promote higher rates of angiogenesis compared to control HPNE macrophages

Figure 4: Panc1 and MiaPaCa2 macrophages induced higher rates of EMT compared to control HPNE macrophages

Figure 5: Panc1 and MiaPaCa2 macrophages augmented extravasation compared to control HPNE macrophages

Figure 6: Inhibition of glycolysis in Panc1 and MiaPaCa2 macrophages using 2-DG inhibited their pro-metastatic phenotype

Chapter 3.2

Figure 7: Macrophages from tumor-bearing mice are highly glycolytic

Figure 8: Macrophage-specific deletion of GLUT1 significantly reduces tumor burden

Figure 9: The pro-inflammatory IL-1 β axis is suppressed in GLUT1 ^{Δ m Φ} macrophages

Figure 10: NK cells and CTLs mediate anti-tumor immunity in GLUT1^{ΔmΦ} mice

Figure 11: The GLUT1 inhibitor WZB117 attenuates tumor burden *in vivo*

Figure 12: The macrophage glycolytic signature is a predictor of poor survival in human PDAC

Chapter 3.3

Figure 13: Pro-tumoral neutrophils infiltrate the pancreas of tumor-bearing mice and their persistent depletion improves tumor outcomes

Figure 14: Pro-tumoral neutrophils exhibit a highly glycolytic signature and are adapted to a hypoxic tumor microenvironment

Figure 15: No reduction in glycolysis and change in tumor growth in neutrophil-specific deletion of GLUT1

Figure 16: Neutrophil-specific deletion of transcriptional factor HIF-1α significantly attenuates tumor burden

Figure 17: HIF-1α^{ΔNΦ} neutrophils exert direct anti-tumoral effects

Figure 18: HIF-1α^{ΔNΦ} neutrophils activate the adaptive immune response

Figure 19: Anti-tumoral effector responses are supported by CD8⁺ T cells and NK cells activation and cytotoxicity

Figure 20: HIF-1α^{hi}, glycolytic marker^{hi} neutrophils found in PDAC patients mirror results from the orthotopic mouse model

Discussion:

Chapter 3.1 Proposed Model

Figure 21. TAMs support metastatic dissemination and colonization at secondary sites

Chapter 3.2 Proposed Model

Figure 22. A metabolic reprogramming in tumor macrophages via conditional knockout of GLUT1 attenuates tumor burden in pancreatic ductal adenocarcinoma.

Chapter 3.3 Proposed Model

Figure 23. Conditional knockout of HIF-1 α in neutrophils promotes anti-tumoral activity via increased ROS and activation of CTL and NK cells cytotoxicity

List of Tables

Materials and Methods:

Table 1: Human primer sequences used for RT-qPCR (Chapter 3.1).

Table 2. Mouse primer sequences used for RT-qPCR (Chapter 3.2).

Table 3. Mouse primer sequences used for RT-qPCR (Chapter 3.3).

Abbreviations

2-DG	2-deoxyglucose
WZB117	2-fluoro-6-(m-hydroxybenzoyloxy) phenyl m-hydroxybenzoate
AMPK	5' adenosine monophosphate-activated protein kinase
AVA	agri-food and veterinary authority
ASC	apoptosis-associated speck-like protein containing a
CARD	
Arg-1	arginase 1
BLI	bioluminescent imaging
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine
CTLs	cytotoxic T lymphocytes
EMT	epithelial-to-mesenchymal transition
FFPE	formalin-fixed paraffin-embedded
ALDOA	fructose biphosphate aldolase A
Gck	glucokinase
GLUT1	glucose Transporter 1
GPI	glucose-6-phosphate isomerase
HGF	hepatocyte growth factor
HK1	hexokinase 1
HK2	hexokinase 2
HPNE	human pancreatic epithelial cell line
HUVECs	human umbilical vein endothelial cells
HIF-1 α	hypoxia-inducible factor 1-alpha
IF	immunofluorescence
IFN γ	interferon gamma
KRAS	kirsten rat sarcoma viral oncogene homolog
KPC	<i>LSL-Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre</i> mouse model
LDHA	lactate dehydrogenase A
LDHB	lactate dehydrogenase B
LPS	lipopolysaccharide
Mgl1/2	macrophage galactose-type lectin-1/2
MACS	magnetic-automated cell sorting

mTOR	mechanistic target of rapamycin
MDSCs	myeloid derived suppressor cells
NACLAR	national advisory committee for laboratory animal research
NK	natural killer
OT	orthotopic transplanted
OXPHOS	oxidative phosphorylation
PDAC	pancreatic ductal adenocarcinoma
PanINs	pancreatic Intraepithelial neoplasia
ppm	parts per million
PBMCs	peripheral blood mononuclear cells
PEC	peritoneal cavity
PFKB1	phosphofructokinase
PGK1	phosphoglycerate kinase 1
PDMS	polydimethylsiloxane
PMNs	polymorphonuclear neutrophils
Akt	protein kinase B
PDK1	pyruvate dehydrogenase kinase isozyme 1
PKM2	pyruvate kinase muscle isozyme M2
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
STAT1	signal transducer and activator of transcription 1
Tregs	regulatory T cells
TCGA	the cancer genome atlas
TGF- β	transforming growth factor beta
TPI1	triosephosphate isomerase
TAMs	tumor-conditioned macrophages
VEGF	vascular endothelial growth factor

Abstract

Myriad mechanisms of immune evasion contribute to therapeutic resistance of pancreatic cancer and poor survival. While it is well established that tumor cells exhibit the Warburg effect for energy production, the contribution of myeloid cell metabolism to disease progression is unknown. In this study, we highlight the importance of immunometabolism, specifically demonstrating that the metabolic signature of an immune cell can instruct downstream effector function. Utilizing a combination of an *in vitro* model of tumor-conditioned human macrophages, an orthotopic pancreatic ductal adenocarcinoma mouse model and patient-derived specimens, we define a pronounced glycolytic signature in tumor-infiltrating myeloid cells that confer them a pro-tumoral phenotype. Inhibiting glycolysis with 2-deoxyglucose, macrophage-specific deletion of glucose transporter 1 or neutrophil-specific deletion of hypoxia-inducible factor 1- α independently ameliorates disease. Our results indicate that glycolysis is a key control point in the immunological reprogramming of tumor-infiltrating myeloid cells, and highlight the therapeutic potential of targeting this metabolic pathway to improve patient outcome.

Pancreatic ductal adenocarcinoma (PDAC)

Pancreatic cancer is the fourth leading cause of cancer death worldwide with a 5-year survival rate of 3-5%¹, with patients usually showing widespread metastases at the point of diagnosis². In Singapore, it is ranked the 5th and 6th most frequent cancer deaths in males and females respectively³. Though not as common as colorectal, breast and lung cancer in Singapore, it is still a local health problem as its high incidence rate almost equals mortality and this is due to the late presentation and aggressive characteristics of the cancer. Pancreatic ductal adenocarcinoma (PDAC) accounts for up to 85% of malignant pancreatic tumor cases^{4,5}. PDAC is a highly aggressive, metastatic and treatment-resistant pancreatic exocrine neoplasm, characterized by a dense desmoplastic reaction composed of both fibrosis and immune cells⁶. Its onset and progression are characterized to be driven by genetic alterations including Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation in early pancreatic Intraepithelial neoplasia (PanINs) and further mutations in TP53/SMAD3/4 in carcinoma cells⁷. Incidence of PDAC increases with age and is dependent on dietary, lifestyle and environmental factors such as working in chemical and electrical equipment manufacturing plants. Pancreatitis and diabetes mellitus are also risk factors leading to the development of PDAC⁸⁻¹⁰.

Due to its poor prognosis and resistance to conventional and targeted therapeutic agents, PDAC incidence almost equals mortality. However, the mechanisms underlying the poor prognosis and highly metastatic nature of this disease still remain poorly understood. To aid in better understanding the dynamics of PDAC development and progression, several genetically engineered mouse models that spontaneously develop tumor have been generated⁵. One such mouse model is the *LSL-Kras*^{G12D/+}; *LSL-Trp53*^{R172H/+}; *Pdx-1-Cre* (KPC) mice where spontaneous PDAC tumors and metastases develop, mirroring many

characteristics of human PDAC¹¹. The KRAS allele activation initiates PanIN early in mice showing similarity with all human histopathologic features of the disease¹². The progression towards PDAC is then associated with accumulation of other genetic mutations including the loss of the p53 tumor suppressor gene activity¹³. Expression of the Lox-STOP-Lox-Kras^{G12D} allele is obtained by breeding this strain with mice expressing the Cre recombinase gene driven by Pdx1 promoter, a pancreatic-specific gene¹⁴. The targeting strategy is shown in Fig. 1.1a, with KPC mice exhibiting a median survival of approximately 5 months (Fig. 1.1b)¹¹.

There have been studies done in animal models of PDAC demonstrating that epithelial-to-mesenchymal transition (EMT), a hallmark of metastasis, is most pervasive at inflammatory sites within the tumor¹⁵. Induction of pancreatitis after tumor progression increased the number of circulating pancreatic tumor cells, whilst immunosuppression using dexamethasone therapy abrogated dissemination¹⁵. In addition, tumor cells undergoing EMT have been identified in precursor lesions, known as PanINs, suggesting that initiation of metastasis occurs prior to the development of frank carcinoma¹⁵.

In PDAC, myeloid cells such as macrophages and neutrophils are recruited from the bone marrow by chemokines to the tumor to establish an immunosuppressive microenvironment¹⁶. With the high influx of immunosuppressive cells in the PDAC microenvironment, effector CD8+ T cells and dendritic cells numbers are reduced. Moreover, the imbalance between the immunosuppressive regulatory T cell and myeloid cell populations prevailing over the Th1 anti-tumoral effector arm is associated with a worse PDAC prognosis¹⁷. Taken together, studies support the notion that inflammation can facilitate tumor dissemination, and may represent one of the early onsets of cellular invasion in PDAC. Hence, studying immune cells infiltration in PDAC may shed light on its particularly metastatic nature.

Figure 1.1. Targeting endogenous *Kras*^{G12D} and *Trp53*^{R172H} expression to the mouse pancreas in a pancreatic cancer mouse model¹¹. (a) Endogenous alleles of *Kras*^{G12D} and *Trp53*^{R172H} are conditionally activated in the pancreata of *LSL-Kras*^{G12D/+};*LSL-Trp53*^{R172H/+};*Pdx-1-Cre* (triple mutant KPC) mice. PCR analysis of genomic DNA from the pancreata of triple mutant KPC mice reveals the “1LoxP” recombination product for each targeted allele. (b) Survival of KPC mice is significantly decreased. Kaplan-Meier survival curve analysis reveals a median survival in KPC mice of approximately 5 months. ($p < 0.001$, log-rank test, for each pairwise combination).

Inflammation-associated cancer development

In the year 1893, Rudolf Virchow observed the presence of immune cells in neoplastic tissues and hypothesized that inflammation may play a contributing role in the development of cancer¹⁸. Inflammation orchestrates host defense to pathogens and tissue injury by mediating tissue repair and regeneration. Although this defense mechanism is essential to protect the host from infection and injury, inflammation may also serve as a double-edged sword, where an overly activated innate immune response could also result in autoimmune diseases such as systemic lupus erythematosus (SLE)¹⁹. Following the early observation by Virchow, many follow-on studies have highlighted the role of inflammation as a trigger to tumor initiation, enhancing tumor progression and facilitating dissemination²⁰⁻²³. Indeed, the past decade has seen a renaissance linking inflammation and cancer as the seventh hallmark of cancer²⁴.

Cancer and inflammation are linked by two pathways, the extrinsic pathway or the intrinsic pathway²⁵. In the extrinsic pathway, tumor initiation and development are triggered by inflammation or infections. On the other hand, in the intrinsic pathway, somatic alterations and genetic mutations activate signaling pathways that lead to inflammatory responses (Fig. 1.2) pathway²⁵. Nevertheless, both pathways converge and regardless of the origin, mediators and effectors of inflammation, such as inflammatory cells, cytokines and growth factors created a conducive environment that aids in tumor cell proliferation and survival as well as metastasis through promoting angiogenesis^{18,26}. More recently, the indispensable role of immune cells in supporting tumor proliferation, survival and metastasis was demonstrated²⁷. PDAC is characterized by a dense fibrosis and stromal infiltrate²⁸, marked by a predominant accumulation of myeloid cells such as macrophages and neutrophils²⁹.

Figure 1.2. Inflammation and cancer²⁵. Cancer-related inflammation begins from either the intrinsic or extrinsic pathway. The intrinsic pathway is driven by genetic events, while the extrinsic pathway is driven by inflammation and infections. Both pathways activate transcriptional factors such as nuclear factor- κ B (NF- κ B) and hypoxia-inducible factor 1 α (HIF-1 α) in tumor cells, resulting in the production of inflammatory chemokines and cytokines. These inflammatory mediators recruit and activate inflammatory cells to produce more chemokines and cytokines which give rise to a cancer-related inflammatory microenvironment.

In the KPC mouse model of PDAC, immunosuppressive immune cells such as myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) have been shown to infiltrate the pancreas as early as the PanIN 1 neoplastic stage and persist through the various stages of PDAC³⁰. Among these immune cells, myeloid cells of which macrophages and neutrophils have been credited to play a key role in tumor progression and metastasis³¹.

Myeloid cells influence anti-tumoral immunity

Macrophage phenotypes in the tumor microenvironment

Macrophages are a prominent immune subset involved in many homeostatic and immune functions. These cells are highly plastic and thus can perform a wide diversity of functions³². Depending on the external stimuli, microenvironment and types of cytokine present, these myeloid cells can polarize into specialized subsets³². Classical (M1) macrophages are activated primarily by IFN- γ and/or lipopolysaccharide (LPS), and produce pro-inflammatory cytokines, nitric oxide or reactive oxygen intermediates (ROI) to mount an immune response against bacteria and viruses. Alternative (M2) macrophages are activated by cytokines, such as interleukin (IL)-4 and IL-10. These macrophages are mainly associated with wound healing and tissue repair³².

Macrophages are phagocytic in nature, and mediate efferocytosis — a process of removing apoptotic tumor cells³³. In prostate cancer, the tumor secreted factor milk fat globule-EGF factor 8 (MFG-E8), facilitates macrophage efferocytosis and also promotes M2 polarization³³. The hypoxic microenvironment, created by highly glycolytic tumor cells, also triggers macrophage polarization towards an anti-inflammatory phenotype³⁴. Consequently, macrophage skewing and polarization is dependent on exposure to external stimuli. As such, strategies that can re-polarize these TAMs towards an anti-tumoral phenotype are advantageous for eradicating tumor cells.

A recent study done in my lab observed that TAMs exhibit a mixed M1 and M2 phenotype³⁵, which underlies their capability to promote tumor progression and metastasis, whilst retaining their anti-tumoral function in the presence of tumor-targeting monoclonal antibodies³⁶. One study demonstrated that in the presence of soluble SIRPαFc protein that blocks CD47, *in vitro* polarized human M2 macrophages produce a superior phagocytic response than M1 macrophages³⁷. Another study, however, reported that only M1, but not M2 macrophages, can engulf head and neck squamous carcinoma cell lines³⁸. While these studies demonstrate the capability of pro-tumoral M2-like TAMs to perform M1-like anti-tumoral functions, it is clear that TAMs cannot be simply classified into distinct M1 or M2 phenotypes. With TAM exhibiting diverse spectrum of differential activation states in the tumor microenvironment, specifically targeting M2 characteristics alone may not be sufficient to re-polarize their pro-tumoral phenotype towards an anti-tumoral phenotype to augment tumor progression and metastasis.

This immunosuppression is at least in part mediated by macrophages but also involves regulatory T cells (Fig. 1.3)³⁹. TAM expression of cell surface receptors, secreted cytokines, chemokines, and enzymes suggest that they have an important role in the recruitment and activation of Tregs and the suppression of effector cells in the tumor microenvironment (Fig. 1.3)³⁹.

Characteristics of neutrophils in tumor sites

It has been well established that macrophages can affect almost all aspects of tumor progression, from tumor initiation and proliferation to angiogenesis and metastasis. However, in the recent years, studies have highlighted that neutrophils, like macrophages, also play a critical role in tumor progression⁴⁰. Polymorphonuclear neutrophils (PMNs) are the most abundant circulating leukocyte in humans. They are innate immune cells involved in the first line of defense against infections, thus playing an indispensable role in the inflammatory response.

Figure 1.3. Pro-tumoral macrophage mechanisms and their effects on effector cells³⁹. TAMs express effector molecules that are pro-tumoral inhibiting the anti-tumoral immune responses. TAMs inhibit T cells and NK cell activities directly with their expression of ligand receptors for PD-1 and CTLA-4. Upon activation, TAMs suppress cytotoxic functions of T cell, NK T cells and NK cells. TAMs also express the ligand for death receptors FAS and TRAIL that triggers a caspase-dependent cell death in target cells. In addition, TAMs secrete IL-10 and TGF-β inhibiting T cells effector functions and inducing regulatory functions and chemokines that recruit regulatory T cells (Tregs).

During an infection, activated neutrophils release proteinases into the microenvironment, damaging surrounding tissues. They also produce cytokines and chemokines that recruit inflammatory cells, altering immune responses⁴¹.

Neutrophils were originally perceived as terminally differentiated effector cells playing a major role in microbial immunity and acute inflammation. However, these short-lived cells are not casual observers in this chronic, progressive disease of cancer. In the past decade, neutrophil recruitment and activation has been observed in tumors, similar to the setting of infection in which they are classically studied. Neutrophils have been reported to infiltrate tumor in large numbers and have been shown to kill tumor cells as early as the 1980s^{42,43}. Neutrophils also secrete cytokines and myeloperoxidase, which is involved in monocyte and macrophage recruitment⁴⁴. Existing literature indicate that tumor-infiltrating neutrophils are a double-edged sword, acquiring both anti-tumoral and pro-tumoral phenotypes depending on the tumor microenvironment, with the majority of the neutrophil's effects are pro-tumoral (Fig. 1.4)^{40,45-47}.

Similar to that of macrophages, neutrophil has also been reported with data supporting a skewing of neutrophils phenotypes⁴⁰. Tumor-infiltrating neutrophils acquire a pro-tumor phenotype to become N2-like neutrophils. N2-like neutrophils favor tumor initiation and progression through the release of vascular endothelial growth factor (VEGF) for angiogenesis, as well as suppress cytotoxic T cell activity through the expression of arginase⁴⁸. This polarization is largely dependent on transforming growth factor beta (TGF- β) and blocking TGF- β will alter neutrophils to acquire an anti-tumor phenotype (i.e. N1-like neutrophils)⁴⁰.

Figure 1.4. Effects of tumor-infiltrating neutrophil-derived products on the tumor microenvironment⁴⁷. Neutrophil-derived factors play critical roles in tumor progression from tumor initiation to metastasis. Neutrophils acquire both anti-tumoral and pro-tumoral phenotypes depending on the tumor microenvironment. MMP-8 plays a role in increasing the tumor cell's ability to bind laminin and collagen, reducing the ability of these cells to metastasize (anti-tumoral, green arrow). However, the majority of the neutrophil's effects are pro-tumoral (red arrows), consistent with poor prognosis associated with neutrophil numbers at the tumor site.

Another subset of immune cells relevant in cancer settings are the myeloid derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages and dendritic cells at different stages of differentiation that are distinct from mature myeloid cells, and can be functionally described by their strong immunosuppressive properties⁴⁹. MDSCs strongly expand as a result of perturbed hematopoiesis in pathological diseases, such as chronic inflammation and cancer, and were originally characterized in tumor-bearing mice as having a CD11b+Gr1+ phenotype⁵⁰. MDSCs accumulate in the blood, bone marrow, and the peripheral lymphoid organs (including the lymph nodes and the spleen of tumor-bearing mice), where they have a causative role in promoting immune suppression and thus tumor progression⁵⁰.

MDSCs may serve as a potential cellular target to control tumor cell growth. MDSCs are comprised of two major subsets: monocytic MDSCs (M-MDSCs) and granulocytic MDSC (G-MDSCs). Both subsets can suppress the cytotoxic activity of cytotoxic CD8+ T lymphocytes (CTLs) and natural killer (NK) cells⁴⁹. In mice, M-MDSCs are classified as CD11b+Ly6ChighLy6G- cells, while G-MDSCs are classified as CD11b+Ly6ClowLy6G+ cells. In humans, M-MDSCs are CD33+CD14+CD15-HLA-DRlow while G-MDSCs are CD33+CD14-CD15+CD66b+HLA-DR-/low⁵¹. In a recent study, Sagiv *et al.* distinguished circulating murine neutrophils according to their density⁴⁵. The researchers characterized high-density neutrophils (HDNs) as N1-like cells and circulating low-density neutrophils (LDNs) as N2-like pro-tumoral cells. LDNs can be further subdivided into mature and immature cells, with the immature cells being referred to as G-MDSCs⁴⁵.

However, despite extensive research on these immune cells, the cellular definition of MDSCs subsets remains to be elucidated. At the morphological level, M-MDSCs and G-MDSCs are identical to monocytes and granulocytes, respectively and there are no specific

markers that can unequivocally differentiate them. Consequently, researchers are reluctant to use the current MDSC nomenclature to identify myeloid cells with immuno-suppressive capabilities^{52,53}. Taken together, data indicate neutrophil plasticity in mediating cancer, and there is now major interest in understanding and characterizing myeloid cells infiltration and polarization in cancer progression.

Arms of anti- and pro-tumoral immune responses

Anti-tumoral immune responses are mainly directed by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. However, their cytolytic functions are suppressed by TAMs and tumor-infiltrating neutrophils within the tumor microenvironment. TAMs repress anti-tumoral cellular activity mainly through (1) suppressing polyclonal T-cell proliferation and (2) promoting an immunosuppressive T-cell phenotype in the tumor. In the hypoxic tumor stroma, up-regulated hypoxia inducible factor-1 alpha (HIF-1 α) and signal transducer and activator of transcription 1 (STAT1) expression in TAMs⁵⁴ triggers nitric oxide secretion^{55,56} and induces arginase activity respectively to induce T-cell apoptosis⁵⁵ and halts their expansion. TAMs also help tumor cells evade immuno-surveillance by suppressing T-cell activity. Tregs are immunosuppressive cells that are critical for maintaining self-tolerance, especially in autoimmune diseases. In the context of cancer, the presence of Tregs is often a negative prognostic factor with respect to overall survival⁵⁷. Su and colleagues demonstrated that Tregs develop from naïve CD4+ T cells *in situ*. More importantly, their study using a human breast cancer xenograft mouse model showed that naïve T-cell recruitment is directed by CCL18 produced by TAMs⁵⁸.

Like TAMs, tumor-infiltrating neutrophils also exert immune suppression via multiple mechanisms, such as nitric oxide production⁵⁹, L-Arg depletion⁶⁰ and L-cysteine sequestration⁶¹. Tumor-infiltrating neutrophils essentially limit nutrient availability and deprive T cells of the energy source required to support their function. Interestingly, reactive nitrogen

species produced by these neutrophils induce post-translational modifications in CCL2 that decrease its affinity to CCR2 and its chemoattractant effects on CD8⁺ T cells into the tumor⁶².

The other arm of host anti-tumor immune response is mediated by NK cells. A previous study by Mattiola and colleagues highlighted the effect of different macrophage subsets on NK-cell activity⁶³. They found that M1 macrophage specifically promoted IL-23 and IFN- β -dependent up-regulation of NKG2D, IL-1 β -dependent up-regulation of NKp44, and trans-presentation of IL-15. Cis-presentation of IL-15 on NK cells together with the 2B4-CD48 pathway utilized by M1 trigger NK cell production of IFN- γ . These synergistic cellular mechanisms lead to enhance NK cell cytotoxicity⁶³. Conversely, contact-dependent interactions between M2 TAMs and NK cells stimulate macrophages to produce immunosuppressive TGF- β ⁶⁴. Exhausted NK cells decrease IFN γ , TNF α , and Ki-67 expression, which contributes to an overall reduction in NK-cell activation and cytotoxicity⁶⁵. Evidently, contact between TAMs and NK cells downplays their cytotoxic function and once again promotes a skew towards a pro-tumoral response.

Immunometabolism

Immunometabolism describes the interplay between immunologic and metabolic processes. There has been burgeoning interest in the field of immunometabolism and their impact in disease pathogenesis^{35,66,67}.

Metabolic reprogramming is a hallmark of cancer⁶⁸. For tumor cells to thrive, they must reprogram their metabolic profiles to fuel their energy needs according to the microenvironment to promote their proliferation, survival and differentiation⁶⁹. One of the main metabolic phenomenon used by tumor cells is the Warburg effect, whereby glucose uptake is increased and fermented to produce lactate even in the presence of oxygen⁷⁰. Several recent studies have observed that stimulated immune cells also exhibit specific metabolic profiles which in turn regulate their

downstream effector functions^{71,72}. For example, immune cells can undergo both glycolysis and oxidative phosphorylation (OXPHOS) to produce adenosine triphosphate (ATP) for their functional requirements. During glycolysis, cells uptake glucose available in the environment via glucose transporters (GLUT), and convert it into pyruvate and ATP; nicotinamide adenine dinucleotide (NAD⁺) is also reduced to produce NADH. HIF-1 α have been shown to induce the expression of several glycolytic proteins including glucose transporters (GLUT1, GLUT3), and enzymes [hexokinase 2 (HK2), LDHA] (Fig. 1.5)⁷³.

In environments where there is a lack of oxygen, HIF-1 α can alter the migration, proliferation and glucose metabolism of a cell^{74,75}. Hypoxic cells tend to utilize a larger amount of glucose to meet their energy needs. HIF-1 α regulates this metabolic requirement through the activation and induction of enzymes involved in the glycolytic pathway such as glucose transporters⁷⁶. Immune cells also adapt to low oxygen tension conditions by activating HIF-1 α , which in turn enhances the transcription of glycolytic enzymatic genes⁷⁷. Here, HIF-1 α regulates immune cell glycolytic capacity and their downstream immune effector functions^{78,79}.

HIF-1 α over-expression in myeloid cells translates towards a highly glycolytic phenotype and decreases OXPHOS in these HIF-1 α -overexpressing cells⁸⁰. Colegio and colleagues also showed that HIF-1 α expression in TAMs is required to induce M2-associated genes such as VEGF, arginase 1 (Arg-1) and macrophage galactose-type lectin-1/2 (Mgl1/2) in a colon carcinoma mouse model⁸¹. Given that HIF-1 α can regulate the metabolic activity that drives immune effector functions, studies on the role of immune cell metabolism in tumor initiation and growth have received renewed attention in the recent years.

Figure 1.5. HIF-1 promotes the expression of glycolytic enzymes and transporters during glycolysis⁷³. Green arrows show HIF-1-target gene products that are directly involved in the glycolytic pathway. Enzymes are represented in italicized blue font and their substrates in bold black.

Metabolic signature of macrophages

Macrophages constitute a prominent subset of immune cells that are involved in many homeostatic and immune functions and as mentioned earlier, large numbers of macrophages have been shown to infiltrate pre-neoplastic lesions of PDAC⁸². These cells are phagocytic in nature and were originally perceived to be involved in anti-tumoral immune responses. However, studies have challenged this notion and indicated that TAMs display pro-tumorigenic properties such as enhancing chronic inflammation, promoting angiogenesis, and invasion^{83,84}. When activated, macrophages undergo metabolic shifts, which fuel their switch from a quiescent to an activated state⁸⁵. Emerging evidence suggests that metabolic signaling pathways such as protein kinase B (Akt), mechanistic target of rapamycin (mTOR) or 5' adenosine monophosphate-activated protein kinase (AMPK) can direct immune cell differentiation and instruct their effector function⁸⁶. Indeed, a study recently shown that modulating glucose metabolism can alter the downstream macrophage phenotype thereby influencing their cytokine production and expression of key surface markers⁸⁷.

Despite the diversity of signals and polarization states subjected to macrophages, M1 macrophages have been shown to upregulate glycolysis, leading to the accumulation of succinate, a metabolite shown to direct IL1 β production⁸⁸, while the metabolic profile of M2 macrophages showed an increase in oxidative phosphorylation and fatty acid oxidation⁸⁹. Moreover, the blocking of glycolysis using 2-deoxyglucose (2-DG), a competitive inhibitor of HK inhibited M1 macrophage activation and suppressed their inflammatory response⁹⁰.

In macrophages, glucose transporter 1 (GLUT1) is the main glucose transporter facilitating glucose uptake⁹¹⁻⁹⁵, and GLUT1 has been shown to be highly expressed on macrophages in inflamed tissues⁹⁵. In addition, T_h1, T_h2, and T_h17 cells also express high expression of GLUT1 correlating to increased levels of glycolysis⁹⁶. In a study conducted by Freemerman and colleagues, the investigators reported

that glucose uptake and metabolism via GLUT-1 induced a ROS-driven proinflammatory phenotype in macrophages⁷¹. They showed that macrophages overexpressing GLUT-1 were highly glycolytic and were forced into a hyper-inflammatory state with high production of inflammatory mediators. This phenomenon is also true for dendritic cells and T cells, where alterations in cellular metabolism pathways occur after stimulation and activation^{97,98}. These studies highlight a role of glycolysis and GLUT1 in driving pro-inflammatory phenotypes of immune cells.

As evidenced from recent literature on this topic, metabolic sensing can critically influence the activation and polarization of macrophages. However, the role metabolic sensing play in tumor progression remains to be elucidated and could potentially be involved in directing immune responses in a tumor microenvironment.

Metabolic alterations of tumor-infiltrating neutrophils

Due to the presence of few mitochondria per cell, neutrophils rely almost exclusively on glycolysis⁹⁹ and exhibit very low rates of oxidative phosphorylation¹⁰⁰. They are strongly committed to anaerobic glycolysis for energy production and effector functions, such as respiratory burst and chemotaxis¹⁰¹. A study conducted by Azevedo *et al.*, showed how the PPP and glycolysis contribute to the formation of neutrophil extracellular traps (NETs). They demonstrated that a metabolic shift towards the PPP is essential, as glucose-6-phosphate dehydrogenase (G6PD) release will fuel NADPH oxidase activity to ultimately produce the superoxide (SO) that induces NET formation¹⁰².

Mechanistically, conceptual progress has been made in understanding the signaling pathways that underlie myeloid cell metabolism reprogramming. One such pathway is the mTOR pathway, which regulates various important cell processes such as protein synthesis, cell growth and metabolism¹⁰³. This pathway is also a key orchestrator

of myeloid cell effector responses to nutrient availability and cellular energy requirements, driving an increase in glucose utilization during glycolysis⁹⁷. In cases where tumor growth exceeds the ability of the host's vascular system to supply the tumor microenvironment with sufficient oxygen, hypoxic regions are established that induce HIF-1 α activation and instruct cells to utilize glucose causing an increase in lactate release¹⁰⁴.

The glucocorticoid receptor (GR) is expressed by almost every cellular organism and is involved in regulating the genes that control energy metabolism and the immune response¹⁰⁵. GR signaling suppresses HIF-1 α and regulates tumor-infiltrating neutrophil function via HIF-1 α -dependent glycolysis, thus revealing a role for GR-HIF-1 α in the metabolism and suppressive activities of these neutrophils¹⁰⁶. As such, studies indicate that the regulation of glycolysis and its metabolites are able to direct downstream neutrophil effector functions.

It was postulated by the Nobel laureate Otto Heinrich Warburg that glycolytic metabolism is essential for tumor progression. Over the past decade, it has been well established that tumor cells undergo aerobic glycolysis, releasing tumor-derived metabolites such as lactic acid into the tumor microenvironment¹⁰⁷⁻¹⁰⁹. Pancreatic tumor cells were also observed to overexpress glycolytic enzymes such as HK2, pyruvate dehydrogenase kinase isozyme 1 (PDK1), lactate dehydrogenase A and B (LDHA, LDHB) compared to normal pancreatic cells¹¹⁰. In line with this, tumor-derived lactate has also been found to provoke inflammation resulting in the high influx of immune cells and their secreted factors such as MDSCs, cytokines and growth factors respectively driving metastasis¹¹¹.

Myeloid cells constitute a large cellular niche in the tumor microenvironment and play critical roles in tumor progression and metastasis²⁴. With myeloid cells forming the basis of the tumor microenvironment in PDAC, it is important to understand whether the

changes in the metabolic fates of these cells regulate their anti- or pro-tumoral properties. To date, whether myeloid cells in the PDAC lesions exhibit a specific metabolic profile and how this in turn affects tumor initiation and progression are unknown.

Understanding how myeloid cell metabolism could potentially lead to their pro-tumoral phenotype may precipitate a novel anti-tumor strategy whereby homeostatic reprogramming of the myeloid cells could limit tumor initiation, growth and metastasis.

Project Outline

Over the past decade, conceptual progress in cancer research has led to the addition of two emerging cancer hallmarks: (1) reprogramming of energy metabolism and (2) the evasion of immune destruction. Here, we address the relevance of these two hallmarks in pancreatic cancer, in which a myriad of mechanisms permit immune-cell evasion, resulting in poor therapeutic efficacies and poor survival rates. While it is well established that tumor cells rely largely on the Warburg effect to support their high-energy demands in fluctuating hypoxic tumor environments for their growth and survival, the utilization of similar metabolic profiles by myeloid cells in the tumor microenvironment and how this metabolic utilization affect their functions are largely unknown. Our study uses mouse models and human pancreatic tissue samples to investigate how myeloid cell metabolism influences pancreatic tumor progression. Here, we seek to understand the metabolic programs utilized by tumor-infiltrating macrophages and neutrophils, and how these programs in turn affect tumor progression. Upon understanding how myeloid cell metabolism could potentially lead to their pro-tumoral phenotype, we will explore novel anti-tumor strategy whereby homeostatic metabolic reprogramming of the myeloid cells could limit tumor initiation, growth and metastasis.

Hypothesis #1

Altered metabolic profile of *in vitro* tumor-conditioned macrophages promotes their polarization towards a pro-metastatic phenotype, facilitating tumor dissemination.

Drawn by H.L.Penny

Hypothesis #2

The pronounced glycolytic signature of macrophages in pancreatic lesions promotes their polarization towards a pro-tumoral phenotype, disrupting anti-tumoral immunity.

Drawn by J.L. Sieow

Hypothesis #3

Pro-tumoral neutrophils infiltrate the pancreas in large numbers and up-regulate glycolysis that influence their downstream effector functions leading to the progression of PDAC.

Drawn by J.L. Sieow

Generation of *in vitro* conditioned media

Conditioned media were generated from a normal human pancreatic epithelial cell line hTERT-HPNE (“HPNE”) (ATCC® CRL-4023) from ATCC and two PDAC human epithelial cell lines Panc1 and MiaPaCa2 (gifts from Dr. Abel Ong Choon Kiat, Singapore General Hospital). Cells were seeded at the same cell density at 1×10^6 cells in 30 ml complete IMDM (“cIMDM” – IMDM supplemented with 5% Human serum and 1x Penicillin-Streptomycin) in a T175 flask, and grown to 70–80% confluency. The conditioned media was then removed from the flasks, sterile-filtered (0.2 μ m), aliquoted, and stored at -20°C.

Development of *in vitro*-generated TAMs

All human blood samples used in this study were approved by the Institutional Review Board, Singapore in accordance to guidelines of the Health Science Authority of Singapore (Reference code: NUS-IRB10-250). Peripheral blood mononuclear cells (PBMCs) were obtained from blood of healthy donors using Ficoll density centrifugation. Monocytes were isolated using α -CD14 conjugated microbeads by magnetic-automated cell sorting (MACS) (Miltenyi Biotec), and cultured with conditioned media (thawed and warmed to 37°C) for 7–9 days to generate macrophages cultured in either HPNE, Panc1, and MiaPaCa2 conditioned media. Panc1, and MiaPaCa2 conditioned macrophages will be referred to as TAMs and HPNE conditioned macrophages are called control macrophages. For macrophage supernatant experiments, the conditioned media were removed from HPNE-, Panc1-, and MiaPaCa2-macrophages after the 7–9-day culture, and replaced with fresh cIMDM for 6 h. This media was then harvested and used as macrophage supernatant.

Flow cytometry

Macrophages were resuspended in FACS buffer (10% Fetal calf serum, 5% Human serum, 0.01% sodium azide in PBS). After Fc blockade with anti-Fc γ RIII/II (BD Biosciences), cells were stained with DAPI, and surface-stained with anti-CD163 APC (BioLegend 333610), anti-CD206 APC-Cy7 (BioLegend 321120), and anti-M-CSFR PE (BioLegend 347304). Intracellular CD68 was stained using anti-CD68 Alexa 488 (BioLegend 333811) as per the manufacturer's protocol. Annexin-V APC (eBiosciences 88-8007) was stained as per the manufacturer's protocol. Flow cytometry analysis was performed on a BD Fortessa flow cytometer (BD Biosciences).

Endocytosis assay

Macrophages were harvested, washed and incubated with 100 μ g/ml of pHrodo-red Dextran (Invitrogen) in cIMDM at 37°C for 2 min or 20 min. The macrophages were washed again with cIMDM and assayed for pHrodo-red Dextran uptake by flow cytometry. pHrodo-Red dextran has a pH-sensitive fluorescence excitation/emission of 565/585 nm that increases in intensity with increasing acidity.

RT-Quantitative polymerase chain reaction (qPCR) – *In vitro*

Cells were lysed with Qiazol (Qiagen), and total RNA was purified using the RNeasy Mini kit (Qiagen), as per the manufacturer's instructions. DNase-treated total RNA (0.5–1 μ g) was reverse-transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad) according to manufacturer's instructions. Expression of *IL-1 β* , *IL-6*, *TNF α* , *CCL3*, *Arg1*, *HK1*, *HK2*, *GPI*, *ALDOA*, *TPI1*, *PGK1*, *VEGFA*, *TGF- β* , *HGF* and *HPRT* were determined by quantitative PCR with KAPA Sybr FAST ABI Prism 2x qPCR Master Mix (KAPA Biosystems) as per the manufacturer's instructions using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Housekeeping gene *HPRT* was used to normalize the data. All qPCR reactions were done in triplicates and CT

values were averaged. List of qPCR primer sequences are listed in Table 1.

HUVEC network formation assay

Human Umbilical Vein Endothelial Cells (HUVECs) were grown in EndoGRO-LS media (Millipore) to 60–80% confluency before harvesting. EC Matrix (Millipore), a form of matrigel, was thawed on ice and left at 4°C overnight prior to use. On the day of assay, 10 µl of EC Matrix was added to the inner well of a µ-angiogenesis slide (Ibidi) and allowed to solidify at 37°C for at least 3 h. HUVECs (1×10^4 cells) in 20 µl of EndoGRO-LS media were seeded into the upper well of the µ-angiogenesis slide together with 20 µl of macrophage supernatant. HUVEC networks were allowed to form over the next 4 h at 37°C before termination of the assay. VEGF-A (200 ng/ml) and the VEGFR inhibitor Sorafenib (10 µM) were used as positive and negative controls, respectively. HUVEC network formation was captured by brightfield imaging using an Olympus epifluorescent microscope at the 4-h timepoint. At least four non-overlapping Fields of view (FOVs) were captured at 10X magnification per well. Images were quantitatively analyzed with WimTube software (Ibidi) to evaluate key network formation metrics, such as total tube length, total number of branching points, loops, and nets.

Name of Primer	Sequence (5' to 3')
IL-1 β – Forward	TTACAGTGGCAATGAGGATGA C
IL-1 β – Reverse	CTGGAAGGAGCACTTCATCTG
IL-6 – Forward	CACAGACAGCCACTCACCTCTTCAGAACGA
IL-6 – Reverse	ACCAGTGATGATTTTCACCAGGCAAGTCTC
TNF α – Forward	CAGCCTCTTCTCCTTCCTGAT
TNF α – Reverse	GCCAGAGGGCTGATTAGAGA
CCL3 – Forward	CCTTGCTGTCCTCCTCTGCACCATGGC
CCL3 – Reverse	GGTCGCTGACATATTTCTGGACCCACT
Arg1 – Forward	GTGATGTGAAGGATTATGGGGACCT
Arg1 – Reverse	GGCTGATTCTTCCGTTCTTCTTGA C
HK1 – Forward	CACCTGTGAGGTTGGACTCA
HK1 – Reverse	CCACCATCTCCACGTTCTTC
HK2 – Forward	AAGCCCTTTCTCCATCTCCT
HK2 – Reverse	CTTCTTCACGGAGCTCAACC
GPI – Forward	GAAGTGCTGGTCCATCCAGT
GPI – Reverse	AACATGTTTCGAGTTCTGGGA
ALDOA – Forward	CAGCTCCCGGACTGACTG
ALDOA – Reverse	ATTCCACGGGCTAGAGGAG
TPI1 – Forward	GGCGAAGTCGATATAGGCAG
TPI1 – Reverse	AGTTCTTCGTTGGGGGAAAC
PGK1 – Forward	CTTGGGACAGCAGCCTTAAT
PGK1 – Reverse	CAAGCTGGACGTTAAAGGGA
VEGF – Forward	CGAACGTACTTGCAG ATGTGACA
VEGF – Reverse	GTCTTTCCTGGTGAGAGATCTGGTT
TGF- β – Forward	GCACCGCCGAGCCCTGGACACCAACT
TGF- β – Reverse	TTGAGGAGCGCACG ATCATGTTGGA
HGF – Forward	GATTGGATCAGGACCATGTGA
HGF – Reverse	CCATTCTCATTTTATGTTGCT CA
HPRT – Forward	CTTTGCTTTCCTTGGTCAGG
HPRT – Reverse	CAAGGGCATATCCTACAACAA AC

Table 1. Human primer sequences used for RT-qPCR (Chapter 3.1).

Epithelial-to-mesenchymal transition (EMT) assay

HPAF-II was selected to use in this assay because it is a pancreatic epithelial tumor cell line with a low EMT score (EMT score = -0.556) (ATCC CRL-1997) 3×10^3 cells per well in 200 μ l of cIMDM were seeded in Nunc MicroWell 96-Well black-wall optical-bottom plates. After 4 days, HPAF-II started to grow in tight self-contained colonies, approximately 10–20 cells in size. Media was aspirated from the wells before addition of macrophage supernatants and EMT-inducing media (a gift from R&D Systems) at a 1:1 ratio. EMT induction was allowed to proceed for 36–48 h before the media was removed and cells were fixed with 4% PFA for 30 min at 4°C. The wells were washed with PBS and blocked with 10% normal goat serum for 10 min before staining with mouse monoclonal anti-human E-cadherin (BD Biosciences 610182) for 30 min. After three washes of PBS, the cells were stained with donkey anti-mouse IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific R37114). Cells were washed with PBS three times and stained with DAPI for 5 min. HPAF-II colonies were imaged using a FV-1000 confocal system with an inverted Olympus IX81 microscope. Internuclear distance was calculated using DAPI alone images ran on an in-house script to determine closest distance between two adjacent nuclei. To plot the mean intensity and area of E-cadherin images were analyzed by TissueQuest software (TissueGnostics).

Extravasation assay

Fabrication of a polydimethylsiloxane (PDMS) microfluidic device to measure extravasation, which consisted of a central collagen gel region and two adjacent fluidic channels, was performed by soft lithography as previously described¹¹². HUVECs (5×10^6 cells/ml) in 50 μ l of EGM-2 media (Lonza) were seeded in one of the fluidic channels pre-coated with 50 μ g/ml fibronectin. After 3 days, a uniform monolayer mimicking a blood vessel wall is formed¹¹³. Panc1 and MiaPaCa2 tumor cells, with or without tumor-conditioned macrophages, were injected in the same microchannel to investigate their extravasation across the endothelial

monolayer into the gel region. Tumor cells and macrophages were seeded at a ratio of 1:1 with a concentration of 1.5×10^5 cells/ml for each cell type in EGM-2 media. Macrophages and pancreatic tumor cells were labeled prior to seeding into the device. Macrophages were labeled with Vybrant DiD (Life Technologies) live cell-labeling solution, while pancreatic tumor cells were labeled with PKH26 Red Fluorescent Cell Linker for Cell Membrane Labeling (Sigma-Aldrich). Nuc Blue Live Cell (Life Technologies) was used to stain for nucleus in both macrophages and tumor cells. For the experiments using macrophage supernatants only, the tumor cells were resuspended in the respective macrophage supernatants, and injected into the device as before. After 24 h, the devices were fixed with 4% paraformaldehyde (PFA) for 12 min, washed, and stored at 4°C before confocal imaging. The images were captured using an Olympus IX81 confocal microscope. Images were analyzed with IMARIS software (Bitplane Scientific Software) to quantify the extravasation of pancreatic tumor cells under the different test conditions. The number of extravasated tumor cells was counted and expressed as a percentage of the total tumor cells present on the monolayer.

Live metabolic flux assay

The mitochondria oxygen consumption rate (OCR, O_2 pmol/min) and extracellular acidification rate (ECAR, mpH/min) of macrophages were analyzed using an XF^e96 extracellular flux analyzer (Seahorse Bioscience) according to the manufacturer's instructions. ECAR is quantified using the Glycolysis Stress Test where macrophages were harvested, washed, and resuspended in ECAR medium (DMEM base [no bicarbonate] with 2 mM L-glutamine, 143 mM NaCl, and 0.5% phenol red [pH 7.35]). This test consisted of four consecutive stages: basal (without drugs), glycolysis induction (10 mM glucose), maximal glycolysis induction (5 μ M oligomycin), and glycolysis inhibition (100 mM 2-DG). Glycolytic capacity was calculated as follows: (Mean of highest ECAR values induced by oligomycin) – (Mean of basal ECAR values).

For the Mito Stress Test OCR analysis, macrophages were harvested, washed, and resuspended in OCR medium (DMEM base, 25 mM glucose, 1 mM pyruvate, 2 mM L-glutamine [pH 7.35]). This test also consisted of four consecutive stages: basal respiration (without drugs), mitochondrial complex V inhibition (5 μ M oligomycin), maximal respiration induction [5 μ M carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)], and electron transportation chain inhibition (1 μ M rotenone and 1 μ M antimycin A). Mitochondrial respiration capacity was calculated as follows: (Mean of highest OCR values induced by FCCP) – (Mean of OCR values after oligomycin addition). Macrophages were resuspended in the respectively media were and seeded at 8×10^4 /well in 96-well flat-bottom plates, and incubated in a non-CO₂ incubator for 1 hour at 37°C prior to the start of each assay. Cells were seeded in at least replicate wells per condition.

Cell line authentication

HPNE, Panc1, MiaPaCa2, and HPAF-II cell lines were sent for authentication by Genetica using short tandem repeat profiling.

Mice

WT C57BL/6, (GLUT1^{fl/fl}; LysM-cre), CXCR2^{-/-}, (HIF-1 α ^{fl/fl}; MRP8-cre) and associated “control” (i.e. LysM-cre, and/or GLUT1^{fl/fl} alone, HIF-1 α ^{fl/fl}, MRP8-cre- littermate control) mice were housed and maintained under specific pathogen-free conditions in the Biological Resource Centre (BRC) of A*STAR, Singapore. GLUT1^{fl/fl} mice were a kind gift from Prof E. Dale Abel at the University of Iowa. HIF-1 α ^{fl/fl} mice were a kind gift from Dr. Subhra K. Biswas, and MRP8-cre mice were a kind gift from Dr. Ng Lai Guan at the Singapore Immunology Network. Both males and females were used and all experimental groups were gender- and age- matched. All experiments were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of the BRC, according to the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore (IACUC protocol #171230). The compounds KI20227 and WZB117 were custom-synthesized by GVK Biosciences. Both compounds were incorporated into standard rodent chow Harlan 2018 at 250 ppm and irradiated (Envigo–Teklad Diets). In some experiments, gemcitabine (Actavis, 53J5044) was administered via intraperitoneal (i.p.) injection at 50 mg/kg twice/week; anti-NK1.1 (Bio X Cell, PK136) or isotype control antibody (Bio X Cell, C1.18.4) was injected i.p. at 250 μ g per 20 g mouse once/week; anti-CD8 α (Bio X Cell, YTS169.4) or isotype control antibody (Bio X Cell, 2A3) was injected i.p. at 500 μ g per 20 g mouse once/week; Ly-6G (Bio X Cell, Clone 1A8, BP0075) or isotype control antibody (Bio X Cell, BP0089) was administered via i.p. at 200 μ g per 20g mouse thrice/week.

Generation of stable luciferase expressing 1242 mouse PDAC cell line

The luciferase gene was PCR amplified from the PGL3 basic vector (Promega, E1751) and sub-cloned into the ITR-CAG-DEST-IRES-puro-ITR plasmid (kind gift from Marc Schmidt-Supprian). ITR-CAG-DEST-

IRES-puro-ITR (Control plasmid) or ITR-CAG-Luciferase-IRES-puro-ITR (Luciferase) plasmid was mixed with SB100X transposase (1:1 ratio) and transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific, 11668027) into the 1242 tumor cell line (kind gift from Dr. Dannielle Engle, Tuveson lab, Cold Spring Harbor Laboratory), derived from the tumor of spontaneous KPC mice¹². Three days post-transfection, transfected tumor cells were selected with 2 µg/ml puromycin for seven days. To validate the stable and constitutive expression of luciferase, cells were lysed and assayed for luciferase activity using the Luciferase Assay kit (Promega, E1483). Stably transfected tumor cells were designated 1242L.

***In vivo* bioluminescent imaging (BLI)**

On day 6, 13, 20, and 27-post injection of 1242L tumor cells, mice were imaged using the IVIS Spectrum Imaging System (Perkin Elmer) to monitor tumor progression *in vivo*. Mice were first anesthetized with isoflurane and then injected intraperitoneally with 50 µl of D-Luciferin (5 mg/ml; Perkin Elmer). After 5 min, mice were positioned in the IVIS Spectrum and imaged at various time exposures. Using LivingImage Software, the bioluminescence signal for each mouse was quantified as Total Flux (photons/second) using an exposure image where the signal was not saturated. BLI images shown in the figures were at 15s exposure.

Tissue dissociation

Tumors and pancreata were dissected from mice, collected in ice-cold 1 X PBS and finely minced with scissors in 15-ml scintillation vials. Enzymatic tissue digestion was performed using collagenase type IV (C5138, Sigma) and 0.5 mg/ml DNase I (Sigma, DN25) in DMEM containing 4500 mg/L glucose with 10% FBS (Life technologies) and 1x penicillin-streptomycin (PAN Biotech) – cDMEM, for 30 min at 37°C. Single-cell suspension was obtained by passing the dissociated

pancreas through a 70- μ m filter. Whole blood was collected by cardiac puncture and lysed in RBC lysis prior to flow cytometry staining.

Flow cytometry

Single-cell suspension of the dissociated pancreas were resuspended in FACS buffer (10% Fetal calf serum, 5% Human serum, 0.01% sodium azide in PBS) with 0.5 mg/ml DNase I. After Fc blockade with anti-Fc γ RIII/II (BD Biosciences), cells were stained with DAPI (Sigma Life Science, D9542), and surface stained with the following mouse antibodies – Myeloid cell panel: CD45 (Biolegend, 103120), CD11b (Biolegend, 101239), I-A/I-E (Biolegend, 107622), Ly6G (Biolegend, 127614), Ly6C (Biolegend, 128026), CD24 (Biolegend, 101820), CD11c (Biolegend, 117318), CD64 (Biolegend, 139304) and F4/80 (Biolegend, 123146), together with lineage markers CD3e (BD Biosciences, 553062), NK1.1 (BD Biosciences, 108706) and CD19 (BD Biosciences, 115506) to exclude lymphoid cells. Lymphoid cell panel: CD45 (BD Biosciences, 564279), CD90.2 (Biolegend, 105328), CD3e (Biolegend, 100312), CD4 (Biolegend, 100547), CD8a (eBiosciences 48-0081-82), CD25 (Biolegend, 102016), CD19 (BD Biosciences, 652291), NK1.1 (BD Biosciences, 108706), CD44 (Biolegend, 103032) and CD62L (Biolegend, 103326). For intracellular (IC) cytokine stain, pancreas single-cell suspensions were stimulated with 100 ng/ml LPS (myeloid cell panel), or with 10 ng/ml PMA and 1 μ M ionomycin (lymphoid cell panel), accompanied with Golgi Plug (BD Biosciences 555029) for 4 h in cIMDM and stained with the following antibodies – Myeloid IC cell panel: IL-12 (Biolegend, 560564) and TNF α (eBiosciences, 12-7321-82). Lymphoid IC cell panel: granzyme B (Biolegend, 515403), perforin (eBiosciences, 12-9392-82) and IFN γ (BD Biosciences, 563854). Caspase 1 activity was measured using the Caspase-1-FAM-FLICA Assay (ImmunoChemistry Technologies 97) according to the manufacturer's instructions.

Measurement of reactive oxygen species (ROS) production

Real time ROS production was measured using 1 μ M of Oxidative Stress Detection Reagent from the Total Reactive Oxygen Species detection kit (Enzo LifeSciences ENZ-51010) according to the manufacturer's protocol. Neutrophils were incubated with the oxidative stress detection reagent for 0 and 15 min, and analyzed using the Flow cytometer (BD Biosciences). Pyocyanin (100 μ M) was used as a positive control to induce ROS.

Cell cytotoxicity assay

The cell cytotoxicity assay was performed using the DELFIA® Technology by Perkin Elmer according to manufacturer's protocol. Briefly, neutrophils were sorted based on Dapi⁻CD45⁺ Lin(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺ and used as effector cells. The target cells, 1242L tumor cells, were washed once with cDMEM and the number of cells was adjusted to 1 x 10⁶ cells/ml. 3ml of cells in cDMEM were added to 5 μ l of DELFIA BATDA reagent (Perkin Elmer, C136-100). The cells were loaded for 25 minutes in an incubator at 37°C and washed three times prior to the assay. Effector to target cells (E:T) were co-cultured in the following ratios: 5:1, 10:1 and 20:1 and measured for cytotoxic activity at 2h, 4h, 6h and 16h. In some experiments, effector cells were treated with 5mM of ROS Inhibitor, N-acetyl-L-cysteine (Enzo Life Sciences, ENZ-51010), for 30 min at 37°C prior to the start of assay. The background, spontaneous release, and maximum release were measured and plated as follows: Background – culture medium alone without cells; Spontaneous release – target cells alone without effector cells; Maximum release – target cells supplemented with 10 μ l of Lysis buffer (Perkin Elmer, 4005-0010). After incubation at the different time points at 37°C, 20 μ l of the supernatant from the sample wells were transferred to a black-well flat bottom plate. 200 μ l of Europium solution (Perkin Elmer, C135-100) was then added to the wells and further incubated for 15 minutes at room temperature on a shaker. The Europium signal was then measured using the EnVision

2104 Multilabel Reader (PerkinElmer, 2104-0020). The percentage of specific release was calculated as follows: (Experimental release - Spontaneous release) / (Maximum release - Spontaneous release) x 100.

Immunofluorescence (IF)

Sorted peritoneal cavity (PEC) macrophages were seeded at 2.5×10^5 cells/200 μ l of complete media per well in an 8-well Ibidi μ -slide. Cells were stimulated with or without 1 μ g/ml of LPS for 3 h at 37°C, before 10 μ M of nigericin were added and incubated for a further 30 min. After stimulation, cells were fixed with 4% PFA for 30 min at 37°C, washed twice with PBS, and permeabilized with Perm/Block buffer (10% goat serum, 1% FBS, 0.5% Triton X-100 in PBS) for 30 min at 37°C. Cells were then stained with 10 μ g/ml of anti-ASC mouse monoclonal antibody (Millipore 04-147) overnight at 4°C, washed, and stained with donkey anti-mouse Alexa Fluor-488 secondary antibody (Invitrogen A21202) for 1 h at room temperature. After washing, cells were counterstained with DAPI to visualize nuclei and imaged at 100x magnification using an FV-1000 confocal system with an inverted Olympus IX81 microscope. At least 10 Z-stacks of 1 μ m thickness each were combined to form the final images.

For paraffin-embedded mouse tumor tissue sections (3-4 μ), slides were dewaxed in xylene and dehydrated through a series of ethanol solution before antigen retrieval using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). Mouse sections were stained in a 4-plex staining protocol (Perkin Elmer) in the following sequence: First cycle, primary antibody GLUT1 (Thermo Fisher Scientific, RB-9052-P1), secondary antibody anti-rabbit HRP (Dako, K4003) followed by OPAL FITC dye (Perkin Elmer Life Sciences) with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). Second cycle, primary antibody S100A9 (Abcam, 105472), secondary antibody anti-rat HRP (Thermo Fisher Scientific, 31470), followed by

OPAL Cy3 dye (Perkin Elmer Life Sciences) with microwave treatment using Tris pH 9 (Dako, S2367). Third cycle, primary antibody HIF-1 α (Novus Biologicals, NB100-105), secondary antibody anti-mouse HRP (Dako, K4001), followed by OPAL Cy5 dye (Perkin Elmer Life Sciences) with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer. Finally, all sections were counterstained with DAPI (Sigma Life Science, D9542-5MG) and mounted. IF images were acquired under a confocal microscope. At least 10 images at regions of interest were taken per mouse section and quantifications were performed using TissueQuest Analysis Software (TissueGnostics GmbH).

Western blot

Cell lysates from sorted PEC macrophages were used for Western blot. 10 μ g of samples were resolved on 15% acrylamide denaturing gels (SDS-PAGE). Proteins were transferred to nitrocellulose membranes (Bio-Rad Trans-Blot Turbo Mini Nitrocellulose Transfer Packs) using the Bio-Rad Trans-Blot Turbo Transfer System for 7 min. The membrane was blocked in 1x Tris buffered saline and 0.1% Tween 20 (TBS-T) containing 5% low-fat milk for 1 h and probed overnight at 4°C with primary antibodies diluted in blocking buffer. Primary antibodies used were: β -actin 1:500 (Merck Millipore, MAB1501), IL1 β 1:250 (Santa-Cruz Biotechnology, sc-7884) to detect pro-IL1 β and IL1 β , and caspase-1 1:500 (Santa-Cruz Biotechnology, sc-56036) to detect pro-caspase-1 and caspase-1, followed by either anti-mouse 1:5000 (Abcam, ab97023) or anti-rabbit 1:5000 (Abcam 97051) HRP-conjugated secondary antibodies. The immunoreaction was detected with SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific) and visualized using BioRad ChemiDoc XRS system.

Transcriptomics

Bone marrow, blood and tumor-infiltrating neutrophils pooled from 10 WT and 5 OT mice per experiment were sorted based on their expression of Lin(CD3/B220/NK1.1)⁻ CD45⁺Ly6G⁺Ly6C⁺CD11b⁺. Total

RNA isolation was subsequently performed following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, Waltham, MA, USA) followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Hilden, Germany). All RNA was analyzed on Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) for quality assessment with RNA Integrity Number (RIN) range from 7.8 to 10 and median RIN 9.3. cDNA libraries were prepared using 2ng of total RNA and 1ul of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion® Thermo Fisher Scientific, Waltham, MA, USA) using SMARTSeq v2 protocol¹¹⁴ except for the following modifications: 1. Use of 20µM TSO and 2. Use of 250pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, San Diego, CA, USA). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip GX system (Perkin Elmer, Waltham, MA, USA). All samples were subjected to an indexed PE sequencing run of 2x151 cycles on an Illumina HiSeq 4000 (32 samples/lane). RNA-Seq data in the form of FASTQ files were subsequently mapped to the mouse genome build mm10 using the STAR alignment software. The mapped reads were counted using featureCounts (Subread) based on GENCODE M7 annotations and the raw counts were used to perform differential gene expression (DEG) analysis using edgeR (R version 3.1.2). Differentially regulated genes with $FDR < 0.05$ and $\log_2FC > 2$ were identified. MultiExperiment Viewer (MeV) was used for hierarchical clustering and the generation of heat maps for visualization.

RT-Quantitative polymerase chain reaction (qPCR) – *In vivo*

Cells were lysed in 1 ml QIAzol Lysis Reagent (Qiagen) prior to the addition of 200 µl chloroform to the mixture. Total RNA was isolated from cells with the miRNeasy Mini Kit (Qiagen) with on-column DNase I digestion using the RNase-Free DNase Set (Qiagen). DNase-treated total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Gene expression quantifications were

performed in 384-well plates using the 7900HT FAST Real-time PCR (Applied Biosystems) instrument. Expression of *GLUT1*, *HK1*, *HK2*, *GPI*, *PFKB1*, *ALDOA*, *PGK*, *PKM2*, *LDHA*, *HIF-1 α* (Table 2), *CCL3*, *CCL2*, *HIF-1 α* , *ALDOA*, *LDHA*, *GLUT1*, *HK1*, *HK2*, *Arg1*, *IL-12*, *CCL5* (Table 3), were determined by quantitative PCR with KAPA Sybr FAST ABI Prism 2x qPCR Master Mix (KAPA Biosystems) using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All qPCR reactions were done in triplicates and CT values were averaged. Calculation of normalized relative expression levels were done with respect to *β -actin*.

Name of Primer	Sequence (5' to 3')
GLUT1 – Forward	CATCCTTATTGCCCAGGTGTTT
GLUT1 – Reverse	GAAGACGACACTGAGCAGCAGA
HK1 – Forward	TGCCATGCGGCTCTCTGATG
HK1 – Reverse	CTTGACGGAGGCCGTTGGGTT
HK2 – Forward	AGCTGTTTGACCACATTGCC
HK2 – Reverse	CACGCCACTGGACTTGAAC
GPI – Forward	GGGACCCCTCATGGTGACT
GPI – Reverse	GTCTTGGAGGCGATTATAAAGAGG
PFKB1 – Forward	GCCACCTGTCCTACATCAAGA
PFKB1 – Reverse	TTCACTCTCACCATGTCGGC
ALDOA – Forward	AGAACACCGAGGAGAACAGG
ALDOA – Reverse	AGTTGTCTCGCCATTGGTTC
PGK – Forward	GGAAGCGGGTCGTGATGA
PGK – Reverse	GCCTTGATCCTTTGGTTGTTTG
PKM2 – Forward	CCCTCGGGCTCCTATCATTG
PKM2 – Reverse	TCCATGGCCAAGTTTACACGA
LDHA – Forward	GTGTAAGTGCGAAGTCCAAGC
LDHA – Reverse	TGGATTGGAGACGATCAGCAG
HIF-1 α – Forward	GTCGGACAGCCTCACCAAACAG
HIF-1 α – Reverse	TAGGTAGTGAGCCACCAAGTGTCC
β -actin – Forward	AGAGGGAAATCGTGCGTGAC
β -actin – Reverse	CAATAGTGATGACCTGGCCGT

Table 2. Mouse primer sequences used for RT-qPCR (Chapter 3.2).

Name of Primer	Sequence (5' to 3')
CCL2 – Forward	CAGGTCCCTGTCATGCTTCT
CCL2 – Reverse	GTCAGCACAGACCTCTCTCT
CCL3 – Forward	ACCATGACACTCTGCAACCA
CCL3 – Reverse	TCAGGCATTGAGTTCCAGGT
HIF-1α – Forward	GTCGGACAGCCTCACCAAACAG
HIF-1α – Reverse	TAGGTAGTGAGCCACCAGTGTCC
ALDOA – Forward	AGAACACCGAGGAGAACAGG
ALDOA – Reverse	AGTTGTCTCGCCATTGGTTC
LDHA – Forward	GTGTAAGTGCCTGAACTCCAAGC
LDHA – Reverse	TGGATTGGAGACGATCAGCAG
GLUT1 – Forward	CATCCTTATTGCCAGGTGTTT
GLUT1 – Reverse	GAAGACGACACTGAGCAGCAGA
HK1 – Forward	TGCCATGCGGCTCTCTGATG
HK1 – Reverse	CTTGACGGAGGCCGTTGGGTT
HK2 – Forward	AGCTGTTTGACCACATTGCC
HK2 – Reverse	CACGCCACTGGACTTGAAC
Arg1 – Forward	GATTGGCAAGGTGATGGAAG
Arg1 – Reverse	TCAGTCCCTGGCTTATGGTT
IL-12 – Forward	CGTGCTCATGGCTGGTGCAAAG
IL-12 – Reverse	GAACACATGCCCACTTGCTG
CCL5 – Forward	ACCATGAAGATCTCTGCAGC
CCL5 – Reverse	TGAACCCACTTCTTCTCTGG
β-actin – Forward	AGAGGGAAATCGTGCGTGAC
β-actin – Reverse	CAATAGTGATGACCTGGCCGT

Table 3. Mouse primer sequences used for RT-qPCR (Chapter 3.3).

Multiplex immunofluorescence (IF) staining and analysis

Three PDAC patient cohorts from the Stanford Medical Center, National Cancer Centre Singapore (SingHealth CIRB 2012/879/B), and National Cancer Institute Singapore (DSRB 2012/00939) were obtained. Collection of Singaporean patient sections was approved by the Institutional Review Board of Singapore. Human PDAC tissue FFPE sections were stained in a 6-plex macrophage staining protocol (Perkin Elmer) in this sequence: 1st cycle – primary antibody anti-CD68 1:150 (Dako, PG-M1), followed by secondary antibody anti-mouse HRP (Dako, K4001), and then TSA-Cy3 1:100 (Perkin Elmer Life Sciences, FP1170), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). 2nd cycle – primary antibody anti-GLUT1 1:250 (Thermo Fisher Scientific, RB-9052-P1), followed by secondary antibody anti-rabbit HRP (Dako, K4003), and then TSA-Cy3.5 1:100 (Perkin Elmer Life Sciences, FP1484), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). 3rd cycle – primary antibody used was anti-CD163 1:200 (Abcam, ab188571), followed by secondary antibody anti-rabbit HRP (Dako, K4003), and then TSA-Cy5.5 1:100 (Perkin Elmer Life Sciences, FP1486), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). 4th cycle – primary antibody used was anti-HK2 1:150 (Abcam, 104836), followed by secondary antibody anti-mouse HRP (Dako, K4001), and then TSA-FITC 1:100 (Perkin Elmer Life Sciences, FP1168), with microwave treatment using Tris pH 9 (Dako, S2367). 5th cycle – primary antibody used was anti-HIF-1 α 1:100 (Novus Biologicals, NB100-105), followed by secondary antibody anti-mouse HRP (Dako, K4001), and then TSA-Cy5 1:100 (Perkin Elmer Life Sciences, FP1171), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). After the 5th cycle, the sections were counterstained with DAPI (Sigma Life Science, D9542-5MG).

Human PDAC tissue FFPE sections were stained in a 6-plex neutrophil staining protocol (Perkin Elmer) in the following sequence: 1st cycle –

primary antibody anti-pan cytokeratin 1:50 (Abcam, 27988), secondary antibody anti-mouse HRP (Dako, K4001), followed by OPAL 520 (Perkin Elmer Life Sciences) with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). 2nd cycle – primary antibody anti-GLUT1 1:200 (Thermo Fisher Scientific, RB-9052-P1), secondary antibody anti-rabbit HRP (Dako, K4003), followed by OPAL 540 (Perkin Elmer Life Sciences) with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer. 3rd cycle – primary antibody anti-HK2 1:150 (Abcam, 104836) 1:200 (Abcam, 188571), secondary antibody anti-rabbit HRP (Dako, K4003), followed by OPAL 620 (Perkin Elmer Life Sciences) with microwave treatment using Tris pH 9 (Dako, S2367). 4th cycle – primary antibody anti-HIF-1 α 1:100 (Novus Biologicals, NB100-105), secondary antibody anti-mouse HRP (Dako, K4001), followed by OPAL 650 (Perkin Elmer Life Sciences, FP1168), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer. 5th cycle – primary antibody CD15 1:50 (Abcam, MY-5), secondary antibody anti-mouse HRP (Dako, K4001), followed by OPAL 690 (Perkin Elmer Life Sciences, FP1171), with microwave treatment using Citrate pH 6 Antigen Retrieval Buffer (eBioscience, 00-4955-58). After the 5th cycle, the sections were counterstained with DAPI (Sigma Life Science, D9542-5MG) and mounted.

At least 15 - 20 multispectral (MSI) images in regions of interest were taken per patient tissue section using the Vectra Workstation (Perkin Elmer). The InForm software (PerkinElmer) was used to phenotype cells and quantify the protein expression levels in these stained sections using multispectral fluorescence analysis and spectral unmixing. All analysis was performed based on the “Entire Cell Total Normalized Counts” generated by the InForm software. Using in-house codes, the association of a cell subset co-localization (for e.g., CD15⁺HIF-1 α ⁺) to survival and cancer stage progression was analyzed. Survival analysis was performed on each of the manually selected cell subsets. All analysis and visualizations were done using the R statistical language (v3.3.1).

Statistics

Data are presented as means $\pm$ standard error of the mean (sem) (Graph Pad Prism v7.0a). Statistical significance was determined by the Student's *t*-test (two-tailed) and *p* values < 0.05 were considered significant, * < 0.05 , ** < 0.01 , *** < 0.001 . Survival curves in mice and patients were measured using the Kaplan-Meier analysis, and significance in survival was determined using the log-rank test. For the IF analysis of PDAC patient cohorts', a Kruskal-Wallis rank sum test was performed to examine if the composition of a cell subset (CD15⁺HIF-1 α ⁺) is significantly different between different cancer stage. A Mann-Whitney test was performed to determine differences between matched normal and PDAC patients. For the survival curves of the PDAC patients, a Kruskal-Wallis rank sum test was used. Multiple testing corrections for the cell subsets were done using the Benjamini & Hochberg method.

Study approval

All mouse studies were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of the ASTAR BRC, in accordance to the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore (IACUC protocol #171230). The Institutional Review Board of Singapore approved collection of the Singaporean patient sections. Three FFPE PDAC patient cohort slides from the Stanford Medical Center, National Cancer Centre Singapore (SingHealth CIRB 2012/879/B), and National Cancer Institute Singapore (DSRB 2012/00939) were approved and obtained.

3.1 Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma

PDAC tumor-conditioned macrophages exhibit a mixed M1/M2 phenotype

An *in vitro* model of tumor-conditioned macrophages was used to address how metabolism in macrophages affects the progression of PDAC. Human CD14 blood monocytes from healthy donors were cultured with conditioned media from control (HPNE) or PDAC (Panc1 or MiaPaCa2) cells for 7 - 9 days to generate HPNE, Panc1 and MiaPaCa2 respectively. Conditioned macrophages were validated based on their morphology, endocytosis and CD68, CD163, M-CSFR, and C206 macrophage marker expression. Results showed that all three conditioned macrophages had similar spindly-like and flatten egg-shaped morphology (Fig. 1a) and endocytosis capacity (Fig. 1b). Panc1 and MiaPaCa2 macrophages exhibited significantly higher expressions of CD163 and CD206 compared with the HPNE control macrophages (Fig. 1c). Interestingly, transcript analysis revealed that Panc1 and MiaPaCa2 macrophages expressed both classically activated M1 markers – *IL-1 β* , *IL-6*, *TNF- α* , and *CCL3*, and the alternatively activated M2 marker – *Arg1* (Fig. 1d). This suggests that tumor-conditioned macrophages exhibit a mixed M1 and M2 macrophage phenotype.

Glycolytic signature of TAMs

To address the metabolic profiles of PDAC tumor-conditioned macrophages, we utilized a live Seahorse metabolic flux assay to determine their rate of glycolysis and OXPHOS capacities. Results showed that Panc1 and MiaPaCa2 TAMs exhibited a 2-fold increase in glycolytic capacity over control HPNE macrophages, showing a preferential use of glycolysis (Fig. 2a).

Figure 1. PDAC tumor-conditioned macrophages exhibit a mixed M1/M2 phenotype. (a) CD14⁺ blood monocytes from healthy donors were cultured with HPNE, Panc1 and MiaPaCa2 conditioned media for 7-9 days. Shown are representative brightfield images of macrophages generated from the respective conditioned media. (b) Shown are histograms of HPNE, Panc1 and MiaPaCa2 macrophages assayed for their ability to endocytose using phrodo-red dextran after 2 and 20 min incubation. (c) Macrophage markers CD68, CD163, M-CSFR and CD206 on the three macrophages types were analyzed by flow cytometry. Dotted lines have been drawn at the peak of HPNE control macrophage signal for comparison. Data are representative of n=4 independent experiments. (d) Macrophages were assayed for transcript expression of M1 and M2 markers IL-1 β , IL-6, TNF- α , CCL3 and Arg1. Bar graphs shown are with relative expression to HPRT. Data are representative of n=3 independent experiments, means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Figure 2. Glycolytic signature of TAMs. Shown are the Seahorse trace of (a) ECAR and (b) OCR values at each timepoint in the Glycolysis Stress test and Mitostress test respectively. On the right are bar graphs of the mean $\pm$ SEM of the (a) glycolytic capacity and (b) maximal mitochondrial respiration capacity. Data are representative of $n=9$ independent experiments. (c) Macrophages were assayed for transcript expression of key glycolysis genes *HK1*, *HK2*, *GPI*, *ALDOA*, *TPI1* and *PGK1* by qPCR. Bar graphs shown are with relative expression to HPRT. Data are representative of $n=4$ independent experiments. Data represent the means $\pm$ SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired student's t-test with a 95% confidence interval.

Consistent with this, Panc1 and MiaPaCa2 TAMs exhibited a 2-fold decrease of mitochondrial respiration capacity (Fig. 2b).

Furthermore, transcript analysis of key glycolytic genes revealed an increased expression of hexokinase 1 (*HK1*), *HK2*, glucose-6-phosphate isomerase (*GPI*), fructose biphosphate aldolase A (*ALDOA*), triosephosphate isomerase (*TPI1*) and phosphoglycerate kinase 1 (*PGK1*) in Panc1 and MiaPaCa2 TAMs as compared to their control counterparts (Fig. 2c). Taken together, data show that Panc1 and MiaPaCa2 TAMs have the tendency to utilized glycolysis for their metabolic needs over OXPHOS. As such, their pro-metastatic phenotype may be dependent on the preferential use of glycolysis.

Panc1 and MiaPaCa2 TAMs promote higher rates of angiogenesis, induced higher rates of epithelial-to-mesenchymal transitions and augmented extravasation as compared to control HPNE macrophages

To determine the pro-metastatic capabilities of Panc1 and MiaPaCa2 TAMs, we assessed their ability to induce angiogenesis, promote extravasation, and boost EMT of tumor cells. Using a HUVEC network-formation assay, we observed that supernatants of Panc1 and MiaPaCa2 TAMs caused an increased in HUVEC networks formation as compared to the control HPNE macrophages (Fig. 3a). VEGF-A and Sorafenib (VEGFR inhibitor) were used as positive and negative controls respectively. Images were quantified and analyzed using the WimTube software for key network formation parameters such as total network length, total branch points, total number of loops and nets (incomplete loops). Notably, we observed a significant increase in total network length (Fig. 3b), total branch points (Fig. 3c) and total number of loops formed (Fig. 3d), and a significant decrease in the total number of nets (Fig. 3e) in TAMs networks as compared to HPNE networks. Following the accelerated angiogenesis observed in TAMs supported networks, we next proposed that TAMs might act in a VEGF-dependent

manner. Consistent with this, transcript analysis of TAMs showed a significant higher expression of *VEGFA* as compared to HPNE control macrophages (Fig. 3f). In addition, neutralization of VEGF-A in the macrophage supernatants using α -VEGF-A antibody showed that the TAMs promote angiogenesis in a VEGF-dependent manner (Fig. 3g).

During metastasis, tumor cells at the primary site increase in cell motility and migrate to secondary organs through a process also known as EMT¹¹⁵. Epithelial markers and cell adhesion markers such as E-cadherin are lost as cells begin to spread. To determine the effect TAMs have on the metastasis of tumor cells, we utilized pancreatic HPAF-II colonies with a low EMT score and seeded them with the addition of the respective Panc1 and MiaPaCa2 TAMs and HPNE control supernatants. Consistent with the increased angiogenesis and extravasation potential of Panc1 and MiaPaCa2 TAMs, Panc1 and MiaPaCa2 TAMs were also observed to induce a greater spread of HPAF-II colonies as compared to the HPNE control (Fig. 4a). Internuclear distance between neighboring cells were calculated and results showed a significant increase in HPAF-II tumor cells incubated with Panc1 and MiaPaCa2 TAMs supernatant (Fig. 4b). Further analysis also revealed a loss of E-cadherin in Panc1 and MiaPaCa2 TAMs as compared to their control counterparts (Fig. 4c).

Figure 3. Panc1 and MiaPaCa2 macrophages promote higher rates of angiogenesis compared to control HPNE macrophages. Macrophages supernatants harvested from HPNE, Panc1 and MiaPaCa2 macrophages were added HUVECs for 4 hours at 37°C. rhVEGF-A and VEGFR inhibitor Sorafenib were used as a positive and negative control respectively. (a) Shown are representative brightfield images of HUVEC networks analyzed by Ibidi's WimTube Image Analysis software. (b) Total network length (tubes identified by red lines, length of tubes identified by blue regions), (c) total branch points, (d) total loops (identified by yellow numbers) and (e) total nets are depicted in bar graphs from n=6 independent experiments. (f) Shown is a bar graph of the transcript expression of VEGFA in the respective macrophages by qPCR. (g) Shown are bar graphs of total network length in which VEGFA is neutralized in the macrophage supernatants by the addition of an anti-VEGF antibody. Data are representative of n=4 independent experiments, means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

To determine the mechanism by which Panc1 and MiaPaCa2 TAMs may induce EMT, we performed transcript analysis on growth factor known to promote EMT such as TGF- β and hepatocyte growth factor (HGF). True enough, as compared to the control HPNE macrophages, Panc1 and MiaPaCa2 TAMs showed a 1.5 to 3-fold increase in *TGF- β* gene expression, as well as a 1.5 to 2-fold increase in *HGF* gene expression (Fig. 4d). Further supporting this hypothesis, neutralizing antibodies to TGF- β and/or HGF, were added to the co-culture of Panc1 and MiaPaCa2 TAMs and HPNE control supernatants with HPAF-II colonies. Results showed that addition of α -TGF- β antibody, and α -TGF- β + α -HGF antibodies were able to inhibit the spreading of HPAF-II colonies (Fig. 4e). α -HGF antibody alone also decreased internuclear distance of HPAF-II colonies, however the decrease did not reach significance (Fig. 4e). Taken together, results show that Panc1 and MiaPaCa2 TAMs may be augmenting EMT of the tumor cells in a TGF- β and HGF-dependent manner.

To determine the extravasation potential of Panc1 and MiaPaCa2 TAMs, we utilized a microfluidic device consisting of a central gel matrix and two fluidic channels by the sides¹¹³. HUVECs were seeded into one of the fluidic channels to form a uniform monolayer representing a blood vessel wall (Fig. 5a). Labeled Panc1 and MiaPaCa2 TAMs, with or without their respective PDAC tumor cells, were seeded into the “lumen” of the blood vessel. Confocal analysis was done to determine the number of extravasated tumor cells through the blood vessel wall into the central gel matrix (Fig. 5b). Interestingly, results showed that both Panc1 and MiaPaCa2 TAMs promoted the extravasation of Panc1 and MiaPaCa2 tumor cells by 3 to 4-fold as compared with control HPNE macrophages (Fig. 5c). Shown in Fig 3f, both Panc1 and MiaPaCa2 TAMs were observed to have an elevated expression of VEGFA. As such, VEGF may also likely be involved in the promotion of vascular permeability to allow the extravasation of tumor cells.

Figure 4. Panc1 and MiaPaCa2 macrophages induced higher rates of EMT compared to control HPNE macrophages. HPAF-II cells were seeded and left to grow into colonies for 4 days. On day 4, macrophages supernatants were added to the HPAF-II colonies to induce EMT. After 36-48 hr, HPAF-II colonies were assayed by confocal microscopy to determine: (i) internuclear distance of cells within each colon and (ii) loss of E-cadherin expression. (a) Shown are representative immunofluorescence images of HPAF-II colonies incubated with the respective macrophage supernatants, stained with E-cadherin and F-actin. (b) Shown is a dot plot of the means $\pm$ SEM of the internuclear distance. Each dot represents a measurement between 2 neighboring HPAF-II cells. (c) Shown is a collated dot plot of the means $\pm$ SEM of the intensity of E-cadherin. Data are representative of n=8 independent experiments. (d) Shown are bar graphs of the means $\pm$ SEM of the transcript expression of TGF- β and HGF of the HPAF-II colonies at the end of the EMT assay by qPCR. Data are representative of n=3 independent experiments. (e) The scatter plots of the internuclear distance in which HGF was neutralized with an anti-HGF antibody (Goat IgG as isotype control), and TGF- β was neutralized with an anti-TGF- β antibody (Mouse IgG1 as isotype control) in the macrophage supernatants, n=4 independent experiments are shown. Data are means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Collectively, this data indicates that Panc1 and MiaPaCa2 TAMs are pro-metastatic given their ability to promote angiogenesis, EMT and extravasation of PDAC tumor cells.

Inhibition of glycolysis in Panc1 and MiaPaCa2 macrophages using 2-DG inhibited their pro-metastatic phenotype

To address whether the pro-metastatic phenotype of TAMs is dependent on their preferential use of glycolysis, macrophages were treated with 2-DG, a competitive inhibitor of HK2. HK2 phosphorylate glucose to glucose-6-phosphate in the first step of glucose metabolism committing the molecule of glucose to glycolysis. With the addition of 2-DG, blocking the production of glucose-6-phosphate from glucose, glycolysis is inhibited. After treatment with 2-DG, we repeated the three assays of metastasis – angiogenesis, extravasation, and EMT.

After confirming that treatment with 2-DG did not affect macrophage viability while inhibiting glycolysis, we showed that remarkably, 2-DG-mediated inhibition of glycolysis reversed the angiogenesis capabilities initially observed with TAMs (Fig. 6a). Treatment with 2-DG significantly inhibited the total network length, total branch points and total number of loops, while increasing the total number of nets in the HUVEC network formation of Panc1 and MiaPaCa2 TAMs as compared to HPNE control macrophages (Fig. 6b).

Figure 5. Panc1 and MiaPaCa2 macrophages augmented extravasation compared to control HPNE macrophages. HUVECs were cultured in a single layer in one of the fluidic channels. Panc1 or MiaPaCa2 tumor cells with or without corresponding macrophages (1:1) were injected into the channel containing HUVECs. After 24h, tumor cell extravasation across the endothelial monolayer into the gel region was quantified by confocal microscopy and expressed as a percentage of the total number of cells. **(a)** Shown is a 3D image of the microfluidic device and the predicted movement of tumor cells and/or macrophages across the HUVEC monolayer into the 3D matrix. **(b)** Shown are representative immunofluorescence images of MiaPaCa2 tumor cells at three different stages – (i) not extravasated, (ii) in the process of extravasating and (iii) extravasated across the HUVEC monolayer. **(c)** Shown are bar graphs of the percentage of extravasated cancer cells, using collated data from at least 6 devices per condition. Data represent the means $\pm$ SEM, ** $p < 0.01$; *** $p < 0.001$ by unpaired student's t-test with a 95% confidence interval.

In the second assay of metastasis, extravasation of PDAC tumor cells was also observed to be significantly reduced in 2-DG-treated Panc1 and MiaPaCa2 TAMs but not 2-DG-treated HPNE macrophages (Fig. 6c). Notably, consistent with the decreased in angiogenesis and extravasation capabilities of 2-DG-treated Panc1 and MiaPaCa2 TAMs, indicators of EMT were also reversed (Fig. 6d). With 2-DG treatment, a significant decrease in internuclear distance was observed in HPAF-II colonies incubated with Panc1 and MiaPaCa2 supernatants (Fig 6e). There was also restoration of the cell adhesion marker E-cadherin (Fig, 6f).

Taken together, these results indicate that the pro-metastatic phenotype of Panc1 and MiaPaCa2 TAMs is strongly dependent on their commitment to the preferential use of glycolysis, also known as the Warburg effect. Inhibition of glycolysis using 2-DG, a competitive inhibitor of HK2, is able to subvert their pro-metastatic phenotype, reversing angiogenesis, extravasation, and EMT of tumor cells.

Figure 6. Inhibition of glycolysis in Panc1 and MiaPaCa2 macrophages using 2-DG inhibited their pro-metastatic phenotype. CD14⁺ monocytes were co-cultured with HPNE, Panc1 and MiaPaCa2 conditioned media to obtain the three macrophages types. Media was aspirated and replaced with fresh media with or without 25mM 2DG for the next 6 h. Macrophage supernatants were then harvested for downstream (a, b) angiogenesis, (c) extravasation and (d-f) EMT assays. (a) Shown are representative brightfield images of HUVEC networks analyzed by Ibidi's WimTube Image Analysis software, along with (b) scatter plots of the WimTube Image Analysis. Data shown is collated from n=6 independent experiments. (c) Shown are bar graphs of the percentage of extravasated cancer cells, using collated data from at least 4 devices per condition. (d) Shown are representative immunofluorescence images of HPAF-II colonies incubated with the respective macrophage supernatants, stained with E-cadherin and F-actin. Shown are scatter plots of the mean $\pm$ SEM of (e) internuclear distance and (f) mean intensity of E-cadherin. Scatter plots are collated data from one experiment with at least 8 colonies per condition. Data are means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval

3.2 A metabolic reprogramming in tumor macrophages via conditional knockout of GLUT1 attenuates tumor burden in pancreatic ductal adenocarcinoma

Macrophages from tumor-bearing mice are highly glycolytic

Orthotopic PDAC tumors were generated in mice through intrapancreatic injections of tumor cells derived from the KPC mice¹² expressing the luciferase gene to allow for the *in vivo* monitoring of tumor progression over time. The weight of the pancreas was recorded to confirm the successful implantation and growth of tumor cells over time (Fig. 7a). Flow cytometry analysis revealed an influx of macrophages into the pancreatic tumors (3.2% of total CD45⁺ cells as compared to 0.4% in sham non-tumor bearing mice) (Fig. 7b). To ascertain the contribution of macrophages in tumor progression, we depleted macrophages using KI 20227, a specific Csf1r inhibitor. Monitoring of tumor growth over time showed that macrophage depletion indeed did result in reduced tumor burden. At day 26 post-tumor transplantation, *in vivo* monitoring by bioluminescence imaging (BLI) showed a significant reduction in tumor growth as compared with untreated control mice (Fig. 7c, d). Though data suggest that macrophage play a role in promoting tumor progression, the overall survival of treated mice did not show a significant improvement as compared to controls (Fig. 7e). This indicates that purely depleting macrophages was not a good enough strategy to increase survival in PDAC.

Following our hypothesis and in our *in vitro* tumor-conditioned macrophages that the metabolic profile utilized by tumor-infiltrating macrophages cells is altered in PDAC leading to tumor progression, we first seek to determine the metabolic state of these macrophages. We assessed macrophages metabolism using a Seahorse live metabolic flux assay where PEC macrophages are sorted based on their expression of Dapi⁻CD45⁺F4/80⁺CD11b⁺. PEC macrophages from tumor

bearing OT mice exhibited a 3-fold higher rate of glycolysis compared with sham non-tumor bearing controls (Fig. 7f, g).

Moreover, gene expression analysis further validate this phenomenal with a significant upregulation of key glycolytic enzymes such as *GLUT1*, *GPI*, phosphofructokinase (*PFKB1*), *ALDOA*, *PGK*, pyruvate kinase muscle isozyme M2 (*PKM2*), *LDHA*, and HIF-1 α (Fig. 7h). Furthermore, intracellular staining of IL-1 β revealed upregulation of this cytokine in tumor-infiltrating macrophages as compared with sham controls (Fig. 7i). Collectively, we show that OT macrophages exhibited a pronounced glycolytic profile with upregulation IL-1 β as compared to sham non-tumor bearing controls.

Figure 7. Macrophages from tumor-bearing mice are highly glycolytic. (a) Scatter plot of orthotopic tumor weights at 6, 14, 21, 28 and 35 days post transplantation in orthotopic transplanted (OT) and sham non-tumor bearing mice (n = 3 mice per experiment at each time point). (b) Pancreata from Day 28 tumor-bearing OT mice were assessed for their myeloid cell populations by flow cytometry. Scatter plot of mΦ (CD45+ Lin- MHCII^{low-int} CD24+ CD11b+ Ly6G- Ly6C^{low} F4/80+) that infiltrate the pancreas/tumor, as a percentage of total CD45+ cells, from OT (black) and age-matched sham controls (grey) (sham, n = 10; OT, n = 8, three independent experiments pooled). (c-d) OT mice were fed either KI20227 (red) or standard rodent chow (black) beginning Day 1 of tumor cell transplantation. Representative scatter plot of the total flux on Day 26 as calculated from the BLI image shown (KI20227, n = 9; Control, n = 10, two independent experiments). BLI images for all figures were acquired at 15-s exposure, and color scale set with lower limit at 3×10^6 and upper limit at 5×10^7 photons/sec. (c) Survival analysis of KI20227-fed mice (n = 9) compared with standard chow-fed mice (n = 10). (e) Sorted peritoneal (PEC) mΦ from OT and sham control mice were assessed for their glycolytic capacity in a live metabolic flux assay. (f) Shown is a representative Seahorse trace of OT-PEC mΦ (black) compared with sham (grey), (g) and a bar graph of glycolytic capacity as calculated from the Seahorse trace (n = 3 mice pooled per group, five independent experiments). (h) PEC mΦ from OT and sham controls were assessed at the transcript level for *GLUT1*, *HK1*, *HK2*, *GPI*, *PFKFB1*, *ALDOA*, *PGK*, *PKM2*, *LDHA*, and *HIF1α* (n = 3 mice pooled per group, three independent experiments). (i) Representative scatter plot of IL-1β production in OT and sham tumor mΦ, (sham, n = 5; OT, n = 8 mice, three independent experiments). Data are means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with 95% confidence interval.

Macrophage-specific deletion of GLUT1 significantly reduces tumor burden

To address the role of macrophage metabolism in disease outcome, we crossed GLUT1^{fl/fl} mice with LysM^{Cre} mice to generate mice with a macrophage-specific deletion of GLUT1 (GLUT1^{ΔmΦ}). To validate the conditional knockout of GLUT1 in GLUT1^{ΔmΦ} mice, we performed a live Seahorse metabolic assay to determine the glycolytic profile of the knockout macrophages. Extracellular flux analysis showed that GLUT1^{ΔmΦ} macrophages were not able to undergo glycolysis, validating this genetic model (Fig. 8a). With the specific knockout of glycolysis in macrophages, we performed orthotopic transplantation of 1242L tumor cells into the pancreas of GLUT1^{ΔmΦ} mice and observed tumor progression. OT GLUT1^{ΔmΦ} mice showed a dramatic reduction in tumor burden following all time points post transplantation determined by BLI imaging as compared to controls (day 28 showed in Fig. 8b). Strikingly, in contrast to KI20227-treated mice, GLUT1^{ΔmΦ} mice displayed a significantly improved overall survival as compared to controls (Fig 8c). We ruled out the possibility that cell viability was affected in GLUT1^{ΔmΦ} mice as the percentage of pancreatic macrophages found in the tumor remain unchanged as compared with controls (Fig.8d).

Given the promising tumor outlook in GLUT1^{ΔmΦ} mice, we next elucidated the downstream immune effector functions in these mice contributing to their reduced tumor burden.

Figure 8. Macrophage-specific deletion of GLUT1 (GLUT1 Δ m Φ) significantly reduces tumor burden. (a) Sorted PEC macrophages from control and GLUT1 Δ m Φ mice were assessed for their glycolytic capacity in a live metabolic flux assay. Shown is a representative Seahorse trace of control (black) compared with GLUT1 Δ m Φ (blue) ($n = 3$ mice pooled per group, three independent experiments). (b) Orthotopic tumors from control and GLUT1 Δ m Φ mice were monitored *in vivo* over time using BLI. Shown is a representative scatter plot of the total flux on Day 28 as calculated from the BLI images (control, $n = 17$; GLUT1 Δ m Φ , $n = 25$, pooled from four independent experiments). (c) Shown is a survival analysis of GLUT1 Δ m Φ mice ($n = 21$) compared with controls ($n = 18$). (d) The scatter plot of tumor infiltrating m Φ are plotted as a percentage of total CD45+ cells. Data are means $\pm$ SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired student's t-test with 95% confidence interval.

The pro-inflammatory IL-1 β pathway is suppressed in GLUT1 $\Delta m\Phi$ macrophages

To determine the pro-inflammatory status of GLUT1 $\Delta m\Phi$ macrophages, we performed intracellular staining of pro-IL-1 β , in IL-6 and TNF α cytokines. Data showed a significant down-regulation of pro-IL-1 β in GLUT1 $\Delta m\Phi$ macrophages as compared with control macrophages (Fig. 9a). In contrast, a slight increase was observed in the production of IL-6 and TNF α cytokines, though percentages did not reach significance (Fig. 9b, c). To dive in further into the reduced production of pro-IL-1 β in GLUT1 $\Delta m\Phi$ macrophages as a possible mechanism for the controlled tumor growth, we assessed the assembly of apoptosis-associated speck-like protein containing a CARD (ASC) specks, and the activity of caspase 1, the catalytic enzyme that cleaves pro-IL-1 β to the active form of IL-1 β . ASC has been shown to bind to procaspase-1 and to adapter proteins involved in caspase-1 activation, thereby regulating cytokine pro-IL-1 β secretion¹¹⁶. Using IF staining for ASC, we observed that the proportion of ASC speck-positive GLUT1 $\Delta m\Phi$ macrophages was reduced by more than 3-fold as compared with controls (7.91% vs. 25.0%) (Fig. 9d). Moreover, upon stimulation with LPS and nigericin, the proportion of ASC speck-positive GLUT1 $\Delta m\Phi$ macrophages remained significantly reduced compared with controls (21.2% vs. 48.3%) (Fig. 9d). In addition, we assessed Caspase 1 activity and observed a down-regulation of its activity in GLUT1 $\Delta m\Phi$ macrophages as compared to controls (Fig. 9e). Furthermore, the reduction in pro-IL-1 β cytokine secretion, ASC speck-positive cells and Caspase 1 activity were confirmed with reduced protein expression of IL-1 β and caspase 1 by western blotting (Fig. 9f). Taken together, data support the notion that the pro-inflammatory IL-1 β pathway is suppressed in GLUT1 $\Delta m\Phi$ macrophages and therefore we conclude that the protection from progression of PDAC seen in GLUT1 $\Delta m\Phi$ mice may be due, in part, to their suppression of IL-1 β -mediated pro-inflammatory pathway.

Figure 9. The pro-inflammatory IL1 β axis is suppressed in GLUT1 Δ m Φ macrophages. (a-c) Intracellular flow cytometry of pro-inflammatory cytokines were performed on control (black) and GLUT1 Δ m Φ (blue) pancreata cell suspensions re-stimulated with 100 ng/ml LPS for 4 h. Shown are scatter plots of the (a) % pro-IL1 β ⁺, (b) % IL6⁺, (c) % TNF α ⁺ of total macrophages (control, n = 16; GLUT1 Δ m Φ , n = 16, pooled from three independent experiments). (d) Immunofluorescence staining of ASC specks was performed in FACS-sorted control (black) and GLUT1 Δ m Φ (blue) PEC macrophages stimulated with 1 μ g/ml of LPS for 3 h, and 10 μ M of nigericin for a further 30 min (+stim, bottom row) or without (-stim, top row). Shown are representative confocal images with nuclei (blue) and ASC (green) overlaid. Each ASC speck is identified as a dot-like perinuclear stain. Magnification bar = 10 μ M. Scatter plots shown are the quantification of % ASC speck⁺ cells for every field of view in unstimulated (open circles) and stimulated cells (filled circles) (n = 3 mice pooled per group, two independent experiments). (e) FACS-sorted control (black) and GLUT1 Δ m Φ (blue) PEC macrophages were stained with Caspase-1-FAM-FLICA to assess caspase-1 activity by flow cytometry. Shown is a representative bar graph of the MFI of caspase-1 (n = 3 mice pooled per group, two independent experiments). (f) Representative western blot of sorted control and GLUT1 Δ m Φ pancreatic tumor macrophage cell lysates probed for pro-IL1 β and pro-caspase 1 (n = 6–8 mice pooled per group, two independent experiments). Data are means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with 95% confidence interval.

NK cells and CTLs mediate anti-tumor immunity in GLUT1^{ΔmΦ} mice

We next went on to further elucidate the pronounced protective phenotype in GLUT1^{ΔmΦ} mice and hypothesized that anti-tumoral activity seen may be due to changes in the adaptive lymphocyte immunity. To determine the anti-tumoral activity in GLUT1^{ΔmΦ} mice, first we characterized the lymphoid populations and observed no significant differences in the population of CD4⁺ T cells between GLUT1^{ΔmΦ} mice and controls (Fig. 10a). Interestingly, though the percentages of CTLs trended upwards in GLUT1^{ΔmΦ}, it did not reach significance (Fig. 10b). Notably, the percentages of NK cells were significantly upregulated in GLUT1^{ΔmΦ} mice compared with controls (Fig. 10c). Moreover, NK cells in GLUT1^{ΔmΦ} mice expressed higher amounts of IFN γ (Fig. 10d), perforin (Fig. 10e) and granzyme B (Fig. 10f) via flow cytometry, indicating enhanced activation and cytotoxicity. In support of our hypothesis that NK cells are involved in mediating anti-tumoral activity in GLUT1^{ΔmΦ} mice resulting in better tumor outcome, we depleted NK cells via I.P. injection of α -NK1.1 (PK136) antibody. As compared to isotype controls, GLUT1^{ΔmΦ} mice treated with α -NK1.1 antibody showed a significant increase in tumor burden (Fig. 10g) indicating that NK cells played a role in controlling tumor growth in GLUT1^{ΔmΦ} mice.

Although the percentages of CTLs were trending higher but did not reach significance, nevertheless, we assessed their anti-tumoral activity via the production of IFN γ , perforin and granzyme B. Similarity to NK cells, CTLs in GLUT1^{ΔmΦ} mice expressed higher amounts of IFN γ (Fig. 10h), perforin (Fig. 10i) and granzyme B (Fig. 10j) via flow cytometry, indicating enhanced activation and cytotoxicity. The depletion of CTLs with anti-CD8 α (YTS 169.4) antibody in GLUT1^{ΔmΦ} mice also showed a significant increase in tumor burden as compared to isotype control mice (Fig. 10k). Data indicates that CTLs are also able to control the tumor growth in GLUT1^{ΔmΦ} mice. In conclusion, results suggest that the overall protection from tumor progression in GLUT1^{ΔmΦ} mice is mediated in part by the anti-tumoral cytotoxic effect of both NK cells and CTLs.

Figure 10. NK cells and CTLs mediate anti-tumor immunity in GLUT1 Δ m Φ mice. (a-f, h-j) Flow cytometry was performed in control and GLUT1 Δ m Φ mice to assess NK and CTL populations. Shown are scatter plots of the (a) % CD4+ T cells, (b) % CD8+ T cells, and (c) % NK cells of the total CD45+ population in the pancreata of control (black) and GLUT1 Δ m Φ (blue) mice (n = 14 mice per group, pooled from four independent experiments). Shown are scatter plots of (d) % IFN γ +, (e) % Perforin+, and (f) % Granzyme B+ of total NK cells in the pancreata of control (black) and GLUT1 Δ m Φ (blue) mice (n = 16 mice per group, pooled from three independent experiments). (g) NK cells were depleted *in vivo* by injecting 250 μ g of either isotype or anti-NK1.1 antibody i.p. per mouse, once a week. Shown are bar graphs of the tumor weight measured at Day 28 of control and GLUT1 Δ m Φ mice (n = 5 mice per group, representative of two independent experiments). (h-j) Shown are scatter plots of (h) % IFN γ +, (i) % Perforin+, and (j) % Granzyme B+ of total CD8+ T cells in the pancreata of control (black) and GLUT1 Δ m Φ (blue) mice (n = 16 mice per group, pooled from three independent experiments). (k) CD8+ T cells are depleted *in vivo* by injecting 500 μ g of either isotype or anti-antibody i.p. per mouse, once a week. Shown are bar graphs of the tumor weight measured at Day 27 of control and GLUT1 Δ m Φ mice (at least n = 5 mice per group). Data are means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with 95% confidence interval.

The GLUT1 inhibitor WZB117 attenuates tumor burden *in vivo*

Given that conditional knockout of GLUT1 in macrophages was successful in significantly reducing tumor burden and improving survival in mice, we decided to take this a step further looking into the therapeutic potential of GLUT1. Although GLUT1 has been shown to be the most widely expressed glucose transporter in many cancers¹¹⁷⁻¹²⁰, it is not until recently where a new generation of GLUT1 inhibitors have been synthesized to overcome its toxicity and specificity¹²¹. One inhibitor of interest is WZB117 [2-fluoro-6-(m-hydroxybenzoyloxy) phenyl m-hydroxybenzoate] which inhibits GLUT1-mediated passive sugar transport by binding reversibly at sugar binding sites thereby limiting glycolysis. We hypothesize that treatment with the WZB117 GLUT1 inhibitor might give us a similar reduction in tumor burden we see in GLUT1^{ΔmΦ} mice. To validate the potency of WZB117 to inhibit glycolysis, 2-week-old orthotopic tumors were treated with increasing concentration of WZB117 daily for seven days via i.p. injection. Seahorse extracellular flux assay was used to analyze the effectiveness of WZB117 in blocking glycolysis and a dose of 40 mg/kg WZB117 was found to be effective in blocking glycolysis in OT WT PEC macrophages (Fig. 11a). Doses of 50 mg/kg WZB117 were well tolerated in mice and thus was use in further WZB117 treatment experiments.

To determine the potential of WZB117 in improving disease outcome, WT OT mice were fed WZB117 at 250 parts per million (ppm) (equivalent to a 50 mg/kg daily dose) from day 1 orthotopic transplantation of 1242L tumor cells into the pancreas till the end of the experiment. At day 26 post-tumor transplantation and treatment, *in vivo* monitoring by BLI showed a significant reduction in tumor growth in WZB117 treated mice as compared with untreated control mice (Fig. 11b). Upon harvest at day 34, as compared to controls, OT mice treated with WZB117 showed a significant decrease in tumor burden (Fig. 11c) confirming our results in GLUT1^{ΔmΦ} mice. However, in contrast to GLUT1^{ΔmΦ} mice, macrophages in WZB117 treated mice displayed an

upregulation of IL-1 β , IL-12p40, and TNF α proinflammatory cytokines (Fig. 11d).

Nevertheless, similarly to GLUT1 $\Delta m\Phi$ mice, we observed an increased in the percentages of CTLs in WZB117 treated mice (Fig. 11e), with significant increase in their levels of IFN γ , perforin and granzyme B as compared to controls (Fig. 11f). In addition, the percentages of NK cells were also upregulated in WZB117 treated mice (Fig. 11g), with increased expression of IFN γ , perforin and granzyme B (Fig. 11h). Results suggest that WZB117 treatment is able to control tumor growth in mice in a similar fashion to GLUT1 $\Delta m\Phi$ mice, acting upon the anti-tumoral activity of CTLs and NK.

To further assess the potency of WZB117 treatment to the current standard-of-care chemotherapy for PDAC patients, we compared mice treated with WZB117 or gemcitabine and looked at their tumor outcomes. In a spontaneous model of PDAC, gemcitabine was previously shown to significantly increase the median survival in mice as compared with placebo controls¹²². Following authors' protocol, mice were treated with low-dose gemcitabine at 50 mg/kg/mouse via i.p. injection twice weekly or WZB117 from day 1 orthotopic transplantation of 1242L tumor cells into the pancreas till the end of the experiment. We observed a similar trend in tumor burden in both WZB117 and gemcitabine treated mice as compared to untreated controls (Fig. 11i). Taken together, results suggest the potential of GLUT1 inhibitors, in particular WZB117, as a potential therapeutic strategy to improve PDAC disease outcome.

Figure 11. The GLUT1 inhibitor WZB117 attenuates tumor burden *in vivo*. WZB117 were fed to mice beginning day 1 of orthotopic transplantation of tumor cells. Disease progression was monitored *in vivo*, and immune parameters assessed in the pancreatic tumors. **(a)** Mice bearing 2-week old orthotopic tumors were injected daily for 7 days i.p. with varying concentrations of WZB117. Shown is the Seahorse trace of a glycolysis stress test on *ex vivo* sorted PEC macrophages from these mice. Representative trace shown of two independent experiments. **(b)** Scatter plot of the total flux on day 26 of mice fed with standard rodent chow (untreated, UT – black) (n = 9) or WZB117-incorporated chow (magenta) (n = 10) as calculated from BLI images. **(c)** Mice were euthanized and shown is a scatter plot of their tumors weighed at day 34. Tumor images shown on right hand side. Scale bar = 1 cm. Shown are scatter plots of the percentages of **(d)** pro-IL1 β +, IL6+, IL12p40+, and TNF α + of total macrophages, **(e)** CD8+ of total CD45+ cells, **(f)** IFN γ +, Perforin+, and Granzyme B+ of CD8+ cells, **(g)** NK+ T of total CD45+ cells, **(h)** IFN γ +, Perforin+, and Granzyme B+ of NK+ T cells. Data representative of two independent experiments, with n = 9–10 mice per group. **(i)** WZB117 was compared with gemcitabine. Shown is a scatter plot of tumors from mice fed standard rodent chow (“UT” – black), WZB117-incorporated chow (magenta) or treated with gemcitabine (brown) weighed at day 28. Data representative of two independent experiments, n = 10 mice per group. Data are means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student’s t-test with 95% confidence interval.

The macrophage glycolytic signature predicts poor survival in human PDAC

To validate our observations *in vivo* translating them to human PDAC, we examined the expression of glycolytic markers on macrophages in formalin-fixed paraffin-embedded (FFPE) sections of PDAC patients from Stanford Medical Center, Singapore National Cancer Centre, and Singapore National Cancer Institute. PDAC patient sections were stained in a sequential IF staining protocol for CD68 (a macrophage marker), CD163 (a tumor-associated, or M2-associated marker), GLUT1, HK2, HIF-1 α (glycolytic markers), and DAPI (nuclear marker) (Fig. 12a).

Representative images of a matched normal section and patients at Stages I, II and IV are shown (Fig. 12b). The expressions of GLUT1, HK2 and/or HIF-1 α were quantified as a percentage of total CD68⁺ macrophages in a patient and referred to as a subset (e.g. CD68⁺GLUT1⁺ subset). Consistent with our mouse data, analysis in PDAC patients revealed that the glycolytic signature in macrophages is able to predict a poorer survival with two subsets correlating with disease outcome – CD68⁺GLUT1⁺ and CD68⁺GLUT1⁺HK2⁺HIF-1 α ⁺ (“4-marker⁺”) subsets. Notably, the percentages of GLUT1⁺ on CD68⁺ macrophages was more than 2-fold higher in PDAC patients (56.4%) compared with matched normal controls (26.6%) (Fig. 12c). The 4-marker⁺ subset showed a smaller, but significant difference between PDAC patients (30.7%) and matched normal controls (17.2%) (Fig. 12d). However, no correlation between the different disease stages was observed for both subsets (Fig. 12e, f).

Figure 12. The macrophage glycolytic signature is a predictor of poor survival in human PDAC. (a) Schematic of the sequential, multiplex, immunofluorescence staining protocol of myeloid markers (CD68 and CD163) and metabolic markers (GLUT1, HIF1α, and HK2) used to stain FFPE sections from multi-center patient cohorts. (b) Shown are representative images from matched normal and patients at different stages of PDAC, CD68 (magenta), GLUT1 (yellow), CD163 (aqua), HK2 (green), HIF1α (red), DAPI (blue). Scale bar = 100 μM. (c-h) The expression of GLUT1, HK2, and HIF1α on CD68+ mΦ was objectively quantified. CD68+ cells co-expressing each glycolytic marker (called “subsets”) were defined as a percentage of total CD68+ cells in individual patients. (c) Shown are pie charts of the mean of the % CD68+GLUT1+ subset, and (d) % CD68+ GLUT1+HK2+HIF1α+ in matched normal versus PDAC (collectively) as a representation of total CD68+ cells. (e) Shown are box plots of the mean ± s.d. of the % CD68+GLUT1+ subset, and (f) % CD68+ GLUT1+HK2+HIF1α+ across the different stages of disease compared with matched healthy controls. (g) The percentage composition of the CD68+GLUT1+ subset and (h) CD68+ GLUT1+HK2+HIF1α+ subset were thresholded at various percentiles and the binary states (patients above the cut-off percentile was defined as “high”, and those below defined as “low”) were used in a log-rank survival analysis. Shown are Kaplan-Meier survival curves for the two subsets in which the percentile cut-offs yielded significant difference between the high and low patient populations.

Our PDAC patients were ranked based on their expression of CD68⁺GLUT1⁺ and CD68⁺GLUT1⁺HK2⁺HIF-1 α ⁺ from low to high, thresholded at various percentiles and the binary states (“low” and “high” based on percentile cut-offs) for each subset were used to perform a log-rank survival analysis. We observed that the CD68⁺GLUT1^{high} (70th percentile) and CD68⁺GLUT1^{high} HK2^{high}HIF-1 α ^{high} (60th percentile) exhibited a significantly poorer survival as compared to their counterparts (Fig. 12g, h). Taken together results show that PDAC patients exhibited a highly glycolytic profile as compared to matched normal controls validating our findings in the mouse model. Furthermore, results in human PDAC revealed that the glycolytic signature of macrophages is able to predict survival in PDAC.

Collectively, our *in vivo* and PDAC patient cohort data suggest that targeting the metabolic signature in macrophages may serve as a potential avenue to control PDAC progression and tumor resistance.

3.3 Conditional knockout of HIF-1 α in tumor-infiltrating neutrophils conveys protection against pancreatic ductal adenocarcinoma

Pro-tumoral neutrophils infiltrate the pancreas of tumor-bearing mice and their targeted depletion improves tumor outcome

To generate an orthotopic model of PDAC, 1242 tumor cells were transfected to overexpress luciferase and transplanted into the pancreas of WT mice. Flow cytometry analysis revealed a significant increase of Ly6G⁺ neutrophils of orthotopic transplanted (OT) mice both in the blood (51.1% of total CD45⁺ cells compared with 6.4% in sham non-tumor bearing controls) and pancreas (25.1% of total CD45⁺ cells compared with 1.3% in sham non-tumor bearing controls) (Fig. 13a, b). Further, immunofluorescent (IF) staining showed that a large number of neutrophils infiltrated the pancreas of OT mice compared with sham controls (Fig. 13c).

To determine the contribution of neutrophils in promoting tumor progression, we assessed the infiltration of neutrophils into the pancreas and showed that BM and blood neutrophils expressed higher levels of C-X-C motif chemokine receptor 2 (CXCR2) as compared to pancreatic neutrophils (Fig. 13d). CXCR2 is expressed by neutrophils and mediates their migration to sites of inflammation. We hypothesize neutrophils in the blood upregulated CXCR2 for homing into the pancreas. Thus, we utilized a CXCR2^{-/-} mouse model, where mice exhibit impaired neutrophil recruitment into the pancreas (Fig. 13e), and generated orthotopic PDAC tumors in these CXCR2^{-/-} mice. Interestingly, CXCR2^{-/-} OT mice displayed a significant reduction of tumor burden at all time points (Fig. 13f) and showed a strikingly improved overall survival as compared with controls (Fig. 13g). We next considered the possible therapeutic potential of depleting neutrophils *in vivo* using α -Ly6G (1A8) antibody. Pancreatic neutrophils were depleted up till day 21-post transplantation, but then repopulated by day 28 (Fig. 13h). Similarly to

the setting of CXCR2^{-/-} mice, neutrophil depletion via α -Ly6G antibody resulted in a significant reduction in tumor burden at all time points measured post tumor transplantation compared with untreated controls (Fig. 13i).

However, in contrast to OT CXCR2^{-/-} mice, regressed tumors progressed at day 35-post transplantation (Fig. 13i). As neutrophils steadily infiltrate the tumor in increasing numbers, we hypothesize that neutrophil depletion via α -Ly6G antibody was insufficient to completely ablate neutrophil infiltration into the tumor. This likely resulted in the escape from tumor control. Despite a delay in tumor progression between the neutrophil-depleted mice and control mice, overall survivals of both groups of mice (treated and untreated) remain unchanged (Fig. 13j). These results strongly suggest that neutrophils are pro-tumoral as the absence of neutrophils in the tumor enhanced survival in PDAC.

a Blood (Singlets⁺Dapi⁻CD45⁺Lin⁻)

b Pancreas (Singlets⁺Dapi⁻CD45⁺Lin⁻MHCII^{lo})

c

d

Figure 13. Pro-tumoral neutrophils infiltrate the pancreas of tumor-bearing mice and their persistent depletion improves tumor outcomes. (a) Pancreatic tumors were harvested from day 28 tumor-bearing OT mice and the composition of the myeloid cell populations was assessed by flow cytometry. Scatter plot of neutrophils [Dapi-CD45⁺Lin(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺] that infiltrate the blood and (b) pancreas were plotted as a percentage of total CD45⁺ cells, from OT (open circles) and age-matched sham non-tumor bearing controls (black circles). OT n = 15-17, sham n = 10; pooled from three independent experiments. (c) OT and control mice pancreata sections were stained for Dapi and Ly6G expression. Representative immunofluorescence images of n = 3 experiments for each group. Scale bar = 100 μ M. (d) Representative flow cytometry histogram showing CXCR2 expression in the bone marrow, blood and pancreas of OT mice. (e-g) Orthotopic tumors were generated in CXCR2^{-/-} (blue circles) or control (black circles) mice. (e) Neutrophil infiltration into the pancreas was monitored at 6, 21 and 28 days post transplantation (CXCR2^{-/-} n = 4, controls n = 7 per experiment for each time point). (f) Scatter plot of orthotopic tumor weights at 6, 14, 21, 28 and 35 days post transplantation (n = 3 mice per experiment for each time point). (g) Kaplan-Meier survival analysis of CXCR2^{-/-} OT mice compared with control OT mice (n = 10). (h-j) OT mice were treated with α Ly-6G antibody (green) or isotype control antibody (black) via intraperitoneal (i.p.) injection thrice per week. (h) Neutrophil infiltration into the pancreas (plotted as a percentage of total CD45⁺ cells) was monitored at 6, 21 and 28 days post transplantation (n = 3-5 mice per experiment for each time point). (i) Scatter plot of orthotopic tumor weights at 6, 14, 21, 28 and 35 days post transplantation (n = 3 mice per experiment for each time point). (j) Kaplan-Meier survival analysis of α Ly-6G treated mice compared with isotype control treated mice (n = 10). Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Pro-tumoral neutrophils exhibit a highly glycolytic signature and are adapted to a hypoxic tumor microenvironment

While depletion of neutrophils delayed the kinetics of tumor growth, it remains unclear how tumor-infiltrating neutrophils contribute to PDAC progression. Studies have shown that tumor-infiltrating neutrophils are significantly different from circulating blood^{123,124}. To further address this, we sorted Dapi⁺CD45⁺Lin(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺ neutrophils from the bone marrow (BM), blood and pancreatic tumor of OT mice and performed transcriptomic analysis in an attempt to decipher the molecular signature of mature neutrophils in these 3 anatomical compartments. Principal component analysis of the transcriptomic data showed differentially expressed transcripts of tumor neutrophils vs both BM and blood neutrophils (Fig. 14a). Data suggest that the tumor microenvironment may have primed tumor-infiltrating neutrophils to display distinct gene signatures as compared to their counterparts. To further understand the biology behind the gene expression differences within the three subsets of neutrophils, we plotted a heatmap clustering of the differentially expressed genes and identified 4 distinct clusters of genes (Fig. 14b).

Notably, genes in cluster 1 were downregulated in tumor neutrophils, and comprised of genes involved in protein transport (GO:0015031), intracellular protein transport (GO:0006886), protein targeting to vacuole (GO:0006623), nucleophagy (GO:0044804), and the adaptive immune response (GO:0002250) (Fig. 14c). In contrast, genes in cluster 4 were upregulated in tumor neutrophils, and consisted of genes involved in the positive regulation of cell division (GO:0051781), chemokine-mediated signaling pathway (GO:0070098), one-carbon metabolic process (GO:0006730), positive regulation of angiogenesis (GO:0045766), and positive regulation of cellular metabolic process (GO:0031325) (Fig. 14c).

Figure 14. Pro-tumoral neutrophils exhibit a highly glycolytic signature and are adapted to a hypoxic tumor microenvironment. (a-e) Bone marrow (BM), blood and tumor neutrophils were sorted based on their expression of Dapi⁺CD45⁺Lin⁺(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺ (orthotopic transplanted [OT] n = 5 mice pooled, three independent experiments). Hierarchical clustering and principal component analysis (PCA) were performed with log₂-transformed values. (a) PCA plots of transcriptomic data of BM, blood and tumor neutrophils respectively. (b) Heat map of the differentially expressed genes selected with Benjamini-Hochberg adjusted p-values <0.05. Four gene clusters (1 to 4) were manually defined and exported for gene ontology (GO) biological process analysis. (c) GO biological process terms enriched in clusters 1 (left) and 4 (right). (d, e) Expression of genes with row/gene-based z-score values (log₂ FPKM) of cluster 4-associated genes across all sorted populations encoding (d) GO: 0006096 – Glycolytic Process and (e) GO: 0071456 – Cellular response to hypoxia. (f-g) Representative immunofluorescence staining of pancreas sections from OT and sham mice stained with Dapi (blue), S100A9 (red) neutrophil marker, and HIF-1 α (green) and GLUT1 (cyan) glycolytic markers. Immunofluorescence images shown are representative images of n = 3 mice, and 10-15 fields of view. Scale bar = 100 μ m. (h) Scatter plots represent the percentage of GLUT1 expressing neutrophils in the tumor and (i) the mean fluorescence intensity (MFI) of HIF-1 α ⁺ neutrophils staining in the nucleus and cytoplasm.

Having established that the tumor-infiltrating neutrophils showed differential transcriptome gene expression as compared to their counterparts, we focused specifically on the groups of genes that were upregulated in tumor neutrophils, and seek to understand how the upregulation of these genes could translate to the biological function of these neutrophils. Interestingly, we observed an upregulation of genes involved with several cellular metabolic processes. This suggests a change in the metabolic signature of neutrophils that infiltrated the tumor. Of note, the expression of genes related to glycolytic process (GO: 0006096) were up-regulated in tumor neutrophils, despite the fact that neutrophils are highly glycolytic by nature⁹⁹ (Fig. 14d). As the tumor microenvironment is hypoxic, we also examined genes related to the cellular response to hypoxia (GO: 0071456) and observed a similar induction of hypoxia related genes in tumor neutrophils as compared to BM and blood neutrophils (Fig. 14e). In addition, the HIF-1 α gene transcript itself was found to be up-regulated in tumor-infiltrating neutrophils (Fig. 14e). Such distinct metabolic signature leads us to hypothesize that tumor-infiltrating neutrophils exhibit a pronounced glycolytic profile that may be contributing to their overall pro-tumoral effect in the progression of PDAC.

To confirm that tumor neutrophils exhibit a pronounced glycolytic signature at the protein level, we assessed the presence of glycolytic markers GLUT1, a glucose transporter and HIF-1 α , a transcriptional factor of glycolysis using immunofluorescence (Fig. 14f, g). As expected, little to no neutrophil infiltration was observed in the sham non-tumor bearing mice controls (Fig. 14g). Notably, neutrophils from OT mice exhibited a profound glycolytic signature with 94% of GLUT1⁺ neutrophils in the tumor (Fig 14h). In addition, high expression of activated HIF-1 α was observed in the nucleus as compared to the cytoplasm (Fig. 14i). Taken together, we show that tumor-infiltrating neutrophils from pancreatic OT mice exhibit a strong glycolytic profile with upregulated GLUT1 and HIF-1 α as compared to non-tumor-infiltrating neutrophils found in the BM and the blood.

No reduction in glycolysis and change in tumor growth in neutrophil-specific deletion of GLUT1

To address the role of neutrophil metabolism in disease outcome, we crossed GLUT1^{fl/fl} mice with MRP8^{Cre} mice to generate mice with GLUT1 specifically deleted in neutrophils (GLUT1^{ΔNΦ}). We performed orthotopic transplantation of 1242L tumor cells into the pancreas of GLUT1^{ΔNΦ} mice and observed for tumor progression. Surprisingly, tumor burden in OT GLUT1^{ΔNΦ} mice were comparable to controls (day 29 showed in Fig. 15a, b) and exhibited a similar survival curves as compared to controls (Fig. 15c). We next performed a live Seahorse metabolic assay to assess the glycolytic profile of the knockout neutrophils. Surprising, extracellular flux analysis showed that GLUT1^{ΔNΦ} neutrophils were still able to undergo glycolysis (Fig. 15d). Taken together, data indicates that deletion of GLUT1 alone in neutrophils could not inhibit glycolysis in neutrophils. This suggests that unlike in macrophages (Fig. 8a), GLUT1 is dispensable in neutrophils probably due to the expression of other GLUT family members such as GLUT3 and GLUT8 (online database, Biogps). Given the unfavorable tumor outlook in GLUT1^{ΔNΦ} mice, we next elucidated how neutrophils exert its effector functions contributing to their pro-tumoral phenotype.

Figure 15. No reduction in glycolysis and change in tumor growth in neutrophil-specific deletion of GLUT1. (a) Representative images of GLUT1 $\Delta N\phi$ OT and control OT mice tumors. (b) Tumors were harvested on day 29 post transplantation and weighed. Scatter plot of orthotopic tumor weights from GLUT1 $\Delta N\phi$ OT (blue clear) and age-matched OT controls (black clear) (GLUT1 $\Delta N\phi$ n = 5, controls n = 5). (c) Sorted neutrophils from GLUT1 $\Delta N\phi$ OT and control OT mice were rested at 37°C for 1 h and assessed for their glycolytic capacity by live metabolic flux assay. A representative Seahorse extracellular acidification rate (ECAR) trace of GLUT1 $\Delta N\phi$ OT neutrophils (blue) compared with control OT neutrophils (black) is shown (left). The glycolytic capacity (right) was calculated from the Seahorse ECAR trace (n = 3-5 mice pooled per group, three independent experiments). (d) Kaplan-Meier survival analysis of GLUT1 $\Delta N\phi$ OT mice (n = 5) compared with littermate GLUT1 $^{fl/fl}$ control OT mice (n = 5). Data represent the means $\pm$ SEM, *p < 0.05; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Neutrophil-specific deletion of transcriptional factor HIF-1 α significantly attenuates tumor burden

HIF-1 α has been showed to regulate the glycolytic capacity and direct the downstream effector functions of immune cells especially in hypoxic environments. To address the role of neutrophils metabolism in directing disease outcome in PDAC, we bred HIF-1 $\alpha^{fl/fl}$ mice with MRP8^{cre} mice, to generate mice with a neutrophil-specific deletion of HIF-1 α (HIF-1 $\alpha^{\Delta N\phi}$). The percentages of infiltrating HIF-1 $\alpha^{\Delta N\phi}$ neutrophils were comparable to control neutrophils (21.5% of total CD45+ cells compared with 26.3% in controls) (Fig. 16a). Notably, no significant changes in gene expression were observed in CCL2 and CCL3, which regulate neutrophil recruitment, between HIF-1 $\alpha^{\Delta N\phi}$ and WT neutrophils (Fig. 16b). These findings support the observation of similar percentages of neutrophils infiltrating the pancreas between HIF-1 $\alpha^{\Delta N\phi}$ and littermate control mice indicating that cell viability and recruitment to the tumor was not affected by loss of HIF-1 α . To prove that HIF-1 α was indeed ablated, we performed a functional glycostress Seahorse assay. Indeed, HIF-1 $\alpha^{\Delta N\phi}$ mice showed a reduction in glycolytic capacity (Fig. 16c). Furthermore, transcript expression of *HIF-1 α* and key glycolytic enzymes *ALDOA*, *LDHA*, *GLUT1*, *HK1* and *HK2* indicated that HIF-1 $\alpha^{\Delta N\phi}$ mice down-regulated the capacity for glycolysis, validating this genetic ablation strategy (Fig 16d).

BLI showed that transplanted HIF-1 $\alpha^{\Delta N\phi}$ mice with OT tumors resulted in a pronounced reduction in tumor burden across all time points as compared to littermate controls (day 27 post-tumor transplantation time point shown in Fig. 16e). When euthanized at day 29-post tumor transplantation, a reduction in tumor weight was confirmed in HIF-1 $\alpha^{\Delta N\phi}$ mice compared with controls (Fig. 16f, g). Moreover, the dramatic delay in tumor progression was also proven by the significantly improved overall survival compared with controls (Fig. 16h).

Figure 16. Neutrophil-specific deletion of transcriptional factor HIF-1 α significantly attenuates tumor burden. (a) Pancreatic tumors from tumor-bearing HIF-1 $\alpha^{\Delta N\Delta}$ orthotopic transplanted (OT) and littermate HIF-1 $\alpha^{fl/fl}$ control OT mice were harvested at day 28 post transplantation and the myeloid cell populations were assessed by flow cytometry. The scatter plot of infiltrating neutrophils [Dapi⁺CD45⁺Lin[−](CD3/B220/NK1.1)[−]Ly6G⁺Ly6C⁺] are plotted as a percentage of total CD45⁺ cells, from HIF-1 $\alpha^{\Delta N\Delta}$ OT mice (red clear) and littermate HIF-1 $\alpha^{fl/fl}$ controls (black clear) (OT n = 23, sham n = 14; of three independent experiments, pooled). (b) Pancreatic neutrophils were sorted from HIF-1 $\alpha^{\Delta N\Delta}$ mice and littermate controls and gene expression of *CCL2* and *CCL3* was assessed by quantitative PCR (n = 3 mice, three independent experiments). (c) Sorted neutrophils from HIF-1 $\alpha^{\Delta N\Delta}$ OT and control OT mice were rested at 37°C for 1 h and assessed for their glycolytic capacity by live metabolic flux assay. A representative Seahorse extracellular acidification rate (ECAR) trace of HIF-1 $\alpha^{\Delta N\Delta}$ OT neutrophils (red) compared with control OT neutrophils (black) is shown (left). The glycolytic capacity (right) was calculated from the Seahorse ECAR trace (n = 3-5 mice pooled per group, three independent experiments). (d) HIF-1 $\alpha^{\Delta N\Delta}$ orthotopic transplanted and littermate HIF-1 $\alpha^{fl/fl}$ control mice pancreatic neutrophils were assessed at the transcript level for glycolytic markers, *HIF-1 α* , *ALDOA*, *LDHA*, *GLUT1*, *HK1* and *HK2* (n = 3 mice per group, three independent experiments). (e). The total flux (right) on day 27 post transplantation was calculated from the BLI image (control, n = 25; HIF-1 $\alpha^{\Delta N\Delta}$, n = 34, three independent experiments pooled). BLI images were acquired at a 15-s exposure, and the color scale was set to a lower limit of 3×10^6 and an upper limit of 5×10^7 photons/sec. (f) Representative images of HIF-1 $\alpha^{\Delta N\Delta}$ OT and control OT mice tumors. (g) Tumors were harvested on day 27 post transplantation and weighed. Scatter plot of orthotopic tumor weights from HIF-1 $\alpha^{\Delta N\Delta}$ OT (red clear) and age-matched OT controls (black clear) (HIF-1 $\alpha^{\Delta N\Delta}$ n = 24, controls n = 17; of four independent experiments pooled). (h) Kaplan-Meier survival analysis of HIF-1 $\alpha^{\Delta N\Delta}$ OT mice (n = 25) compared with littermate HIF-1 $\alpha^{fl/fl}$ control OT mice (n = 12). Data represent the means $\pm$ SEM, *p < 0.05; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Collectively, this data indicates that the neutrophil-specific knockout of HIF-1 α resulting in knockdown of neutrophil glycolysis is able to modulate downstream effector immunity of these cells against the tumor. Given the clear indication that HIF-1 α in neutrophils accelerates the kinetics of tumor progression, we further elucidated how HIF-1 $\alpha^{\Delta N\phi}$ neutrophils contribute to the anti-tumoral immunity.

HIF-1 $\alpha^{\Delta N\phi}$ neutrophils exert direct anti-tumoral effects

ROS has been shown to play a role in anti-tumoral immunity¹²⁵, and one of the main effector functions of neutrophils is oxidative burst¹²⁶. Therefore, we hypothesized that the reduction in tumor burden observed in HIF-1 $\alpha^{\Delta N\phi}$ mice may be, in part, due to increased ROS production by HIF-1 α deficient neutrophils. Indeed, we observed a greater abundance of intracellular ROS expression in tumor-infiltrating HIF-1 α -deficient neutrophils, compared to HIF-1 α -sufficient neutrophils (Fig. 17a). To determine if the higher ROS production correlate with their cytotoxic ability, we performed a cell cytotoxic assay in which we incubate neutrophils (effectors) isolated from pancreas of OT HIF-1 $\alpha^{\Delta N\phi}$ mice and littermate controls, with KPC tumor cells (targets). Remarkably, we observed a significant increase in the cytolytic activity of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils at the 10:1 E:T ratio at all time points (Fig. 17b). Pre-treatment of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils with N -acetyl-L-cysteine, a ROS inhibitor before the cytotoxic assay demonstrated that the anti-tumoral effect was indeed mediated through ROS production (Fig. 17c).

Taken together, these data suggest that HIF-1 $\alpha^{\Delta N\phi}$ neutrophils may elicit direct tumoricidal activity via ROS production, thus improving the overall survival of HIF-1 $\alpha^{\Delta N\phi}$ tumor bearing mice.

Figure 17. HIF-1 $\alpha^{\Delta N\Phi}$ neutrophils exert direct anti-tumoral effects. (a) Scatter plot of reactive oxygen species (ROS) production (MFI) in HIF-1 $\alpha^{\Delta N\Phi}$ orthotopic transplanted (OT) and control OT pancreatic neutrophils (HIF-1 $\alpha^{\Delta N\Phi}$ n = 12 mice, littermate HIF-1 $\alpha^{fl/fl}$ controls n = 10; three independent experiments). (b) Neutrophils were sorted based on their expression of Dapi-CD45⁺Lin⁻(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺. Scatter plot shown indicates the cytotoxic capacity of HIF-1 $\alpha^{\Delta N\Phi}$ neutrophils at a 10:1 effector:target (E:T) cell ratio after 2, 4 and 6 h incubation with 1242L tumor cells (n = 5 mice / group; n = 3 replicates). (c) HIF-1 $\alpha^{\Delta N\Phi}$ neutrophils were assessed for their cytotoxic capacity via ROS production. Neutrophils were treated with 5 mM N-acetyl-L-cysteine for 30 min at 37°C. Scatter plot shown indicates the cytotoxic capacity of HIF-1 $\alpha^{\Delta N\Phi}$ neutrophils at a 10:1 E:T ratio after 4 h incubation with 1242L tumor cells (n = 5 mice / group; n = 3 replicates). Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with 95% confidence interval.

HIF-1 $\alpha^{\Delta N\phi}$ neutrophils activate the adaptive immune response

In line with the anti-tumoral effect observed in HIF-1 $\alpha^{\Delta N\phi}$ mice, our transcriptomic analysis revealed a down-regulation of the adaptive immune signaling pathways (GO:0002250) in tumor neutrophils (Fig. 14c). This could be due to tumor neutrophils being more immunosuppressive. Indeed, we observed that the expression of Arg-1 was significantly up-regulated in tumor compared to blood and bone marrow neutrophils (Fig. 18a). Arg-1 is a well-known immunosuppressor of the immune system, and a critical regulator of both the innate and adaptive immunity by mediating cytotoxic T-cell immune responses¹²⁷. In HIF-1 $\alpha^{\Delta N\phi}$ mice, transcript analysis further revealed that Arg-1 was down-regulated in neutrophils as compared to littermate controls (Fig. 18b). This down-regulation of Arg-1 may also explain the increase in anti-tumoral activity observed in HIF-1 $\alpha^{\Delta N\phi}$ neutrophils by mediating T-cell cytotoxicity. Taken together, data suggest that HIF-1 $\alpha^{\Delta N\phi}$ neutrophils may possess anti-tumoral effects via the production of ROS and down-regulation of Arg-1 resulting in the improved overall survival of HIF-1 $\alpha^{\Delta N\phi}$ mice compared to controls.

We next assessed a second mechanism of action involving the cytokine production of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils known to activate T cell responses. Transcript analysis revealed an upregulation of IL-12 and CCL5 in HIF-1 $\alpha^{\Delta N\phi}$ neutrophils as compared to controls (Fig. 18c). Consistent with the gene expression data, HIF-1 $\alpha^{\Delta N\phi}$ neutrophils significantly upregulated expression of IL-12 (Fig. 18d) and TNF- α (Fig. 18e) cytokines compared to controls, as assessed by flow cytometry.

Taken together, our data suggest that HIF-1 α in neutrophils may polarize these cells towards a more pro-tumoral immunosuppressive phenotype. HIF-1 α -specific ablation activates the neutrophils to adopt an anti-tumoral phenotype. In addition, we hypothesized that the increased production of cytokines such as IL-12 and TNF- α , may promote the activation of the anti-tumoral effector functions by cytotoxic CD8 T cells

and NK cells. These findings led us to characterize the lymphoid populations in these mice.

Anti-tumoral immunity in HIF-1 $\alpha^{\Delta N\phi}$ OT mice is mediated by CD8 $^+$ T cells and NK cells

Following the increased production of IL-12 and TNF- α by HIF-1 $\alpha^{\Delta N\phi}$ neutrophils in the tumor environment, we next proposed that the anti-tumoral activity observed might also involve changes in lymphocyte responses. We assessed the lymphocyte infiltrate in the tumor and observed a significant increase in the percentage of CTLs (5.2% of total CD45 $^+$ cells compared with 3.3% in controls) (Fig. 19a) and NK cells (3.7% of total CD45 $^+$ cells compared with 1.4% in controls) (Fig. 19b). Further elucidating the activation of anti-tumoral phenotype of these cells revealed significant elevated levels of perforin (Fig. 19c), granzyme B (Fig. 19d) and IFN γ (Fig. 19e) production in HIF-1 $\alpha^{\Delta N\phi}$ mice as compared to controls.

Taken together, we show that on top of the anti-tumoral phenotype of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils, protection from tumor progression is mediated in part by the tumoricidal activity of NK and CTLs. Our results strongly indicate that tumor-infiltrating HIF-1 α^+ neutrophils is dependent on a metabolic commitment to high rates of glycolysis to fulfill their functional requirements. We show for the first time that HIF-1 α expression in neutrophils during the progression of PDAC is detrimental to the host and HIF-1 α ablation can subvert their pro-tumoral capacity, leading to protection against the disease.

Figure 18. HIF-1 $\alpha^{\Delta N\Phi}$ neutrophils activate the adaptive immune response. (a) Bone marrow (BM), blood and tumor neutrophils were sorted based on their expression of Dapi·CD45⁺Lin(CD3/B220/NK1.1)⁻ Ly6G⁺Ly6C⁺ (orthotopic transplanted [OT] n = 5 mice pooled, three independent experiments). Gene analysis of *Arg1* was analyzed with log₂-transformed values (log₂RPKM). Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by one-way ANOVA, Tukey's multiple comparisons test with a 95% confidence interval. (b) Pancreatic neutrophils were sorted from HIF-1 $\alpha^{\Delta N\Phi}$ and control mice, and gene expression of *Arg1*, (c) *IL-12* and *CCL5* was assessed by quantitative PCR (n = 3 mice, three independent experiments). (d) Representative flow cytometry plots from HIF-1 $\alpha^{\Delta N\Phi}$ and control neutrophils. Neutrophils were stimulated with 100 ng/ml LPS for 4 h and assessed for IL-12 and (e) TNF- α production by flow cytometry. Scatter plots indicate the percentage of total Ly6G⁺ cells, from HIF-1 $\alpha^{\Delta N\Phi}$ OT (red clear) and age-matched OT controls (black clear) (HIF-1 $\alpha^{\Delta N\Phi}$ n = 12, controls n = 10; of two independent experiments pooled). Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with 95% confidence interval.

Figure 19. Anti-tumoral effector responses are supported by CD8+ T cells and NK cells activation and cytotoxicity. (a-e) Flow cytometry was performed in HIF-1 $\alpha^{\Delta N\phi}$ orthotopic transplanted (OT) and littermate HIF-1 $\alpha^{\Delta N\phi}$ control mice to assess cytotoxic T lymphocytes (CTL) and natural killer (NK) cell populations. Shown are scatter plots of (a) the % CD3+CD8+ T cells, and (b) % NK cells of the total CD45+ population in the pancreata of HIF-1 $\alpha^{\Delta N\phi}$ OT (red clear) and control OT (black clear) mice (HIF-1 $\alpha^{\Delta N\phi}$ n = 22, control n = 14 mice per group, pooled from three independent experiments). CTL and NK cells were stimulated with 10 ng/ml PMA and 1 μ M ionomycin for 4 h. Scatter plots indicate the percentage (c) Perforin+, (d) Granzyme B+, and (e) IFN- γ + cells of total CTL and NK cells in the pancreata of HIF-1 $\alpha^{\Delta N\phi}$ OT (red clear) and control OT (black clear) mice (HIF-1 $\alpha^{\Delta N\phi}$ n = 22, control n = 14 mice per group, pooled from three independent experiments). Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

HIF-1 α^{hi} , glycolytic marker^{hi} neutrophils found in PDAC patients mirror results from the orthotopic mouse model

To determine if our findings in the orthotopic gene knockout models of PDAC is also observed in human PDAC, we assessed the expression of HIF-1 α and glycolytic markers such as GLUT1 and HK2 in neutrophils using FFPE tumor sections from PDAC patients and adjacent matched normal from three different hospitals using a sequential, multiplex staining protocol (Fig. 20a) for six markers: CD15 (neutrophil marker), cytokeratin (CK; tumor marker), HIF-1 α , GLUT1 and HK2 (glycolytic markers), and DAPI (nuclear marker).

Figure 20b are representative multiplex stained images of matched normal and tumor regions from a PDAC patient. We quantified the expression of glycolytic markers, HIF-1 α , GLUT1 and HK2 as a percentage of total CD15⁺CK⁻ cells in each patient. Analysis were performed to determine CD15⁺ cells either co-expressing one or more of the glycolytic marker (e.g. CD15⁺ HIF-1 α^+ subset).

Similar to our mouse *in vivo* data, we observed a significant 6.2-fold increase in infiltrating CD15⁺ neutrophils in patients with PDAC (9.44%) compared with adjacent matched normal tissue sections (1.52%) (Fig. 20c). Given the up-regulation of glycolytic and hypoxia genes involved in our mouse model of PDAC, we sought to determine whether CD15⁺ neutrophils in our PDAC patients co-expressed HIF-1 α^+ GLUT1⁺HK2⁺ hypoxia and glycolytic markers. PDAC tumors showed a significant increase in the proportion of HIF-1 α^+ GLUT1⁺HK2⁺ neutrophils (PDAC patients 19.26% vs matched normal controls 6.31% (Fig. 20d). To determine if co-expression of two markers would be enough to differentiate PDAC patients from matched normal controls, we assessed the proportion of neutrophils that were double positive for glycolytic markers. Only HIF-1 α^+ GLUT1⁺ neutrophils showed a significant increase in the proportion of in PDAC tumor tissue (23.21%) vs adjacent matched normal tissue (8.17%) while the proportion of HIF-1 α^+ HK2⁺ and GLUT1⁺HK2⁺ neutrophils were similar (Fig. 20e). Surprisingly, HIF-1 α

expression alone in neutrophils was apparently sufficient to distinguish between PDAC and adjacent normal (88.97% in tumor vs 64.79% in adjacent normal) (Fig. 20f). As nuclear translocation of HIF-1 α indicates activation, we also analyzed HIF-1 α cellular localization in CD15⁺HIF-1 α ⁺ neutrophils. Here, we observed a higher proportion of CD15⁺ neutrophils with nuclear HIF-1 α ⁺ expression in PDAC patients compared with adjacent matched normal tissue and no difference in the proportion of CD15⁺ neutrophils with cytoplasmic HIF-1 α ⁺ expression (Fig. 20g). These data suggest that HIF-1a is activated in a larger proportion of neutrophils in PDAC patients compared with adjacent matched normal tissue controls. The percentage of GLUT1⁺ neutrophils was also increased in PDAC patients (24.3%) compared with adjacent matched normal tissue (14.72%), but did not reach statistical significance (Fig. 16f).

Finally, there was no difference in the percentage of neutrophils expressing the glycolytic marker HK2 between PDAC and adjacent matched normal tissue (Fig. 20f). In conclusion, data showed that expression of HIF-1 α ⁺ alone on neutrophils was able to differentiate to a higher degree between PDAC tumor tissue and adjacent matched normal control.

Taken together, both the human PDAC results and the orthotopic mouse model showed that tumor-infiltrating neutrophils are highly glycolytic and that play a role in driving the progression of PDAC.

b In situ staining of tumor - Opal IF

Figure 20. HIF-1 α ^{hi}, glycolytic marker^{hi} neutrophils found in PDAC patients mirror results from the orthotopic mouse model. (a) A sequential, multiplex, immunofluorescence staining panel consisting of a neutrophil marker (CD15), pancreatic epithelial cell marker (CK), and metabolic markers (HIF-1 α , GLUT1 and HK2) was used to stain formalin-fixed paraffin-embedded pancreatic sections obtained from multi-center patient cohorts. (b) Images are representative of matched normal adjacent tissue (n = 13) and PDAC patient tumor tissue (n = 42). Dapi (blue), CK (cyan), CD15 (red), HIF-1 α (white), HK2 (magenta), GLUT1 (orange). Scale bar = 100 μ M. (c) Scatter plot showing the total influx of neutrophils in PDAC patient sections (blue clear) compared to matched normal controls (black clear) as a % of total Dapi+ cells. (d-f) The expression of multiple metabolic markers was quantified using Inform Software and assessed in PDAC patient tissue compared to adjacent matched normal control tissue. (d) Scatter plots represent the expression of triple positive HIF-1 α +GLUT1+HK2+ subset as a percentage of total CD15⁺ neutrophils. (e) Scatter plots indicate the proportions of double positive HIF-1 α +GLUT1+, HIF-1 α +HK2+ and GLUT1+HK2+ neutrophils. (f) Scatter plots indicate the proportions of HIF-1 α +, GLUT1+ and HK2+ cells as a percentage of total CD15⁺ neutrophils. (g) Activation and translocation of HIF-1 α was assessed based on HIF-1 α expression in the nucleus. Non-activated HIF-1 α expression was assessed in the cytoplasm. Data represent the means $\pm$ SEM, *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired student's t-test with a 95% confidence interval.

Over the past decade, conceptual leaps in our understanding and progress in the field of immuno-oncology has led to the emergence of an additional two hallmarks of cancer, metabolic reprogramming and the evasion of immune destruction¹⁰⁸. It has become widely appreciated that tumor cells undergo a pronounced metabolic switch having the preferential and exclusively use of glycolysis even under aerobic conditions, also known as the Warburg effect¹⁰⁹. This phenomenon gives them a survival advantage to fulfill their demands for energy production driving their rapid proliferation. As focus has always been on the metabolism of tumor cells, little is known about the metabolic pathways utilized by immune cells in the tumor microenvironment, and much less whether they exhibit similar metabolic profiles as tumor cells. However recently, others and we have begun to explore the role of immunometabolism in diseases revealing the significance of this previously unexplored field^{35,66,67,128-131}.

The tumor environment is a cellular environment made up of a heterogeneous population of cells including immune cells, fibroblasts and blood vessels. Myeloid cells are effector cells recruited to the tumor site in the early events of tumorigenesis and have emerged as key players in stimulating cancer initiation, malignant progression and dissemination¹³². There is a growing body of evidence not only showing that myeloid cells constitute a significant niche in the tumor microenvironment, these immune cells are capable of employing a specific metabolic profile required for their downstream effector functions in various types of tumors including breast and lung cancer^{71,72,133}. However, whether tumor-infiltrating myeloid cells exhibit a metabolic signature similar to that of tumor cells, and how this affects pancreatic tumor dissemination and progression remain largely unknown. Here we show for the first time in the context of pancreatic cancer, that both human and mouse myeloid cells similarly utilize the Warburg effect to promote tumor growth, progression and metastasis.

We demonstrate that blocking specific genes in the glycolytic pathway in each of the myeloid cell types has significant therapeutic effect.

Using *in vitro* tumor-conditioned media to differentiate human blood-derived monocytes to macrophages, our data showed that these *in vitro*-conditioned macrophages exhibit a pro-metastatic phenotype, have the capacity to promote angiogenesis, EMT and extravasation facilitating tumor dissemination³⁵. In parallel, we also observed that these macrophages showed elevated rate of glycolysis, which is a characteristic of the Warburg effect. Notably, inhibiting glycolysis in these *in vitro*-conditioned macrophages using 2-DG resulted in the ablation of their pro-metastatic phenotype, reversing all three angiogenesis, extravasation, and EMT capabilities. The finding is summarized and illustrated in Figure 21.

Also illustrated in Figure 22, we showed that similarly to our *in vitro*-tumor conditioned macrophages data; macrophages from PDAC mice are also highly glycolytic. Metabolomics analysis of macrophages deficient in GLUT1 (GLUT1^{ΔmΦ}) revealed that these macrophages were unable to undergo glycolysis. They reprogramed to utilize fatty acid oxidation instead to fuel their energy needs (preliminary data, data not shown). Of note, the metabolic reprogramming of macrophages in GLUT1^{ΔmΦ} mice was accompanied by a reduction in IL1β cytokine production suggesting that GLUT1^{ΔmΦ} macrophages are anti-inflammatory. With the reduction in pro-IL1β and pro-Caspase 1 activity in GLUT1^{ΔmΦ} macrophages, we concluded that GLUT1^{ΔmΦ} macrophages exhibit an anti-inflammatory phenotype.

Chapter 3.1 Proposed Model

Drawn by H.L.P

Figure 21. TAMs support metastatic dissemination and colonization at secondary sites. Macrophages that infiltrate PDAC lesions adopt a metabolic profile reminiscent of the Warburg effect for which PDAC tumor cells also exhibit. These infiltrating macrophages support tumor cell dissemination by promoting (1) angiogenesis, inducing (2) EMT and augmenting (3) extravasation of tumor cells to potential secondary sites. Sustained inhibition of such high rates of glycolysis using 2-DG in TAMs subverts all three pro-metastatic capacities. Taken together, our results highlight the potential of pharmacologics targeted at modulating metabolic pathways that deliver a one-two punch to both immune cells and tumor cells, thus refining our approach to curtailing metastasis in PDAC.

Chapter 3.2 Proposed Model

Drawn by J.L.S

Figure 22. A metabolic reprogramming in tumor macrophages via conditional knockout of GLUT1 attenuates tumor burden in pancreatic ductal adenocarcinoma. Similarly to *in vitro*-tumor conditioned macrophages, macrophages from PDAC mice are highly glycolytic. Macrophage-specific deletion of GLUT1 (GLUT1^{ΔmΦ}) significantly ablates tumor burden, accompanied by the increased in Natural Killer (NK) and CD8+ T cell (CTL) activity. Treatment of mice with a GLUT1-specific inhibitor ameliorates disease, comparable with gemcitabine, the current standard-of-care.

We showed that TAMs exhibited a mixed M1/M2 macrophage phenotype. They expressed M2 markers like CD163 and CD206 yet up-regulated the expression of classical M1 inflammatory cytokines – *IL-1 β* , *IL-6*, *TNF- α* , and *CCL3*. This is in line with other studies where macrophages have been shown to express both M1 and M2 markers¹³⁴. Such heterogeneous phenotype of tumor-infiltrating macrophages are apparently based on their polarization and the microenvironment^{135,136}. Higher rates of glycolysis observed in TAMs both *in vitro* and *in vivo* may draw a link to a chronic inflammatory state. Studies in mice have suggested a correlation between an increase glucose uptake leading to a hyper-inflammatory response in macrophages⁹⁵.

Freemerman and colleagues showed that expression of GLUT1 on macrophages mediated glycolysis and drive these cells to a pro-inflammatory phenotype⁹⁵. Reports have also describe an enhanced glycolytic signature in LPS-activated macrophages^{95,137}, and studies have also shown that the limiting glycolytic enzyme, PKM2, promotes the expression of HIF-1 α -dependent genes, such as *IL-1 β* , in LPS-activated macrophages^{138,139}. In line with this, our Seahorse extracellular flux assays, transcript analysis and intracellular *IL-1 β* cytokine staining supports the link between inflammation and glycolysis in macrophages of our tumor bearing mice compared with sham controls.

Here, we demonstrated a pronounced glycolytic signature of macrophages within the tumor microenvironment in which inflammation promotes tumor growth. The inhibition of glycolysis, both *in vitro* and *in vivo* established the direct connection between the pro-metastatic and pro-tumoral phenotype of TAMs to their preferential utilization of glycolysis. We showed that metabolic alterations by blocking glycolysis in TAMs were able to direct their downstream effector function towards a more favorable tumor outcome. This has been shown in literature for T cells, where metabolic reprogramming directs T cell fate and function¹⁴⁰. T cells are known to undergo extreme changes in their metabolic profiles as they transition through differentiation stages from a naïve T

cell to memory or regulatory functions. For example, naive T cells utilized OXPHOS for energy production, and undergo metabolic reprogramming towards glycolysis upon T cell receptor activation and clonal expansion¹⁴¹.

Given that GLUT1^{ΔmΦ} mice were significantly protected from tumor burden compared to wild-type tumor-bearing mice, we hypothesized GLUT1 as a potential drug target. We identified WZB117, a GLUT1-specific inhibitor, and treated mice with the drug day 1 following OT of tumor cells. We showed that similarly to GLUT1^{ΔmΦ} mice, WZB117 was also able to ameliorate disease with upregulation of NK and CTLs cell activation. However, we noticed that the tumor size was not as significantly reduced as compared to those in GLUT1^{ΔmΦ} mice. This is not unexpected as the WZB117 drug was given to mice orally and may have transient effects as compared to the genetically knockout animals where macrophages were completely not able to undergo glycolysis. This effect may also be masked as WZB117 may also have a direct effect on tumor cells and a comprehension study should be conducted in future to analyze the effect WZB117 has on tumor cells. Alternatively, there may be tolerization of the drug or compensation with other GLUT isoforms over time.

Notably, the expression of GLUT1 on macrophages may have prognostic value. In our PDAC patient cohort IF staining, we show that two subsets of CD68⁺ macrophages, i.e. GLUT1⁺ and GLUT1⁺HK2⁺HIF-1α⁺ are better predictors of survival in patients compared with the individual metabolic markers expression alone. While studies have shown that high expression of HK2¹⁴², HIF-1α¹⁴³ or GLUT1^{144,145} expression correlates with poor patient outlook, we show for the first time that the triple positive glycolytic signature of CD68⁺ macrophages is more closely linked to PDAC disease outcome. This signature could be used as a diagnostic tool in predicting the survival of patients with PDAC.

There are various metabolic inhibitors in the market currently undergoing testing in clinical trials¹⁴⁶, however as of date, inhibitor of glucose transporters have not passed Phase II¹⁴⁷. Notably, 2-DG has been tested in mouse models of SLE without toxicity^{148,149}, and similarly in our study, mice fed orally with WZB117 were able to safely tolerate the drug throughout the course of our experiment. However, it has been shown that the functions of the human central nervous system are dependent on glucose transport and metabolism, and the inhibition of glucose transport into and within the central nervous system would cause side effects¹⁵⁰. As such, Ojelabi *et al.* suggested a combination therapy of ketogenic diet (used to manage GLUT1 deficiency) and sugar transport inhibition that would deem a more successful therapy¹⁵⁰. Taken together, we propose that treatment with the WZB117 drug may be a potential avenue to specifically target GLUT1 in patients in the future.

That being said, a number of unanswered questions remain. The generation of our *in vitro*-conditioned macrophages requires the incubation of healthy blood monocytes in Panc1/MiaPaca2 tumor-conditioned and HPNE normal-conditioned media. An obvious question to ask would be what factors secreted in the tumor-conditioned media differentiate them from HPNE normal-conditioned media? To answer this, further analysis of the conditioned media is required to identify the factor(s) responsible for driving the pro-metastatic phenotype of tumor-conditioned macrophages. Secondly, though having shown that TAMs exhibit a mixed M1/M2 phenotype with the preferential use of glycolysis, we cannot rule out the possibility that there are other type of metabolic pathways utilized by our TAMs. There is currently a growing body of evidence suggesting that immune cells can undergo a global metabolic switch in cancer¹⁵¹. Studies have also shown metabolic reprogramming in macrophage polarization as a consequence of their activation¹⁵², and these polarization may be related to characteristic of lipid and iron metabolism^{135,153,154}. Whether our TAMs utilized other metabolic pathways such as of lipid, iron, or amino acid metabolism when

glycolysis is blocked remains to be explored.

Other questions include, how does blocking glycolysis promote anti-tumoral immunity? What alternative metabolic pathway(s) is the macrophage utilizing when glycolysis is inhibited? What are the downstream signaling pathways involved? Studies done in other cell types suggest that PI3K/Akt¹⁵⁵, mTOR¹⁵⁶, AMPK¹⁵⁶, and/or the Nlrp3 inflammasome¹⁵⁷ may be involved in metabolic reprogramming. We proposed that the IL-1 β inflammatory signaling axis may be involved having observed a decreased IL-1 β production in GLUT1 $\Delta m\Phi$ macrophages. However, other nutrient sensors and metabolic regulators such as mTOR and AMPK signaling pathways may also explain the anti-tumoral activity in GLUT1 $\Delta m\Phi$ macrophages^{156,158,159}. In a study by Kishton and colleagues, authors showed that AMPK was required to balance glycolysis. AMPK deficiency also resulted in T-cell acute lymphoblastic leukemia tumor cell death and ablation of the disease⁶⁷. Future works include identifying proteins involved in the mTOR and AMPK signaling pathways and to specifically overexpress or knock them down in GLUT1 $\Delta m\Phi$ macrophages in order to elucidate the mechanism behind the anti-tumoral phenotype. In addition, though key metabolic changes that occur between differentially activated macrophages are widely appreciated, the molecular mechanism behind how metabolic reprogramming is orchestrated remains largely unknown. As such, future works would also include detailed exploration into the mechanisms involved in these tumor macrophages, understanding exactly how the cell's metabolic status regulates its polarization.

Another question arises pertaining to what metabolites may be taken up and secreted within the tumor microenvironment, and the potential effect they may have on inducing the metabolic reprogramming of macrophages towards a glycolytic profile? It has been shown that altered metabolism of tumor cells produces extracellular metabolites that orchestrate the phenotypic diversity of cells in the tumor microenvironment¹⁶⁰. One factor found to play a role in the signaling to

induce the polarization of TAMs and consequently leading to tumor growth is tumor-cell-derived lactic acid¹⁶¹. Lactic acid is a by-product of aerobic and anaerobic glycolysis. Colegio *et al.* showed that lactic acid has critical function in signaling, through inducing the expression of VEGF. It was also reported that lactate-induced expression of Arg 1 by macrophages has an important role in tumor progression. These two studies show the interaction between TAMs and tumor cells and how their metabolism can have functional consequences. Hence, future studies assessing the presence of lactate in our tumor models would help to shed light on the mechanism(s) of communication between TAMs and tumor cells.

A main feature of metabolic pathways is in their plasticity, and changes in nutrient or oxygen levels in the environment are drivers of metabolic reprogramming of immune cells. In the microenvironment of GLUT1^{ΔmΦ} mice, macrophages are knockout of GLUT1 and unable to undergo glycolysis. As a result, there is a surplus of glucose in the tumor microenvironment and a change in nutrient availability. This increase of glucose in the environment may encourage the utilization of glycolysis by infiltrating CTLs and NK cells causing an increase in their activation and anti-tumoral effector functions. In support of this hypothesis, studies have shown that the competition of glucose with tumor cells are able to inhibit anti-tumoral T cell effector functions, promoting tumor progression¹⁶². Thus, we cannot rule out the fact that tumor-infiltrating T and NK cells of GLUT1^{ΔmΦ} mice, having a surplus of glucose, are able to drive their activation towards enhanced effector functions leading to the reduction of tumor burden observed.

Our data showed that macrophage-specific deletion of GLUT1 alone was able to ameliorate disease in our orthotopic-transplanted model of PDAC. Our study highlighted the paramount importance of immunometabolism *in vivo*, and showing that reprogramming of the metabolic profile of a macrophage away from glycolysis can instruct downstream effector functions towards better tumor outcome. Moreover,

the pronounced glycolytic signature in pancreatic patients could be used as a diagnostic tool in predicting survival and provides a strategy for future pharmacologic modulation.

While deleting GLUT1 on macrophages led to a dramatic reduction in tumor burden seen in the GLUT1^{ΔmΦ} mice, specifically deleting GLUT1 on neutrophils, a highly glycolytic immune cell did not result in reduction in tumor burden in GLUT1^{ΔNΦ} mice. This suggests that unlike macrophages, GLUT1 is dispensable in neutrophils probably due to the expression of other GLUT family members such as GLUT3 and GLUT8 (online database, Biogps).

In an oxygen-deprived microenvironment, HIF-1α is a well-known driver of glycolysis and is a major transcriptional factor of hypoxia. Under these hypoxic environments, immune cells have to rely on glycolysis to generate ATP for their energy needs. Depending on the environment, HIF-1α can alter vascularization, survival and cell metabolism^{74,75}. In the context of cancer, HIF-1α appears to be a master transcriptional factor controlling cellular and developmental processes in response to the metabolic phenotype of the tumor cell⁷⁵. Altered metabolism in tumor cells resulting in tumor initiation and growth has received renewed attention with more recent studies linking HIF-1α to a functional role in the metastatic process in tumors⁷⁷. HIF-1α has also been shown to metabolically reprogram immune cells to become inflammatory cells by promoting expression of inflammatory genes. In LPS activated macrophages⁸⁸, or in Th17 T cells¹⁶³, HIF-1α signaling together with aerobic glycolysis are two key features in these activated immune cells.

Neutrophils have distinct bioenergetic programs that rely specifically on aerobic glycolysis for energy production¹⁶⁴. Being highly glycolytic by nature and in a oxygen-deprived hypoxic tumor environment, it is not unexpected that we observed a high infiltration of HIF-1α-expressing neutrophils in the core of the pancreatic tumor. Consistent with

literature, neutrophils make up a significant proportion of infiltrating myeloid cells in cancer^{47,165,166}. As glycolysis is not the most effective way to generate ATP (one molecule of glucose to two molecules of pyruvate with the net production of only two molecules of ATP), the increase in glycolysis observed in tumor-infiltrating neutrophils may enable them to increase their generation of ATP. To further validate our hypothesis, transcriptomic analysis was performed on OT BM, blood and tumor-infiltrating neutrophils. Data revealed an upregulation of glycolytic genes and HIF-1 α in tumor-infiltrating neutrophils and not in the BM or blood of the same mouse. Similar to our *in vivo* PDAC mouse model, a high influx of neutrophils is also observed in the tumors of PDAC patients that correlated to the onset of disease. These tumor-infiltrating neutrophils are highly glycolytic, i.e. CD15⁺HIF-1 α ⁺GLUT1⁺. We provide compelling evidence demonstrating the potential and importance of HIF-1 α and glycolysis in modulating neutrophil metabolism to target tumor progression and also provide a link between the increased glycolytic energy metabolism and progression of PDAC.

Upon genetic ablation of HIF-1 α in neutrophils, we observed a reduction in the rate of glycolysis and a decrease in Arg 1, an immunosuppressive molecule. Our data suggest that the down-regulation of glycolysis in HIF-1 $\alpha^{\Delta N\phi}$ neutrophils conveys them an anti-tumoral phenotype. Indeed, HIF-1 $\alpha^{\Delta N\phi}$ mice were significantly protected from tumor burden as compared with WT OT controls. The mechanism of action was in part dependent on the differential effector functions and cytokine production of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils, as well as the anti-tumoral activity of CTLs and NK cells (illustrated in Fig. 23).

Chapter 3.3 Proposed Model

Drawn by J.L.S

Figure 23. Conditional knockout of HIF-1α in neutrophils promotes anti-tumoral activity via increased ROS and activation of CTL and NK cells cytotoxicity. Neutrophils infiltrate pancreatic tumor lesions in large numbers and adopt a metabolic signature similar to that of PDAC tumor cells and the Warburg effect. These infiltrating neutrophils support tumor progression by up-regulating HIF-1α and Arg-1, thereby inhibiting anti-tumoral CTL and NK cell activity. Loss of HIF-1α by conditional knockout in neutrophils subverts the pro-tumoral activity of these neutrophils by up-regulating ROS, and increasing the production of CTL/NK cell stimulating cytokines such as IL-12 and TNF-α. This mechanism opens new avenues for potential pharmacologics that target neutrophil metabolic pathways to improve disease outcomes.

Previous reports showed that anti-tumoral neutrophils could promote the recruitment and activation of CTLs through the production of T-cell attracting cytokines and chemokines such as IL-12, TNF- α , VEGF and CCL3¹⁶⁷, while pro-tumoral neutrophils produce large amounts of Arg-1 that would inactivate T cell effector functions¹⁶⁸. Our data showed that the ablation of HIF-1 α in neutrophils confers an anti-tumoral phenotype, as illustrated by the increased ROS production leading to cytotoxic activity and numerous studies have reported the involvement of ROS in anti-tumoral immunity¹²⁵. Alongside, the production of IL-12 and TNF- α cytokines by HIF-1 $\alpha^{\Delta N\phi}$ likely recruited CTLs into the tumor.

Our results suggest that HIF-1 $\alpha^{\Delta N\phi}$ neutrophils and control neutrophils differ in terms of their anti-tumoral activity with the former being anti-tumorigenic, and the latter pro-tumorigenic. This change in neutrophil phenotypes has been reported in the literature with studies showing the possibility of at least two different polarized populations of tumor-infiltrating neutrophils⁴⁰. Fridlender *et al.* showed that TGF- β in the tumor microenvironment is able to induce a population of neutrophils with a pro-tumoral phenotype with reduced cytotoxicity activity. Inhibition of TGF- β resulted in the recruitment and activation of neutrophils with an anti-tumoral phenotype paired with increased CTL activity⁴⁰. In line with this, we showed that reduced glycolysis and the inhibition of HIF-1 α in tumor-infiltrating neutrophils similarly conveys them an anti-tumoral effect.

To date, there are HIF-1 inhibitors targeting upstream of HIF-1 α protein synthesis, HIF-1 α stabilization, their dimerization as well as interactions with other proteins¹⁶⁹. Given the multiple failures of Phase II and Phase III trials for these HIF-1 inhibitors, targeting cancer metabolism remains complex and difficult¹⁷⁰. Moreover, HIF-1 α has been shown to be involved in multiple signalling pathways, which makes it a complex molecule to target¹⁶⁹. As such, no selective HIF-1 α inhibitor that has been clinically approved. Though targeting HIF-1 α has its challenges, it is clear that modulating HIF-1 α , or downstream effectors of HIF-1 α , has

proven to be therapeutically advantageous in pre-clinical tumor models^{171,172}. From our findings, we propose that altering the expression of HIF-1 α in infiltrating, pro-tumoral, hypoxic neutrophils may be the best validated target that has yet to be exploited in tumor biology but the challenge would be to find ways of specifically delivering the drug to the target cell of interest which is the neutrophils in this case.

A number of unanswered questions remain. Firstly, apart from the down-regulation of glycolysis, are there any other metabolic programs that are activated in HIF-1 $\alpha^{\Delta N\phi}$ neutrophils, to compensate for the glycolytic knockdown? Do they utilize fatty acid oxidation, oxidative phosphorylation or amino acid metabolism as an alternative source of energy production once glycolysis is shut down? In order to answer this question, a full metabolomics analysis has to be done. Secondly, it has been reported that HIF-1 α is able to promote angiogenesis, directing the migration of endothelial cells towards a hypoxic region via VEGF^{74,75}. In our study, we showed that down-regulated glycolysis in HIF-1 $\alpha^{\Delta N\phi}$ neutrophils switch them from a pro-tumoral phenotype to an anti-tumoral phenotype. Future work includes elucidating the other functional output of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils in terms of how they affect angiogenesis by looking at their ability to produce VEGF or IL-8. With the knockout of HIF-1 α in neutrophils, we propose a down-regulation of VEGF and the pro-metastatic phenotype of HIF-1 $\alpha^{\Delta N\phi}$ neutrophils.

Conclusion

Tumor cells thrive by relying largely on the Warburg effect to support their high energy demands in fluctuating hypoxic tumor environments. Targeting metabolism for cancer therapy have provided novel therapeutic strategies to exploit the altered glucose metabolic pathway as effective cancer treatments^{173,174}. More recently, the field of immunometabolism received renewed attention with studies suggesting the metabolic reprogramming of macrophages for cancer interventions^{175,176}. As immune cells require similar metabolic needs as tumor cells to exert their effector functions, the interplay between tumor and immune cells is not as simple as it seems. Drugs that aim to target tumor and/or immune cell metabolism should therefore optimally target metabolic pathways specific to the differential pro- and anti-tumoral cell populations. Despite these limitations, new insights into the differential metabolic profiles of immune cells have provided novel therapeutic strategies to exploit their altered metabolism and potentially circumvent tumor-resistance to current therapy.

Given that all immune cells, like tumor cells, rely on metabolic reprogramming to support their proliferation and survival tailoring to the different microenvironments, targeting immunometabolism has the potential to alter differential immune cell effector functions. Hence, exploiting the body's own natural defense machinery to recognize and destroy tumor cells through targeting immune cells may be a potentially promising therapeutic approach. Taken together, our studies suggest that targeting metabolic pathways in myeloid cells, such as macrophages and neutrophils to modulate their pro-tumoral phenotype to an anti-tumoral one represents a potential strategy to keep cancer cells in check. Ultimately, targeting immune cells serves as an alternative approach in our battle to overcome the multifaceted mechanisms of tumor resistance, especially in the aggressive disease of pancreatic cancer.

Appendix

I. Chapter 3.1 – Published

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ORIGINAL RESEARCH

Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma

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ABSTRACT

Patients with pancreatic ductal adenocarcinoma (PDAC) face a clinically intractable disease with poor survival rates, attributed to exceptionally high levels of metastasis. Epithelial-to-mesenchymal transition (EMT) is pronounced at inflammatory foci within the tumor; however, the immunological mechanisms promoting tumor dissemination remain unclear. It is well established that tumors exhibit the Warburg effect, a preferential use of glycolysis for energy production, even in the presence of oxygen, to support rapid growth. We hypothesized that the metabolic pathways utilized by tumor-infiltrating macrophages are altered in PDAC, conferring a pro-metastatic phenotype. We generated tumor-conditioned macrophages *in vitro*, in which human peripheral blood monocytes were cultured with conditioned media generated from normal pancreatic or PDAC cell lines to obtain steady-state and tumor-associated macrophages (TAMs), respectively. Compared with steady-state macrophages, TAMs promoted vascular network formation, augmented extravasation of tumor cells out of blood vessels, and induced higher levels of EMT. TAMs exhibited a pronounced glycolytic signature in a metabolic flux assay, corresponding with elevated glycolytic gene transcript levels. Inhibiting glycolysis in TAMs with a competitive inhibitor to Hexokinase II (HK2), 2-deoxyglucose (2DG), was sufficient to disrupt this pro-metastatic phenotype, reversing the observed increases in TAM-supported angiogenesis, extravasation, and EMT. Our results indicate a key role for metabolic reprogramming of tumor-infiltrating macrophages in PDAC metastasis, and highlight the therapeutic potential of using pharmacologics to modulate these metabolic pathways.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer mortality globally. With an overall 5-year survival rate of 3–5%, incidence almost equals mortality.¹ However, the mechanisms underlying the highly metastatic nature of this malignancy remain poorly understood. Studies in mouse models of PDAC demonstrated that epithelial-to-mesenchymal transition (EMT), a hallmark of metastasis, is most pervasive at inflammatory foci within the tumor.² Induction of pancreatitis increased the number of circulating pancreatic tumor cells, while immunosuppression using dexamethasone therapy abrogated dissemination.² In addition, cells already having undergone EMT have been identified in precursor lesions, known as Pancreatic Intraepithelial Neoplasia (PanINs), suggesting that initiation of metastasis occurs prior to the development of frank carcinoma.² Taken together, these results support the notion that inflammation can facilitate tumor dissemination, and may represent one of the early events of cellular invasion in PDAC. Hence, studying the immune cell infiltrate in PDAC may shed light on its particularly metastatic nature.

Highly predominant in many tumors, macrophages were originally perceived to be involved in antitumor immunity. However, overwhelming clinical and experimental evidence now indicate that in many cases, tumor-associated macrophages (TAMs) enhance chronic inflammation, stimulating angiogenesis, migration, and invasion.³ Using mouse models of mammary carcinoma, Qian and colleagues showed that a distinct population of host macrophages was recruited to extravasating pulmonary metastases.⁴ Systematic ablation of this population via both genetic and chemical approaches indicated that these macrophages were necessary for efficient metastatic seeding. Building upon this, Kitamura and colleagues from the same group recently reported that in the same animal model, the macrophages that were not only recruited, but also retained at the metastatic site (lung), promoted extravasation of tumor cells.⁵

During inflammation, macrophages adopt distinct metabolic signatures as they switch from quiescent to activated states.⁶ Emerging evidence suggests that these metabolic pathways themselves can direct immune cell differentiation and instruct effector function. For instance, Haschemi and colleagues have

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shown that modulation of a kinase involved in glucose metabolism can alter macrophage phenotype, influencing cytokine production and the expression of key surface receptors.⁷ Several studies have reported the contribution of other key glycolytic enzymes such as PDK1,⁸ PKM2,⁹ PFKFB3^{10,11} to the inflammatory state of M1, LPS-activated and atherosclerotic macrophages, respectively. Thus, we postulated that metabolic states in TAMs might play a role in directing their tumor response. Large numbers of macrophages have been reported to infiltrate the pre-neoplastic lesions of PDAC,¹² but the metabolic program utilized by these cells is unclear. How these programs, in turn, affect tumor growth, dissemination, and seeding at distal sites is completely unknown. Here, we ask how macrophage commitment to specific metabolic fates in PDAC influences their capacity to promote tumor dissemination.

Results

Generation of TAMs

To address how macrophage metabolism affects tumor outcomes, we utilized an *in vitro* model of tumor-conditioned macrophages. Conditioned media from normal (HPNE) or PDAC (Panc1 or MiaPaCa2) cell lines were incubated with peripheral blood monocytes from healthy donors to generate normal pancreatic “resident” macrophages and TAMs, respectively. These conditioned macrophages were validated as *bona fide* macrophages on the basis of morphology, endocytic capacity, and marker expression (Fig. 1A–C). All three cell types exhibited characteristic spindly- and “fried-egg”-shaped morphology (Fig. 1A), with similar endocytic capacity (Fig. 1B). Classical macrophage markers were also expressed, including CD68, CD163, M-CSFR, and CD206, although Panc1 and MiaPaCa2 macrophages expressed significantly higher levels of CD163 and CD206 compared with their HPNE counterparts (Fig. 1C). A higher proportion of Annexin-V-positive HPNE macrophages were detected, but all three macrophage types had similar viability as assessed by DAPI (Fig. 1D). Interestingly, Panc1 and MiaPaCa2 macrophages expressed higher transcript levels of the “classically-activated M1” markers, *IL-1 β* , *IL-6*, *TNF- α* , and *CCL3*, and the “alternatively-activated M2” marker, *Arg1*, suggesting that these TAMs exhibit a mixed macrophage phenotype (Fig. 1E). Other M1 and M2 genes, such as *IL-4* and *iNOS*, were assayed, but expression levels were too low to be of functional significance.

Pro-metastatic capabilities of TAMs

To determine the pro-metastatic capabilities of these TAMs, we collected the supernatants from the macrophage cultures to assess their capacity to induce new blood vessel formation (angiogenesis), promote tumor cells to seed secondary sites (extravasation), and boost EMT. They will be hereafter referred to as HPNE mac-sup and TAM-sup for macrophages differentiated in HPNE- and PDAC- conditioned media, respectively.

In a classical human umbilical vein endothelial cell (HUVEC) network-formation assay, TAM-sup consistently supported 1.5–2-fold higher formation of HUVEC networks compared with their HPNE counterparts (Fig. 2). Quantitative

analysis of key metrics detected significant increases in total network length, total branch points, and total loops formed, with a concomitant decrease in the number of total nets (incomplete loops) in TAM-sup supported networks (Fig. 2B–E). Therefore, we hypothesized that TAMs may accelerate angiogenesis in a VEGF-dependent manner. Consistent with this, TAMs were found to express significantly higher transcript levels of *VEGFA* (Fig. 2F). Furthermore, neutralization of VEGFA in the macrophage supernatants with an anti-VEGFA antibody demonstrated that the TAMs promote angiogenesis in a VEGF-dependent manner (Fig. 2G).

To mimic cancer cell extravasation in 3D, a microfluidic device as shown in Fig. 3 consisting of a central gel channel flanked by two fluidic channels was utilized. Similar microfluidic platforms have been used for investigating angiogenesis,^{13,14} EMT,^{15,16} cancer cell–macrophage interactions,¹⁷ intravasation¹⁸ and extravasation.^{19,20} For the present work, GFP-expressing HUVECs were seeded into one of the fluidic channels to form a uniform monolayer mimicking a blood vessel wall (Fig. 3A). Labeled PDAC tumor cells, with or without their corresponding conditioned macrophages, were then seeded into the “blood vessel” and the number of tumor cells that extravasated through the blood vessel wall into the matrix was measured by confocal microscopy (Fig. 3B). Remarkably, both Panc1 and MiaPaCa2 macrophages promoted the extravasation of Panc1 and MiaPaCa2 tumor cells, respectively, by 3–4-fold compared with control HPNE macrophages, indicating that these TAMs promote mobilization of tumor cells (Fig. 3C). As VEGF has also been shown to increase vascular permeability to permit extravasation of tumor cells,²¹ the elevated production of *VEGFA* in the TAMs (as shown in Fig. 2F) is a likely explanation for the promotion of both extravasation and angiogenesis.

During development, EMT is a naturally occurring process by which epithelial cells undergo profound changes in gene expression and cell morphology in preparation for increased cell mobility through tissues.²² During the process of EMT, epithelial markers, including tight junction and adherens junction proteins, such as E-cadherin, desmoplakin, and ErbB3, are lost as the cells begin to move apart. Mesenchymal markers, such as Snail, Vimentin, and Zeb1/2 are upregulated, and columnar or cuboidal cells adopt a flattened, spindly morphology in preparation for crossing the tissue layers.

During the initiation of metastasis, it is thought that tumor cells at the leading edge of the tumor undergo a similar process of EMT, facilitating migration to secondary organs.²¹ Consistent with the detected increases in angiogenesis and extravasation, Panc1 and MiaPaCa2 mac-sup induced greater spreading of pancreatic epithelial HPAF-II colonies compared with their HPNE counterparts (Fig. 4A). This result is quantitatively illustrated by the significantly increased internuclear distance between two neighboring cells in the HPAF-II colonies incubated with TAM-sup (Fig. 4B). In addition, compared with HPNE mac-sup, TAM-sup promoted a greater loss of the adherens junction protein E-cadherin (Fig. 4A, C). Staining of HPAF-II cells with phalloidin, which binds polymerizing F-actin, detected an increase in the cell area of HPAF-II colonies incubated with TAM-sup compared to HPNE mac-sup, although this did not reach statistical significance (Fig. 4D).

Figure 1. Validation of *in-vitro*-generated mΦ. (A) CD14⁺ MACS-isolated peripheral blood monocytes from healthy donors were cultured with HPNE-, Panc1- and MiaPaCa2-conditioned media for 7–9 d. The representative bright-field images of mΦ generated from the respective conditioned media are shown. (B) The three mΦ types were assayed for their ability to endocytose using pHrodo-Red Dextran beads. The histograms indicating uptake of the pH-sensitive red fluorescence after 2 and 20 min incubation with the beads are shown. (C) The histograms of the expression of classical mΦ markers CD68, CD163, M-CSFR and CD206 on the three mΦ types by flow cytometry are shown. Dotted lines through CD163 and CD206 histograms have been drawn to demarcate peak of HPNE mΦ stain. (A–C) First (green), second (red) and third (blue) rows denote data for HPNE, Panc1 and MiaPaCa2 mΦ, respectively. Data are representative of *n* = 4 independent experiments. (D) The histograms of Annexin-V staining by flow cytometry on the mΦ types are shown. Bar graphs depict the mean $\pm$ SEM of the proportion of Annexin-V⁺ apoptotic cells and DAPI⁺ live cells of total cells for the three mΦ types. Data are collated from *n* = 2 independent experiments. (E) MΦ are assayed for transcript expression of M1 and M2 markers *IL-1β*, *IL-6*, *TNF-α*, *IL-10* and *Arg1* by qPCR. Bar graphs shown are the mean $\pm$ SEM of the relative expression of that gene over HPRT. Data are representative of *n* = 3 independent experiments. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Several classic mesenchymal markers, including N-cadherin, Zeb1/2, Snail, Slug, and Vimentin, were assayed by qPCR, but little or no upregulation in expression could be detected in these HPAF-II colonies (data not shown).

We therefore hypothesized that Panc1 and MiaPaCa2 macrophages may induce EMT via production of growth factors known to promote EMT, such as TGFβ, HGF, EGF,

and/or FGF. Indeed, compared to HPNE macrophages, we observed a 1.5–3-fold increase in *TGFβ* gene expression, and a 1.5–2-fold increase in *HGF* gene expression in MiaPaCa2 and Panc1 macrophages, respectively. Expression of *EGF* and *FGF* was negligible in all three macrophage types.

To determine whether TAMs induced EMT via an HGF and/or TGFβ-dependent manner, neutralizing antibodies

Figure 2. Panc1 and MiaPaCa2 mΦ promote higher rates of angiogenesis compared to HPNE mΦ. MΦ supernatants (sups) harvested from the three mΦ types were added to 1×10^4 HUVECs grown on top of matrigel per well of Ibidi's μ -angiogenesis slide for 4 h at 37°C . 200 ng/mL rhVEGF-A and 10 μM of VEGFR inhibitor Sorafenib were used as a positive and negative control, respectively. (A) The representative bright-field images of HUVEC networks analyzed by Ibidi's WimTube Image Analysis software are shown. (B) Total network length (networks identified by red lines, length of tubes identified by blue regions), (C) total branch points, (D) total loops (identified by small yellow numbers) and (E) total nets are depicted in bar graphs as mean $\pm$ SEM of collated data from $n = 6$ independent experiments. (F) A bar graph of mean $\pm$ SEM of the expression of VEGFA by qPCR in the respective mΦ is shown. Data are representative of $n = 3$ independent experiments. (G) Shown are bar graphs of total network length in which VEGFA is neutralized in the macrophage supernatants by the addition of an anti-VEGF antibody. Data is collated from $n = 4$ independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

against HGF and/or TGF β were added in the macrophage supernatants when performing the EMT assay. While neutralizing HGF did not result in a significant change in HPAF-II colony spreading (as shown by internuclear distance) compared to isotype control antibody, neutralizing TGF β resulted in a pronounced reduction in colony spreading (Fig. 4F). Neutralizing both HGF and TGF β at the same time did not have a synergistic effect in reducing colony spreading further, compared to neutralizing TGF β alone (Fig. 4F). This suggests that TGF β and not HGF is the key factor in promoting EMT by TAMs.

Taken together, our observations from these key parameters of metastasis strongly indicate that infiltrating PDAC TAMs have the propensity to promote a more aggressive, pro-metastatic phenotype in tumor cells.

Glycolytic profile of TAMs

To ascertain whether the pro-metastatic phenotype of TAMs was associated with a specific metabolic pathway required for such energetic needs, we utilized a live

metabolic flux assay to determine their glycolytic and mitochondrial respiration (oxidative phosphorylation, OXPHOS) capacities. Notably, compared with control HPNE macrophages, Panc1 and MiaPaCa2 macrophages preferentially utilized glycolysis over OXPHOS, as observed by a 2-fold increase in glycolytic capacity (Fig. 5A) and a concomitant 2-fold decrease of mitochondrial respiration capacity (Fig. 5B). Consistent with this result, transcript analysis of key glycolytic genes revealed upregulation of *HK2*, *GPI*, *ALDOA*, *TPI1* and *PGK1* in TAMs compared with HPNE macrophages (Fig. 5C). In addition, L-lactate measured in macrophage lysates revealed 1.4–1.6-fold higher production of this metabolite in TAMs compared to HPNE macrophages (Fig. 5D). ROS production was also assessed, but all three macrophage types appeared to produce little ROS, with no comparable differences at the basal state (Fig. 5E). Taken together, our results show that TAMs preferentially utilize glycolysis to fulfill their energetic needs, which correlates with their pro-metastatic phenotype.

Figure 3. Panc1 and MiaPaCa2 mΦ augmented extravasation compared to HPNE mΦ. A PDMS microfluidic device consisting of a central gel region flanked by two side fluidic channels was utilized. HUVECs were cultured in a uniform monolayer mimicking a blood vessel wall in one of the fluidic channels. Panc1 or MiaPaCa2 tumor cells with or without corresponding mΦ (1:1) were co-injected into the channel containing HUVECs. After 24 h, tumor cell extravasation across the endothelial monolayer into the gel region was quantified by confocal microscopy and expressed as a percentage of the total number of cells present on the monolayer. (A) 3D drawing of the microfluidic device and an illustration of the expected movement of tumor cells and/or mΦ across the HUVEC monolayer into the 3D matrix are shown. This mimics the extravasation of cells from blood vessels into potential secondary metastatic sites. (B) The representative immunofluorescence images of MiaPaCa2 tumor cells in three different conditions after 24 h are shown: (i) not extravasated, (ii) in the process of extravasating and (iii) extravasated across the HUVEC monolayer. (C) Shown are bar graphs of mean $\pm$ SEM of the percentage of extravasated cancer cells, using collated data from at least six devices per condition. ** $p < 0.01$; *** $p < 0.001$.

Effect of inhibition of glycolysis on pro-metastatic capabilities of TAMs

To address whether this pro-metastatic phenotype in TAMs is dependent on their preferential utilization of glycolysis, we inhibited glycolysis in the macrophages by targeting HK2. The enzyme HK2 catalyzes the first rate-limiting step of glycolysis, in which glucose is converted to glucose-6-phosphate, to commit each molecule of glucose to enter glycolysis in an irreversible reaction. We treated the macrophages with a competitive inhibitor of HK2, 2-deoxyglucose (2DG), and repeated the series of angiogenesis, extravasation, and EMT assays.

We first confirmed that 2DG treatment did not adversely affect cell viability. Bright-field images of the macrophages cultured for 6 h without or with 25 mM 2DG clearly show that the macrophages are alive after 2DG treatment (Fig. S1A). Inhibition of glycolysis by 2DG treatment was also verified in all three macrophage types by live metabolic flux assay (Fig. S1B).

2DG inhibition persisted throughout the 6-h treatment period and for a further 7 h after removal of 2DG from the medium (Fig. S1C).

Remarkably, 2DG-mediated inhibition of glycolysis reversed the pro-metastatic capabilities initially observed with TAM-sups (Fig. 6). HUVEC network formation was inhibited in TAM-supported networks, as shown by decreases in total network length, total branch points, and total loops, along with an increase in the total number of nets, whereas HPNE-supported network formation was unaffected (Fig. 6A, B). A significant reduction in tumor cell extravasation was also observed in the presence of 2DG-treated TAM-sups but not in the presence of HPNE mac-sup (Fig. 6C). Indicators of EMT were also reversed, as shown by a decrease in the inter-nuclear distance between HPAF-II colonies (Fig. 6D, E), restoration of E-cadherin expression (Fig. 6F), reduced polymerizing F-actin mean intensity, and a decrease in HPAF-II area per cell (Fig. 6G).

Figure 4. Panc1 and MiaPaCa2 mΦ induced higher rates of EMT compared to HPNE mΦ. 3×10^3 HPAF-II (epithelial pancreatic tumor cell line) cells in single cell suspension were seeded per well in 96-well optical bottom plates and allowed to grow into tight self-contained colonies for 4 d. On day 4, media was aspirated from wells and mΦ supernatants were added to the HPAF-II colonies to induce EMT. After 36–48 h, HPAF-II colonies were assayed by confocal microscopy to determine three key parameters: (i) colony spreading (increase in internuclear distance of neighboring cells within each colony), (ii) loss of epithelial marker (E-cadherin) expression, (iii) morphological “flattening” of cells (increase in area) (A) The representative immunofluorescence images of HPAF-II colonies incubated with the respective mΦ supernatants, stained with E-cadherin and phalloidin are shown. (B) A collated scatter plot of mean $\pm$ SEM of the internuclear distance is shown. Each dot represents a measurement between two neighboring HPAF-II cells. (C, D) The collated scatter plots of mean $\pm$ SEM of the intensity of E-cadherin (C) and area of F-actin (D) where each dot represent a single HPAF-II cell are shown. (B–D) Data for scatter plots were tabulated from TissueQuest-analyzed images of at least eight colonies per condition. Data are representative of $n = 8$ (A–D) independent experiments. (E) The bar graphs of mean $\pm$ SEM of the transcript expression of *TGFβ* and *HGF* of the macrophages by qPCR are shown. Data are representative of $n = 3$ independent experiments. (F) The collated scatter plots of the mean $\pm$ SEM of the internuclear distance from $n = 4$ independent experiments are shown, in which HGF was neutralized with an anti-HGF antibody (Goat IgG as isotype control), and *TGFβ* was neutralized with an anti-*TGFβ* antibody (Mouse IgG1 as isotype control) in the macrophage supernatants. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant.

2DG has been reported to induce ER stress.²³ To rule out the possibility that ER stress induced by 2DG affected the pro-metastatic capabilities of the TAMs, we supplemented 2DG-treated macrophages with exogenous mannose. We observed that mannose supplementation did not affect loop formation in angiogenesis

assays (Fig. S2A), or significantly affect HPAF-II colony spreading in EMT assays (Fig. S2B), compared to 2DG alone. Taken together, these results demonstrate that 2DG inhibition of pro-metastatic capabilities of TAMs is a direct result of diminished glycolysis, and not due to downstream effects of ER stress.

Figure 5. Panc1 and MiaPaCa2 mΦ exhibit pronounced glycolytic profile compared to HPNE mΦ. The representative line graphs of the mean $\pm$ SEM of ECAR (A) and OCR (B) values at each timepoint in the Glycolysis Stress test and Mitostress test, respectively, performed on the three mΦ types are shown. Beside the line graphs are bar graphs of the mean $\pm$ SEM of calculated glycolytic capacity (A) and maximal mitochondrial respiration capacity (B). Data are representative of $n = 9$ independent experiments. (C) mΦ are assayed for transcript expression of key glycolysis genes *HK1*, *HK2*, *GPI*, *ALDOA*, *TPI1* and *PGK1* by qPCR. Bar graphs shown are the mean $\pm$ SEM of the relative expression of that gene over HPRT. Data are representative of $n = 4$ independent experiments. (D) A bar graph of the mean $\pm$ SEM of the fold change of L-lactate measured by LC-MS of the Panc1 and MiaPaCa2 mΦ lysates with respect to HPNE mΦ lysates is shown. Data is collated from $n = 4$ independent experiments. (E) The histograms of ROS production, from mΦ induced with a known ROS inducer as a positive control (left panel) and from the three mΦ types at basal level (right panel) are shown. Bar graph below shows the mean $\pm$ SEM of ROS MFI collated from $n = 2$ independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6. 2DG inhibition of glycolysis in Panc1 and MiaPaCa2 mΦ reverses pro-metastatic phenotype. After 7–9 d of monocytes co-cultured with HPNE, Panc1 and MiaPaCa2 conditioned media to obtain the three mΦ types, media was aspirated and replaced with fresh media with or without 25 mM 2DG for the next 6 h. MΦ supernatants were then harvested and used for downstream angiogenesis (A, B), extravasation (C) and EMT (D–G) assays. (A) The representative bright-field images of HUVEC networks analyzed by Ibi's WimTube Image Analysis software are shown, along with (B) scatter plots of the mean ± SEM of the key metrics as described in Fig. 2. Data shown is collated from $n = 6$ independent experiments. (C) The bar graphs of the mean ± SEM of the percentage of extravasated cancer cells, using collated data from at least four devices per condition are shown. *** $p < 0.001$. (D) The representative immunofluorescence images of HPAF-II colonies incubated with the respective mΦ supernatants, stained with E-cadherin and phalloidin are shown. (E–G) The scatter plots of the mean ± SEM of internuclear distance (E), mean intensity of E-cadherin (F) and F-actin (G) and the area of F-actin (G) are shown. (E–G) Scatter plots are data of one experiment from at least eight colonies per condition. Data are representative of $n = 6$ independent experiments.

Given that 2DG can abrogate the pro-metastatic capacities of TAMs, we hypothesized that the reciprocal experiment—an upregulation of glycolysis in the TAMs—could further promote these capacities. We induced glycolysis transiently by

overexpressing HK2 (Fig. S3) and GLUT1 (data not shown) by mRNA electroporation and plasmid DNA transfection. Despite clear upregulation of *HK2* transcript expression by qPCR (Fig. S3B), this does not translate to functional upregulation of

glycolysis in live metabolic flux assays (Fig. S3A) or of key glycolytic genes by qPCR (Fig. S3C).

Taken together, these results strongly indicate that the pro-metastatic phenotype of TAMs is dependent on a metabolic commitment to high rates of glycolysis to fulfill such functional requirements, and that inhibition of glycolysis can subvert their pro-metastatic capacities almost entirely.

Discussion

It is increasingly appreciated that tumor cells undergo pronounced metabolic reprogramming to fulfill their bioenergetic and biosynthetic demands in an ongoing effort to support rapid proliferation.²⁴ Indeed, metabolic reprogramming has recently acquired status as a core hallmark of cancer progression.²⁵ One of the earliest signs of such profound metabolic changes is the Warburg effect, a phenomenon in which tumor cells almost exclusively use glycolysis to generate energy, even under aerobic conditions.²⁴ Indeed, these metabolic alterations form the basis for the use of ¹⁸Fluoro-Deoxyglucose Positron-Emission Tomography (¹⁸FDG-PET) to image and visualize growing tumors in the human body.

The tumor microenvironment is made up of a heterogeneous population of cells in the tumor stroma, including immune cell infiltrates. Macrophages have emerged as key players involved in altering tumor progression and metastasis, and a growing body of evidence indicates that these immune cells are themselves capable of adopting specific metabolic signatures for effector function.²⁶ However, whether tumor-infiltrating macrophages exhibit the type of metabolic reprogramming reminiscent of tumor cells, and how this, in turn, affects tumor

outcomes remains to be elucidated. Here, we show, for the first time in the context of pancreatic cancer, that human *in-vitro*-derived TAMs utilize Warburg metabolism to promote tumor growth and dissemination.

In a model of our summarized findings (illustrated in Fig. 7), we propose that macrophages infiltrating tumor lesions in a chronic “smoldering inflammation” response²⁷ promote angiogenesis and augment extravasation of tumor cells out of blood vessels in a VEGF-dependent manner. In addition, these TAMs induce EMT, facilitating tumor dissemination to secondary sites, such as the lung and liver, in a TGF β -dependent manner. This pro-metastatic phenotype correlates with high rates of glycolysis characteristic of the Warburg metabolism. Inhibiting glycolysis in the TAMs using 2DG, a specific inhibitor of HK2, results in significant reversal of all three pro-metastatic capabilities. To our knowledge, our results represent the first direct proof-of-concept that alterations in macrophage metabolism can promote tumor dissemination.

Characterization of our *in-vitro*-generated macrophages revealed that Panc1 and MiaPaCa2 macrophages exhibit a mixed M1 and M2 phenotype,²⁸ with increased expression of the M2 markers, CD163 and CD206, as well as increased expression of the inflammatory cytokines, IL-1 β , IL-6, and TNF- α . This is not surprising, as tumor-infiltrating macrophages are known to be heterogeneous in terms of polarization, location, and function.²⁹ Indeed, Helm and colleagues also report that freshly isolated TAMs from human PDAC lesions express both M1 (HLA-DR, IL-1 β , and TNF- α) and M2 (CD163 and IL-10) markers.³⁰

We observed consistently higher rates of glycolysis in TAMs compared with controls, which is in line with findings in the

Figure 7. TAMs support metastatic dissemination and colonization at secondary sites. M Φ that infiltrate PDAC lesions adopt a metabolic profile reminiscent of the Warburg effect for which PDAC tumor cells also exhibit.⁵⁰ These infiltrating m Φ support tumor cell dissemination by promoting (1) angiogenesis, inducing (2) EMT and augmenting (3) extravasation of tumor cells to potential secondary sites. Sustained inhibition of such high rates of glycolysis using 2DG in TAMs subverts all three pro-metastatic capabilities. Taken together, our results highlight the potential of pharmacologics targeted at modulating metabolic pathways that deliver a one-two punch to both immune cells and tumor cells, thus refining our approach to curtailing metastasis in PDAC.

setting of obesity-induced inflammation, which frequently draws parallels to the chronic smoldering inflammation state in tumors. Freerman and colleagues demonstrated that in mice fed a high-fat diet, macrophages found in crown-like structures and inflammatory foci stained positive for GLUT1, suggesting a connection between increased glucose uptake and a hyperinflammatory state.³¹ In addition, stable overexpression of GLUT1 in the RAW264.7 mouse macrophage cell line led to elevated secretion of inflammatory mediators.³¹ Several other studies have also reported a link between key glycolytic enzymes and an M1, or LPS-activated macrophage phenotype.^{8,9,11,32} Thus, recent evidence supports the notion that upregulation of glycolysis in macrophages confers a pro-inflammatory phenotype.

The transcription factor Hypoxia-inducible factor 1 α (HIF1 α), which is upregulated during glycolysis, also activates the transcription of VEGF,³³ providing a connection between increased glycolysis in TAMs to its ability to promote angiogenesis. Although we could detect VEGFA by qPCR in all three macrophage types, VEGFA was barely detected at the protein level in the macrophage supernatants, as were several other angiogenesis-associated factors such as Angiogenin, Angiopoietin-1, -2 etc. (data not shown). This suggests that in addition to VEGF, a combination of other factors could together be promoting angiogenesis.

While there is currently little evidence describing how glycolysis directly affects TGF β signaling, activation of this pathway in breast-cancer-associated fibroblasts has been reported to increase oxidative stress and glycolysis in these stromal cells,³⁴ suggesting a connection between TGF β signaling and glucose metabolism. Indeed, we observed that 2DG inhibition resulted in a 2- to 3-fold reduction in transcript expression of TGF β in TAMs (data not shown). Taken together, our results are consistent with existing literature linking VEGF and TGF β to enhanced glucose metabolism.

In our hands, we were unable to increase glycolytic rates of our *in-vitro*-conditioned macrophages further after glucose addition, despite trying several approaches such as HK2/GLUT1 overexpression and using drugs that target the ETC such as oligomycin, metformin, phenformin and rotenone (latter three, data not shown). Oligomycin, used as a positive control in the Glycolysis Stress Test to shift energy production from OXPHOS to glycolysis and reveal the maximum glycolytic capacity of the cells, invariably failed to upregulate glycolysis in these macrophages (Fig. 5; Fig. S1B and S3A, 2nd stage of Glycolysis Stress Test). We hypothesize that in these *in-vitro*-conditioned macrophages, induction of glycolysis have possibly reached a functional maximum and could not be further increased.

Others have shown that overexpression of signaling mediators in myeloid cells, such as Akt in mouse bone-marrow-derived dendritic cells³⁵ and CARL in the mouse RAW264.7 macrophage cell line,⁷ are able to sustain induction of glycolysis. However, to our knowledge, there are no reports thus far in primary myeloid cells demonstrating the effects of induction of glycolysis on functional polarization. We observed that incubation with 2DG for longer than 8 h results in over 50% cell death in our macrophage populations (data not shown), suggesting that these cells may be sensitive to such prolonged metabolic

changes. It has also been reported that HK2 overexpression in cardiomyocyte and noncardiomyocyte cells induces autophagy in glucose-limiting conditions.³⁶ Thus, macrophages may also display resistance to upregulation of glycolysis as a compensatory mechanism to prevent autophagy and conserve energy resources.

Apart from glucose metabolism, it is possible that other types of metabolic programs involved in macrophage polarization may also be utilized in our *in-vitro*-generated TAMs. M1 and M2 polarization has been related to distinct metabolic characteristics pertaining to lipid and iron metabolism.^{29,37,38} Similarly, reprogramming in glucose and amino acid metabolism was reported for dendritic cells and T cells upon activation.^{26,39} Indeed, there is accumulating evidence for global metabolic changes in immune cells in the context of cancer.⁴⁰ In PDAC, a new study by Son and colleagues has recently shown that PDAC tumor cells are highly dependent on a glutamine-based series of reactions to support tumorigenic growth.⁴¹ Whether this glutamine “addiction,” or other types of lipid, iron, or amino acid metabolism, is preferentially utilized by tumor-infiltrating macrophages remains to be explored.

A number of other unanswered questions remain. Which factors in Panc1 and MiaPaca2 tumor-conditioned media differentiate it from HPNE normal-conditioned media? Deconvolution of tumor-conditioned media is required to elucidate which growth factors or cytokines are responsible for promoting the pro-metastatic phenotype observed. The signaling pathways downstream of glycolysis that mediate the pro-metastatic phenotype in these TAMs are also unknown. Prevailing evidence in other immune cell types suggest a variety of possibilities. PI3K/Akt,³⁵ mTOR,⁴² AMPK,⁴² and the Nlrp3 inflammasome⁴³ have all been shown to mediate downstream signaling pathways upon nutrient-sensing. Incio and colleagues report that activating AMPK signaling pathways with metformin is able to reduce the M2 markers Arg1 and IL-10 in TAMs isolated from orthotopic models of mouse PDAC.⁴⁴ This is associated with an alleviation of desmoplasia at the tumor site, and reduction of both EMT and systemic metastasis.⁴⁴ These results point toward possible AMPK signaling in Panc1 and MiaPaCa2-TAMs. However, detailed exploration into the signaling pathways involved in these TAMs is beyond the scope of this study.

Finally, do our findings extend to other cancers? In unrelated, currently unpublished studies, other investigators have found evidence for Warburg metabolism in myeloid cells in the context of mouse ovarian and human renal and thyroid carcinomas, indicating that our observations are not restricted to the setting of PDAC (personal communication).

Taken together, our results suggest that targeting metabolic pathways in immune cells, such as macrophages, represents a potential strategy to keep tumor metastasis in check. Modulating glycolysis to reshape the immune response in the context of cancer is not without precedent. A recent report by Chang and colleagues demonstrated in a mouse sarcoma model that enhancing tumor cell glycolysis in an antigenic “regressor” tumor is sufficient to override the cytotoxic ability of T cells to destroy tumor cells in an IFN γ -dependent manner.⁴⁵

Since all tumor cells are dependent on metabolic reprogramming to maintain growth and survival, targeting metabolism

raises the possibility of affecting cancers arising from diverse tissue types. Indeed, the enzyme L-asparaginase has been used to treat acute lymphoblastic leukemia (ALL) and related lymphomas,⁴⁶ thus illustrating how the distinct metabolism of tumor cells can be successfully exploited for cancer therapy.

Because normal proliferating cells have the same metabolic needs as cancer cells, it will be essential to determine an appropriate therapeutic window, which is clinically useful while still ensuring safety.⁴⁶ Despite these challenges, the burgeoning field of immunometabolism has renewed interest in the exploration of metabolic enzymes as viable pharmacological targets to deliver a simultaneous hit to both tumor and pro-metastatic immune cells.

Materials and methods

Generation of conditioned media

Conditioned media were generated from a normal pancreatic epithelial cell line hTERT-HPNE (“HPNE”) (ATCC® CRL-4023) and the PDAC epithelial cell lines Panc1 (ATCC® CRL-1469) and MiaPaCa2 (ATCC® CRL-1420). Cells were seeded at the same cell density, 1×10^6 cells in 30 mL of complete IMDM (“cIMDM,” IMDM supplemented with 5% Human serum and $1 \times$ Penicillin-Streptomycin) in a T175 flask, and grown to 70–80% confluency. The conditioned media was then removed from the flasks, centrifuged for 10 min at 1500 rpm, sterile-filtered (0.2 μ m), aliquoted, and stored at -20°C .

Development of *in vitro*-generated TAMs

All blood samples used in this study were approved by the Institutional Review Board, Singapore in accordance to guidelines of the Health Science Authority of Singapore (Reference code: NUS-IRB10-250). Peripheral blood mononuclear cells (PBMCs) were obtained from blood of healthy donors using Ficoll density centrifugation. Monocytes were isolated with α -CD14-conjugated microbeads by magnetic-automated cell sorting (MACS) (Miltenyi Biotec), and cultured with conditioned media (thawed and warmed to 37°C) for 7–9 d. Each donor represents one independent experiment. Macrophages generated using these conditioned media were then assayed to verify the differentiation of *bona fide* macrophages. For macrophage supernatant experiments, the conditioned media were removed from the mature HPNE–, Panc1–, and MiaPaCa2–macrophages after the 7–9-d culture, and replaced with fresh cIMDM for 6 h. This media was then harvested and used as macrophage supernatant (labeled mac-sup or TAM-sup as detailed in the *Results* section).

Flow cytometry

Macrophages were resuspended in FACS buffer (10% Fetal calf serum, 5% Human serum, 0.01% sodium azide in PBS). After Fc blockade with anti-Fc γ R1/II (BD Biosciences), cells were stained with DAPI, and surface-stained with anti-CD163 APC (BioLegend 333610), anti-CD206 APC-Cy7 (BioLegend 321120), and M-CSFR PE (BioLegend 347304). Intracellular CD68 was stained using anti-CD68 Alexa 488 (BioLegend

333811) as per the manufacturer’s instructions. Annexin-V APC (eBiosciences 88-8007) was stained as per the manufacturer’s instructions. Flow cytometric analysis was performed on a BD Fortessa flow cytometer (BD Biosciences).

Endocytosis assay

Macrophages were harvested by gentle scraping, and washed with warm cIMDM three times prior to incubation with 100 μ g/mL of pHrodo-red Dextran beads (Invitrogen) in cIMDM at 37°C for 2 or 20 min. The macrophages were washed again with warm cIMDM three times and immediately assayed for pHrodo-red Dextran uptake by flow cytometry. pHrodo-Red dextran has a pH-sensitive fluorescence emission that increases in intensity with increasing acidity, and thus acts as an indicator of active endocytosis.

RT-qPCR

Cells were lysed with Qiazol (Qiagen), and total RNA was purified using the RNeasy Mini kit (Qiagen), as per the manufacturer’s instructions. DNase-treated total RNA (0.5–1 μ g) was reverse-transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad). Expression of *IL-1 β* , *IL-6*, *TNF α* , *CCL3*, *IL-10*, *Arg1*, *HK1*, *HK2*, *GPI*, *ALDOA*, *TPI1*, *PGK1*, *HIF1 α* , *LDHA*, *VEGFA*, *TGF- β* , and *HGF* were determined by quantitative PCR with KAPA Sybr FAST ABI Prism 2 $\times$ qPCR Master Mix (KAPA Biosystems) as per the manufacturer’s instructions using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Levels of the housekeeping gene *HPRT* were measured in a separate reaction, and used to normalize the data. List of qPCR primer sequences are listed in Table S1.

HUVEC network formation assay

HUVECs were grown in EndoGRO-LS media (Millipore) to 60–80% confluency before harvesting. EC Matrix (Millipore), a form of matrigel, was thawed on ice and left at 4°C overnight prior to use. On the day of assay, 10 μ L of EC Matrix was added to the inner well of a μ -angiogenesis slide (Ibidi) and allowed to solidify at 37°C for at least 3 h. HUVECs (1×10^4) in 20 μ L of EndoGRO-LS media were seeded into the upper well of the μ -angiogenesis slide together with 20 μ L of macrophage supernatant. HUVEC networks were allowed to form over the next 6 h at 37°C before termination of the assay. VEGF-A (200 ng/mL) and the VEGFR inhibitor Sorafenib (10 μ M) were used as positive and negative controls, respectively. Network formation was captured by bright-field imaging using an Olympus epifluorescent microscope at the 4-h time-point. At least four non-overlapping images were captured at $10\times$ magnification per well. Images were quantitatively analyzed with WimTube software (Ibidi) to evaluate key network formation metrics, such as total network length, total number of branching points, loops, and nets. Following WimTube metrics, a segment is considered to be part of the segmental structure between two branching points, or a branching point and a loose end (identified by red lines in the bright-field images of Figs. 2A and 6A). Total network length refers to the total number of segments multiplied by the individual segment length in

pixels (identified by blue regions in the bright-field images of Figs. 2A and 6A). Branching points refer to parts of the skeleton where three or more segments converge/diverge. A loop refers to an area of the background enclosed (or almost enclosed) by the segmental structures. A net refers to an isolated region of segments that contains at least one branching point; thus, isolated segments are not considered nets. A net is essentially an incomplete loop, and is inversely proportional to loop formation.

Extravasation assay

The microfluidic device consists of a polydimethylsiloxane (PDMS) layer bonded to a glass coverslip by plasma treatment to form a central gel region and two adjacent fluidic channels. The PDMS replica was fabricated by soft lithography and a poly-D-lysine (PDL) coating of the microchannels was applied to improve the hydrogel attachment to the channel walls as previously described.⁴⁷ Each of the three channels has a width of 1 mm, a height of 120 μm , and the central gel region is in contact for about 9 mm in length with the two lateral fluidic channels. To create the three-dimensional matrix, a type-I collagen gel solution (2.5 mg/mL, pH 7.4) was injected into the central region and polymerized at 37°C for 30 min. GFP-HUVECs (5×10^6 cells/mL) in 50 μL of EGM-2 media (Lonza) were seeded in one of the fluidic channels pre-coated with 50 $\mu\text{g}/\text{mL}$ fibronectin. After 3 d, a uniform monolayer covering the four walls of the channel was formed mimicking a blood vessel. Panc1 and MiaPaCa2 tumor cells, with or without macrophages, were injected in the same microchannel to investigate their extravasation across the endothelial monolayer into the gel region. Tumor cells and macrophages were seeded at a ratio of 1:1 with a concentration of 1.5×10^5 cells/mL for each cell type in EGM-2 media. Macrophages and pancreatic cells were labeled prior to seeding into the device. Macrophages were stained with Vybrant DiD (Life Technologies) live cell-labeling solution, while pancreatic cells were labeled with PKH26 Red Fluorescent Cell Linker for Cell Membrane (Sigma-Aldrich). Nuc Blue Live Cell Stain for Nucleus (Life Technologies) was used for both macrophages and tumor cells. For the experiments using macrophage supernatants only, the tumor cells were resuspended in the macrophage supernatants, and injected into the device as described before. After 24 h, the devices were fixed with 4% paraformaldehyde (PFA) for 12 min, washed, and stored at 4°C before confocal imaging. The images were captured using an Olympus IX81 confocal microscope. Images were analyzed with IMARIS software (Bitplane Scientific Software) to quantify the extravasation of pancreatic cells under the different test conditions. The number of extravasated tumor cells was counted and expressed as a percentage of the total number of cells present on the monolayer.

EMT assay

Pancreatic epithelial tumor cell line HPAF-II (EMT score = -0.556) (ATCC CRL-1997) was seeded at 3×10^3 cells per well in 200 μL of cIMDM in Nunc MicroWell 96-Well black-wall optical-bottom plates. After 4 d, HPAF-II colonies were in tight self-contained colonies, approximately 10–20 cells in size.

Media was aspirated from the wells before addition of macrophage supernatants and EMT-inducing media (a gift from R&D Systems, CCM017) at a 1:1 ratio. EMT induction was allowed to proceed for 36–48 h before the media was removed and cells were fixed with 4% PFA for 30 min at 4°C. The wells were washed with PBS twice and blocked with 10% normal goat serum for 10 min before staining with mouse monoclonal anti-human E-cadherin (BD Biosciences 610182) and Alexa Fluor 647-conjugated phalloidin (Life Technologies A22287). After three washes of PBS, the cells were stained with donkey anti-mouse Alexa Fluor 488 (ThermoFisher Scientific R37114). Cells were washed with PBS three times and stained with DAPI for 5 min. PBS (100 μL) was added to each well and plates were stored at 4°C before imaging. HPAF-II colonies were imaged using an FV-1000 confocal system with an inverted Olympus IX81 microscope. Internuclear distance was calculated using DAPI alone images ran on an in-house script to determine closest distance between two adjacent nuclei. To plot the mean intensity and area of E-cadherin and actin, images were analyzed by TissueQuest software (TissueGnostics).

Live metabolic flux assay

The mitochondria oxygen consumption rate (OCR, O_2 pmol/min) and extracellular acidification rate (ECAR, mpH/min) of macrophages were analyzed using an XF⁹⁶ extracellular flux analyzer (Seahorse Bioscience) according to the manufacturer's instructions. For the Glycolysis Stress Test ECAR analysis, macrophages were harvested, washed, and resuspended in ECAR medium (DMEM base [no bicarbonate] with 2 mM L-glutamine, 143 mM NaCl, and 0.5% phenol red [pH 7.35]). This test consisted of four consecutive stages: basal (without drugs), glycolysis induction (10 mM glucose), maximal glycolysis induction (5 μM oligomycin), and glycolysis inhibition (100 mM 2DG). Glycolytic capacity was calculated as follows: (Mean of highest ECAR values induced by glucose or oligomycin) – (Mean of basal ECAR values). For the Mito Stress Test OCR analysis, macrophages were harvested, washed, and resuspended in OCR medium (DMEM base, 25 mM glucose, 1 mM pyruvate, 2 mM L-glutamine [pH 7.35]). This test also consisted of four consecutive stages: basal respiration (without drugs), mitochondrial complex V inhibition (5 μM oligomycin), maximal respiration induction (5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)), and electron transport chain inhibition (1 μM rotenone and 1 μM antimycin A). Mitochondrial respiration capacity was calculated as follows: (Mean of highest OCR values induced by FCCP) – (Mean of OCR values after oligomycin addition). Macrophages were resuspended in the respective media were and seeded at 8×10^4 /well in flat-bottom 96-well Seahorse Microculture plates, and incubated in a non- CO_2 incubator for 1 h at 37°C prior to the start of each assay.

Metabolomics analysis

Metabolites from 5×10^6 macrophages of each phenotype were extracted based on a two-phase liquid extraction protocol and subsequently analyzed using reverse phase liquid chromatography, in tandem with high-resolution mass spectrometry as

previously described.⁴⁸ Data pre-processing was performed using an in-house software incorporating the XCMS peak finding algorithm⁴⁹ and differential metabolites with *p* values less than 0.05 (Student's *t*-test with Welch correction) were identified.

Inhibition and overexpression of HK2

To inhibit glycolysis in the fully conditioned macrophages (after 7–9 d of incubation in conditioned media), media was removed and replaced with fresh cIMDM with or without 2DG, a competitive inhibitor of HK2, to a final concentration of 25 mM for 6 h at 37°C. In some experiments, 5 mM mannose was supplemented to 2DG-treated wells for the same time period. To promote glycolysis, HK2 was overexpressed by transfection with an HK2 plasmid (Origene SC309063). Fully conditioned macrophages were harvested and re-plated at 0.8×10^6 cells/well in 900 μ L of Opti-MEM media (Gibco) in a 12-well plate and allowed to re-adhere to the well surface for 30 min. Purified HK2 plasmid DNA (1.25 μ g) was pre-incubated with 7.5 μ L of Lipofectamine 2000 (Life Technologies) in 100 μ L of Opti-MEM for 5 min at room temperature before adding to the macrophages for 6 h at 37°C. Mock transfections were set up without DNA. Inhibition and induction of glycolysis was determined using live metabolic flux assays and RT-qPCR.

Reactive oxygen species (ROS) production

ROS was measured using 1 μ M of Oxidative Stress Detection Reagent from the total Reactive Oxygen Species/SuperOxide detection kit (Enzo LifeSciences ENZ-51010) according to the manufacturer's instructions. Macrophages were incubated with the oxidative stress detection reagent for 0, 10, 20, and 30 min, and analyzed using a BD Fortessa (BD Biosciences). Pyocyanin (100 μ M) was used as a positive control to induce ROS.

Neutralization of VEGF, HGF and TGF β

0.2 μ g/mL anti-VEGFA-neutralizing antibody (R&D AF-293-NA) was incubated along with the macrophage supernatants in angiogenesis assays as indicated. 20 μ g/mL anti-HGF neutralizing antibody (Abcam ab10679) and 50 μ g/mL anti-TGF β neutralizing antibody (eBiosciences 16-9243) were incubated along with macrophage supernatants in EMT assays, where indicated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

H.L.P. conceived, designed, performed, analyzed, interpreted all experiments, and wrote the manuscript. J.L.S., W.H.Y., P.S., B.S.L. assisted with experimental work and analyzed data. G.A. designed, performed, and interpreted all the extravasation experiments, analyzed data, and wrote the parts in the manuscript relevant to extravasation. B.L. and T.L. analyzed data. Y.S.H. and S.Y.M. performed the metabolomics experiments. C.K.O. provided material support. R.Y.H. assisted with the development of methodology. O.R., R.D.K., and F.G. reviewed the manuscript. S.C.W. supervised the project, analyzed, interpreted data, and wrote the manuscript.

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II. Chapter 3.2 – In revision

Hweixian Leong Penny, **Je Lin Sieow**, Sin Yee Gun, Mai Chan Lau, Bernett Lee, Jasmine Tan, Yvaguencia Michel, Cindy Phua, Florida Toh, Yvonne Nga, Wei Hseun Yeap, Baptiste Janela, Dilip Kumar, Hao Chen, Joe Yeong, Justin A. Kenkel, Angela Pang, Diana Lim, Han Chong Toh, Tony Lim Kiat Hon, Christopher I Johnson, Hanif Javanmard Khameneh, Alessandra Mortellaro, Edgar G. Engleman, Olaf Rotzschke, Florent Ginhoux, Jean-Pierre Abastado, Jinmiao Chen, Siew Cheng Wong. Targeting glycolysis in macrophages confers protection against pancreatic ductal adenocarcinoma. (2018).

III. Chapter 3.3 – In revision

Je Lin Sieow, Hweixian Leong Penny, Sin Yee Gun, Ling Qiao Tan, Kaibo Duan, Joe Poh Sheng Yeong, Angela Pang, Diana Lim, Han Chong Toh, Tony Kiat Hon Lim, Edgar Engleman, Olaf Rotzschke, Lai Guan Ng, Jinmiao Chen, Suet Mien Tan, Siew Cheng Wong. Conditional knockout of hypoxia-inducible factor 1-alpha in tumor-infiltrating neutrophils protects against pancreatic ductal adenocarcinoma. 2018.

IV. Review Article – In revision

Je Lin Sieow, Sin Yee Gun and Siew Cheng Wong. The sweet surrender: how myeloid cell metabolic plasticity shapes the tumor microenvironment. (2018).

Other Publications

- I. Maximilien Evrard, Immanuel W.H. Kwok, Shu Zhen Chong, Karen W.W. Teng, Etienne Becht, Jinmiao Chen, **Je Lin Sieow**, Hweixian Leong Penny, Goh Chi Ching, Sapna Devi, José Maria Adrover, Jackson L.Y. Li, Ka Hang Liong, Leonard Tan, Zhiyong Poon, Shihui Foo, Jia Wang Chua, I-Hsin Su, Karl Balabanian, Françoise Bachelerie, Subhra K. Biswas, Anis Larbi, William Y.K. Hwang, Vikas Madan, H. Phillip Koeffler, Siew Cheng Wong, Evan W. Newell, Andrés Hidalgo, Florent Ginhoux, Lai Guan Ng: Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity*, 48:2, 364 - 379.e8 (2018).
- II. Rohimah Mohamud, Jeanne S. LeMasurier, Jennifer C. Boer, **Je Lin Sieow**, Jennifer M. Rolland, Robyn E. O’Hehir, Charles L. Hardy & Magdalena Plebanski: Synthetic Nanoparticles That Promote Tumor Necrosis Factor Receptor 2 Expressing Regulatory T Cells in the Lung and Resistance to Allergic Airways Inflammation. *Frontiers in Immunology*, 8:1812. doi: 10.3389/fmmu.2017.01812 (2017).
- III. Hweixian Leong Penny, **Je Lin Sieow**, Giulia Adriani, Wei Hseun Yeap, Peter See Chi Ee, Boris San Luis, Bernett Lee, Terence Lee, Shi Ya Mak, Ying Swan Ho, Kong Peng Lam, Choon Kiat Ong, Ruby Y. J. Huang, Florent Ginhoux, Olaf Rotzschke, Roger D. Kamm & Siew Cheng Wong: Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma. *Oncot Immunology*, 5, <http://dx.doi.org/10.1080/2162402X.2016.1191731> (2016).

- IV. Tong Seng Lim, Valerie Chew, **Je Lin Sieow**, Siting Goh, Joe Poh-Sheng Yeong, Ai Ling Soon & Paola Ricciardi-Castagnoli: PD-1 expression on dendritic cells suppresses CD8 T cell function and antitumor immunity. *Oncoimmunology*, 5, e1085146, doi:10.1080/2162402X.2015.1085146 (2016).

Poster Presentations

- I. **Je Lin Sieow**, Hweixian Leong Penny, Sin Yee Gun, Lai Guan Ng, Siew Cheng Wong
Neutrophil metabolism drives the progression of pancreatic ductal adenocarcinoma. Myeloid Cells, Breckenridge, Colorado, USA (8 Apr – 12 Apr 2018)
- II. **Je Lin Sieow**, Hweixian Leong Penny, Siew Cheng Wong
The role of neutrophils in the progression and metastasis of pancreatic ductal adenocarcinoma. Frontiers in Cancer Science, Singapore (7 Nov – 9 Nov 2016)
- III. **Je Lin Sieow**, Hweixian Leong Penny, Siew Cheng Wong
The role of neutrophils in the progression and metastasis of pancreatic ductal adenocarcinoma. 8th International Singapore Symposium of Immunology, Singapore (26 May – 7 May 2016)
- IV. **Je Lin Sieow**, Hweixian Leong Penny, Baptiste Janela, Florent Ginhoux, Siew Cheng Wong
Glut1 expression on myeloid cells in pancreatic ductal adenocarcinoma. Immunometabolism in Immune Function and Inflammatory Disease / New Frontiers in Understanding Tumor Metabolism, Banff, Alberta Canada (21 Feb – 25 Feb, 2016)
- V. **Je Lin Sieow**, Hweixian Leong Penny, Baptiste Janela, Florent Ginhoux, Siew Cheng Wong
Glut1 expression on myeloid cells in pancreatic ductal adenocarcinoma. International congress of the Federation of Immunological Societies of Asia-Oceania, Singapore (30 Jun – 3 Jul 2015)

VI. **Je Lin Sieow**, Hweixian Leong Penny, Baptiste Janela, Florent Ginhoux, Siew Cheng Wong

Glut1 expression on myeloid cells in pancreatic ductal adenocarcinoma. Cell Symposia: Cancer, Inflammation and Immunity, Melia Sitges, Spain (14 – 16 Jun 2015)

Awards

- I. Conference Sponsorship from the Cancer Science Institute (CSI) to present at the Frontiers in Cancer Science Conference (7-9 November 2016, Singapore).
- II. A*STAR Graduate Scholarship (Local), SIgN-NTU Immunology PhD Program

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