5-2023

Irreversible electroporation potentiates beta glucan induced trained innate immunity: a novel combination treatment for pancreatic adenocarcinoma.

Matthew Woeste
University of Louisville

IRREVERSIBLE ELECTROPORATION POTENTIATES BETA GLUCAN INDUCED TRAINED INNATE IMMUNITY: A NOVEL COMBINATION TREATMENT FOR PANCREATIC ADENOCARCINOMA

By

Matthew R. Woeste
B.S., Northern Kentucky University 2013
M.D., University of Louisville School of Medicine 2017

A Dissertation
Submitted to the faculty of the
School of Medicine of the University of Louisville
in partial fulfillment of the requirements
for the Degree of

Doctor of Philosophy
in Microbiology and Immunology

Department of Microbiology and Immunology
University of Louisville
Louisville, KY

May 2023

IRREVERSIBLE ELECTROPORATION POTENTIATES

BETA GLUCAN INDUCED TRAINED INNATE IMMUNITY: A NOVEL

COMBINATION TREATMENT FOR PANCREATIC ADENOCARCINOMA

Matthew R. Woeste, MD
B.S., Northern Kentucky University 2013
M.D., University of Louisville School of Medicine 2017

A dissertation approved on

04/14/2023

By the following dissertation committee

_____

Jun Yan, M.D., Ph.D.
Dissertation director

_____

Haribabu Bodduluri, Ph.D.
Dissertation co-director

_____

Venkatakrishna Jala, Ph.D.

_____

Huang-Ge Zhang, Ph.D.

_____

Jason Chesney, M.D., Ph.D.

_____

Kelly M. McMasters, M.D., Ph.D.

DEDICATION

This dissertation is dedicated to my wife,

Anna Christine Woeste, R.D.H

Whose love, support, and encouragement
sustained me through this challenge.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and instruction of my primary research mentor, **Dr. Jun Yan**. His generosity of time, patience, advice, and resources have transformed me into a better scientist, leader, and person. His standard of research and scientific inquiry should be the model to which all others are held. It has been my privilege to work under his direction and I look forward to our future investigations. I next must thank my mentor **Dr. Kelly M. McMasters** who inspired me to pursue a career in academic surgery. By his direction, the Division of Immunotherapy was created within the Department of Surgery, which afforded me this opportunity. It is an incredible honor to be one of his surgery residents at the University of Louisville. I strive to conduct myself by his philosophies pertaining to life and surgery. I also thank the members of my dissertation committee, **Dr. Haribabu Bodduluri, Dr. Venkatakrishna Jala, Dr. Huang-Ge Zhang,** and **Dr. Jason Chesney,** for their invaluable instruction and recommendations to this body of work. This dissertation was also heavily supported by collaboration with **Dr. Robert Martin, Dr. Yan Li,** and **Dr. Min Tan**. They were each instrumental in completing this dissertation and I sincerely thank them for their contributions. I also owe extensive thanks to my peers and fellow members of Dr. Yan's lab whose daily support and encouragement allowed me to persist through the often-expected frustrations and failures of research. The examples set forth by **Dr. Samantha M. Morrisey, Dr. Anne E. Geller, Dr. Rejeena Shrestha, Dr. Diego Montoya-Durango,**

Yunke Wang, Dr. Chuanlin Ding, and Xiaoling Hu are unmatched, and I will forever cherish their friendships. Thank you all for being a constant source of information, for teaching me the fundamentals, and intellectually challenging me. These acknowledgements would also not be complete without giving gratitude to my parents, **Robert and Kathy Woeste.** They raised me with an appreciation for hard work, dedication, and persistence without such skills none of my accomplishments would be possible. I am forever in debt to their sacrifices that afforded me the opportunities to become a surgeon-scientist. Finally, to my friends, family, and especially my wife and daughters, thank you for enduring the sacrifices made in pursuit of this goal—only they understand the burden of this vocation. Amelia, Gwen, and Eleanor—you can do anything!

ABSTRACT

IRREVERSIBLE ELECTROPORATION POTENTIATES
BETA GLUCAN INDUCED TRAINED INNATE IMMUNITY: A NOVEL
COMBINATION TREATMENT FOR PANCREATIC ADENOCARCINOMA

Matthew R. Woeste, MD

April 14th, 2023

Pancreatic cancer (PC) is a challenging diagnosis yet to benefit from advances in immune oncologic treatments. Irreversible electroporation, a non-thermal method of tumor ablation, is used in treatment of select patients with locally advanced unresectable PC and has potentiated the effect of certain immunotherapies. Yeast-derived particulate β-glucan induces trained innate immunity and successfully reduces murine PC tumor burden. This study tests the hypothesis that IRE may augment β-glucan-induced trained immunity in the treatment of PC. β-Glucan-trained pancreatic myeloid cells were evaluated *ex vivo* for trained responses and anti-tumor function after exposure to ablated and un-ablated tumor conditioned media. β-Glucan and IRE combination therapy was tested in an orthotopic murine pancreatic cancer model in WT and $Rag^{-/-}$ mice. Tumor immune phenotypes were assessed by flow cytometry. Effect of oral β-glucan in the murine pancreas was evaluated and utilized in combination with IRE to treat PC. The peripheral blood of patients taking oral β-glucan after IRE in human PC patients was evaluated by mass cytometry. IRE-

ablated tumor cells elicited a potent trained response *ex vivo* and augmented anti-tumor functionality. *In vivo*, β-glucan in combination with IRE reduced local and distant tumor burden prolonging survival in a murine orthotopic PC model. This combination augmented immune cell infiltration to the PC tumor microenvironment and potentiated the trained response from tumor-infiltrating myeloid cells. The anti-tumor effect of this dual therapy occurred independent of the adaptive immune response. Further, orally administered β-glucan was identified as an alternative route to induce trained immunity in the murine pancreas and prolonged PC survival in combination with IRE. β-Glucan *in vitro* treatment also induced trained immunity in peripheral blood monocytes obtained from treatment naïve PC patient. Finally, orally administered β-glucan was found to significantly alter the innate cell landscape within the peripheral blood of stage III locally advanced PC patients who had undergone IRE. These data highlight a relevant and novel application of trained immunity within the setting of surgical ablation that may stand to benefit patients with PC.

TABLE OF CONTENTS

PAGE

.

LIST OF FIGURES

Chapter III

INTRODUCTION

Pancreatic cancer; overview, epidemiology, and risk

The pancreas is an essential digestive organ possessing dual exocrine and endocrine function situated posterior to the stomach within the retroperitoneal compartment of the abdomen. The exocrine pancreas releases digestive enzymes from pancreatic acini, which follow its ductal system toward the second portion of the duodenum. The islets of Langerhans constitute the endocrine component of the gland and release hormones to the blood stream that regulate blood glucose metabolism.[1, 2] Aberrant growth of epithelial ductal and acinar cells give rise to one of the deadliest forms of cancer—pancreatic ductal adenocarcinoma (PDAC).[3] Clinically speaking, diagnosis of, "pancreatic cancer" (PC) is broadly accepted to be adenocarcinoma, unless otherwise specified, as PDAC accounts for nearly 90% of all PC cases.[4] In 2022, greater than 62,000 people will have been diagnosed with PC in the United States accounting for 3% of new cancer diagnoses. However, more concerning is that the mortality of PC approaches its incidence and almost 50,000 people will die of PC in the same year making it the third most common cause of cancer related death.[5,6]

Global trends in PC incidence and mortality are increasing.[7] PC is primarily a diagnosis of older age, with the majority (> 90%) diagnosed after age 55.[8] Further, risk of death from PC also increases with age with < 2 deaths per 100,000 person-years for

individuals aged 35-39 and > 90 deaths per equivalent person years in those > 80 years. The elderly patient population is rapidly growing, therefore, PC stands to place an increasingly significant burden on healthcare systems universally.[7, 9] From 1990 to 2017, there was a 2-3 times increase in PC diagnoses and mortality reported by collaborators of the Global Burden of Disease and was thought to reflect growing populations and ageing. These trends were correlative with socioeconomic status with high-income countries reporting 3 times higher rates of PC than low to middle income countries. Additionally, although not completely understood, the incidence and mortality of PC was consistently higher in males than females.[10] These findings are likely explained by differences in the prevalence of certain environmental and modifiable risks as well as inherent regional health care disparities.

Progression of PC is complex, however at the most basic histologic form local duct obstruction, inflammation, and epithelial injury lead to non-invasive epithelial precursor lesions referred to as pancreatic intraepithelial neoplasia (PanIN).[11] The estimated life time probability of progression from PanIN to PC is < 2% and has an indolent course, supporting further efforts at early detection.[12] While advancements of effective screening and detection markers ensue, it is important to have a firm knowledge of PC development and current defined risk factors. This will ultimately help to identify the most vulnerable patient populations. Several behavioral and inheritable risk factors may contribute to abnormal epithelial growth, but the exact cause of pancreatic cancer remains at large.

Inhaled tobacco products, alcohol consumption, and obesity are important modifiable risks for development of PC. In a 2009 pooled cohort analysis, from the

international Pancreatic Cancer Cohort Consortium, smokers had a nearly 2 times increased odds of a PC diagnosis, which positively correlated with tobacco use intensity.[13] However, PC risk is considered comparable to 'never-smokers' after 15-20 years of cessation, highlighting the importance of this behavior modification.[14] Epidemiologic investigations have also linked alcohol consumption with risk of PC, though may be limited to heavy use.[15, 16] A meta-analysis of 21 independent prospective studies published in 2007, including nearly 3.5 million people and over 8 thousand PC diagnoses, found a 12% increased relative risk of PC for every 5 kg/m^2 gain in body mass index.[17, 18] The effect of obesity on the pancreas is multifactorial yet putative mechanisms include release of pro-inflammatory cytokines, insulin resistance, and stimulation of proliferative pathways. The rising prevalence of obesity especially within developed countries may help to explain the increasing incidence of PC in these respective regions. As previously mentioned, increased age and male gender are two non-modifiable risk factors for development of PC, however several other host factors are well described. It is reported that up to 80% of new PC diagnoses may present with new onset diabetes mellitus (DM) or impaired glucose metabolism.[19] Numerous studies corroborate this association and patients with DM of increasing duration (10-20 years) seem to be at greatest risk.[20, 21] Prior investigations have also observed increased relative risk of developing PC in the setting of DM postulating whether DM is a risk or manifestation of disease.[22, 23] Pancreatitis, acute or chronic inflammation leading to fibrosis, scarring, and loss of acinar and islet cell function, is also heavily linked to development of PC. Acute pancreatitis (AP) has been reported as a potential early symptom of PC. In a matched cohort analysis of over 41 thousand patients in Denmark, AP was associated with an increased risk of PC even after 5 years.[24] Repeated

bouts of AP, lead to progressive and irreversible acinar cell destruction ultimately sustaining a state of pathologic gland fibrosis referred to as chronic pancreatitis. CP may be hereditary, idiopathic, or driven by prolonged exposure environmental toxins (i.e. smoking, alcohol) and is a well-known risk for PC.[25, 26] Similarly, each of these medical comorbidities lead to sustained states of pancreatic inflammation which promote neoplasia and eventual progression to carcinogenesis.

Inherited genetic syndromes and somatic mutations complement 5-10% of all PC diagnoses.[27] However, this phenomenon was only initially described as recently as the 1980s where prior knowledge relied solely on few case reports and observational studies.[28, 29] Although much understanding of the genetics behind PC remains in its infancy, a thorough family history will only continue to gain importance in patient care. Large cancer registry data and collaborations have clearly helped to recognize aggregates of PC in certain families.[30] In the broader sense, familial pancreatic cancer (FPC), is now being recognized as having two or more first degree relatives with PC diagnoses with exponential increases in risk with the number of relatives involved. Further complicating matters, FPC may be associated with various familial cancer syndromes and their underlying germline mutations such as; hereditary breast and ovarian cancer (BRCA 2, BRCA 1, PALB2), Peutz-Jeghers syndrome (STK11/LKB1), cystic fibrosis (ΔF508), familial adenomatous polyposis, Li-Fraumini syndrome (p53), Lynch syndrome (hMSH2), and hereditary pancreatitis (PRSS1).[23] Though more often, > 80% of PC arises due to sporadic and cumulative oncogenic mutations. Exosome sequencing has established KRAS as the most frequently mutated gene in PC (95%). Pyrosequencing methodology has revealed KRAS

mutations are present in more than 99% of the low grade PanIN-1 lesions. Further molecular profiling supports PanIN to PC progression through KRAS mediated inactivation of tumor suppressor genes CDK2NA, p53, and SMAD4 orchestrating a complex template for tumor maintenance. Despite the prevalence of KRAS mutation in PC no targeted therapies have translated to the clinic underscoring the difficulty in PC treatment.

Diagnosis, staging, and conventional treatment

Diagnosis and management of PC is challenging and presenting signs and symptoms of disease are highly dependent on location within the gland. Anatomic location and tumor biology often allow PC to remain symptomatically inconspicuous during early stages of disease (stage I-II). Therefore, many patients are diagnosed when local invasion or metastasis to distant organs has already occurred (stage III-IV).[31] Approximately 60-70% of PC is located within the head of the gland where outward growth may cause biliary or duodenal obstruction leading to jaundice, abdominal pain, weight loss, and anorexia. Clinical suspicion for PC should be investigated by dedicated multiphase computed tomography [32] imaging along with quantitation of the serum tumor marker CA19-9. Pathologic confirmation of PC is often achieved via endoscopic ultrasound and fine needle aspiration. After initial diagnosis and staging are complete, patients should be managed in a multidisciplinary setting comprised of medical oncologists, radiation oncologists, surgeons, and palliative care.

Prognostically, PC remains inferior to all other cancers with consistently worse overall survival (OS).[1] Adjusting for all stages of disease, the 5-year OS of PC is only 10% and outcomes have remained stable over the past two decades, demonstrating a desperate need for scientific advancements in treatment options.[33] Currently, the only chance for cure is early detection of localized disease amenable to complete surgical resection.[5] Yet, early detection is uncommon as PC has a propensity for local invasion and distant spread. To date, no effective or recommended screening tool exists capable of detecting asymptomatic disease making management of PC difficult as it is so often met at advanced and incurable stages.[34]

Complete surgical resection offers the best chance for long term survival, however, only 20% of patients are operative candidates at the time of diagnosis.[35, 36] Even with neoadjuvant (preoperative) chemotherapy followed by complete (R0) resection, the risk of recurrence is considerably high.[37] Tumors within the head of the pancreas are removed via a pancreaticoduodenectomy (Whipple procedure), while cancer in the body or tail of the gland require distal pancreatectomy and concomitant splenectomy. Localized tumors are said to exist on a "continuum" from surgically resectable to unresectable depending on adjacent vascular involvement.[38] Although feasible in select patients, more aggressive surgical approaches including lymphadenectomy, *en bloc* resections, and arterial reconstructions remain controversial.[39]

Unfortunately, even within the subset of patients who are candidates for resection many will experience pathologically positive margins or be found to have lymph node (LN)

involvement prompting further adjuvant (postoperative) treatment. Recently, preoperative systemic chemotherapy has been increasingly utilized in early-stage PC in effort to limit micrometastases, decrease LN spread, and potentially downstage certain patients.[40] However, systemic chemotherapy options remain extremely toxic and are based on modest improvements in patients with metastatic (stage IV) disease. Multiagent fluorouracil or gemcitabine-based chemotherapy are the current standard of care based on two landmark clinical trials. Median OS and objective response rates are significantly prolonged for stage IV patients treated with FOLFIRINOX compared to gemcitabine alone yet is associated with increased toxicity.[41] Von Hoff *et al.* found the addition of nab-paclitaxel (GEM/NP) significantly improved OS compared to gemcitabine alone.[42] Although an improvement, modern systemic chemotherapy treatment of metastatic PC leaves much to be desired with only 10% 2-year OS.

A major unmet need in the field of oncology is in treatment of stage III locally advanced PC (LAPC). 35% of patients will have unresectable LAPC at the time of diagnosis. LAPC is deemed unresectable due to degree of tumor contact or encasement with the adjacent superior mesenteric artery, celiac artery, superior mesenteric vein, or portal vein. Studies have shown that LAPC patients who receive resection with macroscopically positive margins have similar OS to those who are not resected. Palliative chemotherapy with either FOLFIRINOX or GEM/NP continue to define care in LAPC (median OS of 6 to 24 months).[43] Radiation therapy has been utilized in LAPC yet consistent data to support improvements in primary outcomes are lacking.[44] Although many patients with PC will die of distant metastasis (liver, lung) a third of patients die of PC due to local progression supporting efforts to consolidate local disease control.[45]

Immunotherapy in pancreatic cancer

Paul Ehrlich was first to hypothesize neoplastic cells could be eradicated by host immune cells. The "immunosurveillance hypothesis," stated by Lewis Thomas and Sir Frank McFarland, supposed that tumor associated neoantigens can be recognized and targeted by the immune system to prevent tumorigenesis at an early stage.[46-48] However, it would take over one century for scientific advancements to have real clinical significance. Presently, 'immunotherapies' are poised to take precedent in many cancer treatment algorithms owing to incredible treatment responses observed in previously fatal malignancies. Unfortunately, the success of immunotherapy has not been consistent across all cancer subtypes, especially when applied to PC.[49]

Melanoma was the first tumor subtype for which immune based oncologic treatments were approved and serves as a model for the potential value of immunotherapy in other cancer types. In 2011, the immune checkpoint inhibitor (ICI), Ipilimumab, targeting cytotoxic T lymphocyte antigen- 4 (CTLA-4) was approved by the Food and Drug administration [50].[51] CTLA-4 is a cell surface protein that acts to regulate T cell priming by outcompeting the co-stimulatory receptor CD28. CTLA-4 delivers inhibitory signals to reduce T cell activation. Therefore, monoclonal antibody blocking of CTLA-4 allows for de novo T cell activation and anti-cancer responses.[52] Shortly thereafter Pembrolizumab, the ICI targeting programmed death receptor 1 (PD-1), was approved in 2014.[53] PD-1 acts to limit effector T cell activity by binding to its ligands PD-L1 and PD-L2 to reduce T cell function. Anti-PD-1 treatments allow for enhanced T cell activation, cytotoxicity, and

cytokine production.[54] Current studies estimate a 20% increase in median overall survival and 50% durable survival benefit of patients with melanoma since the advent of these treatments.[55] Despite such promise and illustrative examples in melanoma, PC remains particularly resistant to immune based oncologic therapy pointing to inherent differences in tumor biology.

Clinical trials of conventional immunotherapy in PC are underwhelming and it is particularly evident that monotherapy is inadequate. In a phase II trial comprised of locally advanced (LA) and metastatic PC patients, there were no tumor responses from 27 participants who received Ipilimumab.[56] Similar lackluster responses in PC have been documented with PD-1/PD-L1 trials. In a multicenter phase I trial of anti-PDL-1 antibody in treatment of advanced stage cancers, which included 14 patients with PC, there were no objective responses.[57] CTLA-4 and PD-1 therapies in combination treatment of PC have met equal resistance. Duffy *et al.*, reported their pilot study of pre-treated metastatic PC patients treated with Durvalumab (IgG1 monoclonal antibody [mAb] targeting PD-L1) or Durvalumab plus Tremelimumab (IgG2 mAb against CTLA-4) and stereotactic body radiation therapy (SBRT). No objective responses were reported and only 21% had stable disease during the study period.[58] Systemic chemotherapy combined with ICI has also been disappointing. For example, only two of 28 patients treated with CTLA-4 and gemcitabine had partial responses.[59] In another study evaluating gemcitabine with CTLA-4, two patients had PR and five had stable disease.[32] Perhaps the only positive ICI results in PC have been those with mismatch repair deficiency (MMR) treated with PD-1 blockade. In 2017, Le *et al.* published their findings in evaluating patients with MMR

across 12 subtypes of advanced stage cancers. Although, the study only included eight patients with PC, there was a 62% ORR.[60] Unfortunately, subsequent results of PD-1 inhibition in 22 patients with PC from KEYNOTE-158 were not as promising with an ORR of 18.2% and median survival of 4 months.[61] These studies provide clinical evidence of the resistance to ICI in PC and demonstrate that a "one size fits all" mentality clearly will not work in PC treatment.

Pancreatic cancer tumor microenvironment

PC's resilience to conventional immunotherapy has deemed it an "immunologically cold tumor."[62] A notoriously immunosuppressive tumor microenvironment (TME) with complex and multifaceted resistance mechanisms make PC a model for cancer immune evasion.[49] Intrinsic immunosuppressive properties including physical barriers, T cell exhaustion, and low neoantigen abundance all contribute to the molecular difficulty treating this disease. However, efforts to understand the PC TME are increasing and ultimately will allow for better immune oncologic treatment alternatives.[63]

PC is well known to possess a dense and heterogeneous desmoplastic stroma that physically limits systemic drug delivery.[64] The stroma occupies approximately 70% of the tumor mass and is comprised of extracellular matrix (ECM) components and non-neoplastic cells.[62, 65] Pancreatic stellate cells (PCSs) are quiescent peri-acinar fibroblasts that regulate ECM deposition, store fat droplets, and vitamin A under homeostatic conditions. During periods of acute and chronic inflammation, PSCs undergo morphologic changes leading to dysregulated ECM deposition and increasing fibrosis of the TME. This

desmoplastic response increases tumoral interstitial fluid pressure thereby decreasing tumor perfusion via small vessel collapse.[66] Thus, a structural and functional barrier is created via accompanying hypoperfusion and hypoxia driving tumorigenesis. Importantly, this dense scaffold of stroma is also characteristic of PC metastases complicating treatment beyond the primary tumor.[67]

PC's hypoxic TME is self-sustaining and perpetually recruits immunosuppressive cell populations including myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), and T regulatory cells (Tregs).[68] MDSCs are immature myeloid cells which have been shown to promote tumor growth through several mechanisms including Arginase-1 production, iNOS and ROS upregulation, and recruitment of Tregs.[69-71] PC mobilizes MDSCs from the bone marrow and their quantity has been positively correlated to increased disease stage.[72-74] Bone marrow myeloid progenitors are influenced by granulocyte monocyte colony stimulating factor (GM-CSF) to differentiate into MDSCs and migrate into the periphery towards the tumor. Interestingly, tumor-derived GM-CSF is produced in large amount by malignant pancreatic epithelial cells, which suppresses anti-tumor T cell activity.[75] Furthermore, GM-CSF has been expressed *in vivo* from PC patient tumor cells and efforts to block GM-CSF has demonstrated anti-PC tumor effect.[75, 76] Intimately associated with MDSCs are TAMs whose quantity has also been correlated with worsening outcome in PC.[77] Under homeostatic conditions macrophages are normally tasked with clearing debris and antigen presentation. However, PC disease progression influences TAMs to obtain an M2 anti-inflammatory phenotype, expressing CD163 and IL-10. M2 macrophages then release cytokines that activate STAT3 and NF-κB—

stimulating PC stem cells. In similarity to MDSCs, M2 TAMs promote tumor progression and dampen host anti-tumor immune activity through release of TFGF-β, IL-10, and Arginase-1.[78-80] TAMs may also promote phenotypic switching (M1 to M2) of tumor infiltrating macrophages and downregulate effector CD8+ T cells and NK cell function. Several studies have shown that MDSCs can directly differentiate into TAMs within the TME creating an unending cycle of tumor progression.[81] Further, these innate cells are capable of cross-talk with FOXP3+ Tregs promoting their differentiation, development, and accumulation in the TME. Tregs portend worse outcomes in PC. Given this data, these cells have become important targets when considering novel PC therapies.

Further contributing to an exuberant TME is a low quality and quantity of neoantigens for cell surface presentation. One reason for this is due to a lack of tumor mutational burden in PC. Comparatively, the number of somatic mutations in PC is approximately 1 per DNA megabase (Mb), whereas melanoma may produce up to 10 mutations per Mb. Whether such mutation leads to an immunogenic epitope is an even more rare event.[82, 83] Neoantigen status is important as it positively correlates with overall survival.[84] Tumor infiltrating lymphocytes (TILs) are also associated with mutant specific neoantigens, which are more prominent in ICI responsive tumors such as renal cell carcinoma (RCC) and melanoma. MMR deficient PC increases mutational load and positively impacts TILs, however, represents only 1% of PC. Abundance of TILs may not be the problem in PC, but rather the absence of T cell activation status. TCGA analysis of LCK T cell expression in PC has been noted to be high, whereas IFNγ production is low meaning there is a robust although inactive TIL population.[85]

Thus far we have appreciated the complexity of PC as a disease. PC portends a grim prognosis with multifarious risk and is reliant on systemic treatments which are toxic, non-specific, and largely ineffective. We have also identified a large percentage of patients who present with unresectable locoregional disease. Traditional immunotherapy and monotherapies have failed to help patients with PC. Therefore, novel combination therapies that positively alter the TME and immunosuppressive cell populations should be considered.

Irreversible electroporation in pancreatic cancer

Given the significant number of patients who present with LAPC, intense efforts to harness local growth and progression of PC have ensued. Electroporation is the act of permeabilization of the cell membrane using a pulsed electrical field. Depending on voltage amplitude or duration the electroporation effect may be reversible or irreversible. Electroporation has originally been used in drug delivery and genes into tumor cells for several decades. In 2005, Davalos et al proposed the novel application of IRE for use on tumor tissue.[86] IRE delivers targeted high voltage electric pulses across the tumor inducing an apoptotic cell death via creation of permanent cell membrane porosity. Compared to alternative ablation techniques, IRE is said to be 'non-thermal' and elicits cell death without disruption of collagenous tissue structures.[87] This provides advantage in the setting of LAPC whose tumors often encompass unresectable abdominal vessels providing the opportunity to inhibit tumor growth without disrupting vital blood flow. Initial efficacy of IRE demonstrated in cutaneous and subcutaneous tumors percolated interest in applying this ablation modality to abdominal tumors.[88-91]

Clinical applications of IRE in the setting of pancreatic cancer are promising. In 2012 Martin *et al.* first published on the safety and efficacy of IRE utilized in 27 patients with LAPC.[92] These results were then followed by potential increases in progression free and overall survival when compared to LAPC patients treated with standard chemotherapy or radiotherapy alone.[93] Subsequently, there have been a number of retrospective and prospective studies reporting on survival benefit of IRE in PC, however no randomized clinical trials exist, which have hampered enthusiasm for widespread application of IRE in LAPC as standard clinical practice.[94, 95] Furthermore, there is no agreed upon treatment protocol leading to large variances in treatment applications.[96] Despite these limitations, IRE has potential to dramatically benefit LAPC patients and warrants further investigations as we await more definitive data.

Recently, studies have demonstrated the ability of IRE to circumvent the immunosuppressive PC TME improving the efficacy of immunotherapy, delivery of chemotherapy, and positively alter the peripheral immune landscape in patients. Comparisons of gemcitabine concentration in an orthotopic murine PC model were higher in mice also treated with IRE.[97] IRE in combination with anti- programmed death protein-1 (anti-PD-1) significantly prolonged survival in a murine orthotopic PC model by alleviating tumoral hypoxia.[98] IRE has also potentiated dendritic cell (DC) therapy previously shown to be ineffective in PC.[99] IRE in conjunction with a toll-like receptor-7 (TLR-7) agonist and anti-PD-1 eliminated untreated distant tumors.[100] Analysis of peripheral blood in two small clinical cohorts of PC patients treated with IRE demonstrated

positive yet transient alterations in Treg populations.[101, 102] Although promising, there remain large gaps in knowledge regarding local and systemic immune effects of IRE.[103] These studies clearly highlight the complementary role of IRE in treatment of PC, perhaps holding a key to overcoming PC's resistance to immune oncologic treatments.

Trained immunity in cancer therapy

Classic immunology has traditionally described the immune system as an academic oversimplification to be composed of two separate and independent arms—innate and adaptive. Where the innate immune system is rapid, non-specific, and primitive the adaptive immune system is more evolved, specific, and has capacity for memory. However, contrary to this classic immune dichotomy, innate immune cells have been recently shown to possess a 'de facto' immunologic memory. Early research involving vaccines in the 19th century helped to uncover this phenomenon. Bacillus-Calmette-Guérin (BCG) vaccination in children improved survival and protected against subsequent infections beyond its intended target Mycobacterium tuberculosis.[104] Further epidemiologic studies touted similar off target effects.[105, 106] Netea *et al.* were the first to synthesize the available data recognizing that innate cells remained in an enhanced reactive state after an initial insult. This process, was coined "trained immunity," and is defined as an enhanced cellular response (also known as the trained response) of trained innate cells following a secondary stimulus.[107, 108] These early epidemiologic vaccination studies were then followed by findings of increased cytokine production after non-specific restimulation of the blood in vaccinated individuals.[109, 110] Whole genome histone modification studies have elucidated that trained immunity is composed of epigenetic, transcriptomic, and

metabolic reprogramming at the molecular level. For example, changes in histone acetylation markers (H3K4me1, H3K4me, and H3K27Ac) and chromatin accessibility lead to altered gene expression allowing for augmented secondary innate responses. This feature is now considered a hallmark of trained immunity.[111, 112]

In addition to the BCG vaccine, there are many other biologics that may induce trained immunity, however by far the most studied agonist is the *Candida* cell wall component β-1,3-(D)-glucan (β-glucan). Initially utilized by Netea's group as a negative control, fate would have it that *C. albicans* provided a robust monocyte IL1-β and TNF-α response in BCG vaccinated individuals stimulating a myriad of further studies.[113] Thereafter, exposure of mice to *C. albicans* provided protection against their reinfection in the absence of adaptive immune cells.[114] Mechanistically, β-glucan has been shown to activate the mammalian target protein of rapamycin, mTOR, via a dectin-1 dependent HIF-1α pathway ultimately increasing aerobic glycolysis.[115, 116] Long-term effects of trained immunity are regulated by reprogramming of myeloid central progenitors in the bone marrow, termed central trained immunity.[117-119] This is a particularly important concept with potential benefits when applied in the context of deranged cancer induced myelopoiesis. Perhaps, β-glucan may offer a way to regulate aberrant bone marrow hematopoiesis.

Trained immunity agonists, β-glucan and BCG, have previously been shown to exert anti-tumor activity.[120, 121] BCG has long been used in the treatment of bladder cancer, where vesicular instillation of BCG have demonstrated up to 70% response rates of non-

muscle invasive bladder cancer.[122, 123] Eastern medicine practice has also utilized the natural compound β-glucan in treatment of cancer for centuries.[124] Anti-tumor activity of β-Glucan is mediated by CR3 complement activation, thereby increasing cytotoxicity and phagocytosis of opsonized tumors.[125, 126] Keeping in mind the scope of this dissertation, yeast-derived β-glucan has previously exhibited many immunologic effects that may be beneficial if applied to the PC immunosuppressive TME. First, β-glucan has been shown to convert inflammatory/suppressive M2 macrophages into an M1 phenotype, which occurs through C-type lectin dectin-1 pathway.[127] Tian *et al.* described treatment of mice with orally administered β-glucan which downregulated MDSCs, increased infiltrating DCs, macrophages, and slowed tumor progression.[128] Prior work from our lab has supported the anti-tumor effects of β-glucan via activation of DCs expanding antigen specific CD4+ and CD8+ T cells, increasing IFN-γ production and reducing tumor burden.[129] β-Glucan has even allowed for conversion of Tregs into T-helper cells allowing for a more primed T cell response.[130] These studies strongly argue for the use of trained immunity in rebalancing the dysregulated PC TME.

Scope of the current study

Serendipitously, while studying the trafficking of β-glucan we recently reported that intraperitoneal (IP) yeast-derived particulate β-glucan infiltrates the murine pancreas in large quantity and incites trained immunity controlling PC tumor progression in an aggressive orthotopic PC mouse model. The infiltrating myeloid cells had epigenetic histone modifications consistent with trained immunity and increased cytokine TNF-α production. Further, treatment of tumor-bearing mice with IP β-glucan cooperated with

anti-PDL-1 therapy and prolonged survival.[131] These findings have huge translational potential and inspired much of the current work. At the University of Louisville, as a tertiary referral center, we provide care to many patients with LAPC and have extensive experience in utilizing IRE in this patient subset.[132] In this study, the hypothesis that IRE would enhance the anti-PC efficacy of β-glucan by inducing potent trained response is investigated. The work herein first set out to determine whether IRE could provoke and augment the trained response and functionality in β-glucan-trained pancreatic myeloid cells. Second, we wanted to determine whether this combination therapy would have *in vivo* treatment benefit. Third, would oral β-glucan which has an easier route of administration and translatability also incite trained immunity in the murine pancreas? Finally, we sought evidence for trained immunity in combination with IRE in LAPC patients. In this study, the hypothesis that IRE would enhance the anti-PC efficacy of β-glucan by inducing potent trained response is investigated. We demonstrate an effective and novel PC treatment combination whereby β-glucan-mediated trained immunity is augmented by IRE. This combination involves a pronounced and activated myeloid immune cell population within the PC TME. These findings suggest that the combination of trained immunity with IRE warrants further investigation by clinical trials.

CHAPTER I

IRE OPTIMIZES THE β-GLUCAN TRAINED RESPONSE IN PANCREATIC MYELOID CELLS AND ENCHANCES ANTI-TUMOR FUNCTIONALITITY *EX VIVO*

INTRODUCTION

Trained immunity only recently emerged as a cancer therapeutic, expanding its relevance beyond host vaccination responses and protection against secondary infection.[133] Mounting evidence exists supporting anti-cancer mechanisms underlying trained immunity.[131, 134, 135] However, there is a dearth of information practically applying trained immunity to relevant tumor models and clinical treatment scenarios. Additionally, etiologies to provoke the trained response against carcinogenesis have not been reported, despite secondary stimuli being a critical component to engage the benefit of trained immunity.

Our previous findings of the pancreas as a specific target of IP β-glucan trafficking and induction of peripheral trained immunity have meaningful implications in translating alternative immunotherapies to PC patients.[131] Despite these encouraging findings, much of this data utilized prophylactic β-glucan prior to tumor challenge and all mice eventually succumbed to disease prompting further investigations. For example,

how may the trained response be better activated or engaged in the setting of cancer? What level of trained response is beneficial for cancer therapy? What factors have the capacity to act as a secondary stimulus in the setting of β-glucan induced trained immunity? Can the trained response be actively engaged, while also controlling for tumor progression?

The immune modulatory potential of IRE in synergy with immunotherapy is becoming recognized in treatment of solid tumors.[136] The release of tumor antigens, pro-inflammatory factors, and damage-associated molecular patterns (DAMPs) by IRE provides a potential avenue to engage the trained response.[100] Cell exposure to IRE is well known to increase synthesis and release of DAMPs and other danger signals.[137] Our previous study showed that β-glucan trained pancreatic myeloid cells elicit a trained response upon stimulation of tumor-derived DAMPs. Therefore, we first reasoned that IRE could be utilized to trigger a trained response via release of DAMPs that may potently active trained myeloid cells derived from the murine pancreas.

IRE results in DAMP release and elicits a potent trained response.

Enhanced TNF-α cytokine responses from β-glucan trained pancreatic CD11b+ myeloid cells after secondary exposure to tumor conditioned media have been previously observed.[131] Macrophage migration inhibitory factor (MIF) is one example of a DAMP released by PC cells capable of eliciting the trained response. However, it is unknown how increasing doses of MIF affect trained pancreatic CD11b+ TNF-α expression or if the level of trained response may be augmented. To address this, pancreatic myeloid cells from IP β-glucan trained mice were secondarily exposed to recombinant MIF (rMIF) at increasing doses *ex vivo*. As demonstrated in Figure 1.1A, rMIF increased β-glucan trained pancreatic CD11b+ myeloid cell TNF-α production in a dose dependent manner. Prior studies have shown that IRE induces an immunogenic cell death. Further, exposure of PC cells to IRE releases DAMPs and other pro-inflammatory factors.[98, 99] We then compared MIF levels by ELISA from the tumor conditioned media of cultured KPC cells to IRE ablated KPC cells. After IRE, the concentration of MIF within the IRE ablated supernatants increased nearly 80 times that of KPC conditioned media alone (Figure 1.1B). We next examined whether IRE conditioned media may better serve as a secondary stimulus compared to tumor conditioned media alone. Indeed, trained pancreatic CD11b+ myeloid cells released more TNF-α (Figure 1.2A) and IL-6 (Figure 1.2B) when exposed to ablated IRE conditioned media compared to unablated KPC media alone. We then validated these findings by quantifying intracellular TNF-α by flow cytometry after short term culture (4

hours). Again, β-glucan-trained myeloid cells stimulated with supernatants derived from IRE treated KPC cells increased intracellular TNF-α cytokine production compared to KPC media alone in both percent and mean fluorescent intensity (MFI) (Figure 1.3). This data further supports that soluble factors released from IRE ablated PC cells can act as the secondary stimulus within the setting of trained immunity and that the specific levels of trained response can be augmented by increased doses of secondary stimulus.

IRE enhances phagocytosis and cytotoxicity in β-glucan trained myeloid cells.

Pancreatic CD11b+ myeloid cells also have an enhanced anti-tumor functional state after β-glucan training.[131] We next sought to test whether β-glucan trained CD11b+ myeloid cells re-exposed to IRE ablated media may demonstrate enhanced anti-tumor characteristics. Secondary *ex vivo* re-exposure of β-glucan-trained CD11b+ cells with IRE conditioned media compared to KPC conditioned media led to an increase phagocytosis of KPC^{GFP+} tumor cells in a dose dependent manner as demonstrated by increased frequency and MFI of CD11b+GFP+ cells (Figure 1.4). Next, we co-cultured trained CD11b+ cells in the presence of luciferase expressing, IRE ablated or un-ablated, KPC cells and quantified cytotoxicity via resulting luciferase expression within the cell culture supernatants. Trained CD11b+ myeloid cells demonstrated an enhanced cytotoxicity via measured dead cell luminescence and percent cytotoxicity when co-cultured with IRE treated KPC cells compared to untreated KPC cell controls (Figure 1.5). Taken together, these results suggest that IRE ablation results in increased release of DAMPs such as MIF in PC cells that induces a potent trained response and better promote anti-tumor innate immune responses.

CHAPTER I FIGURES

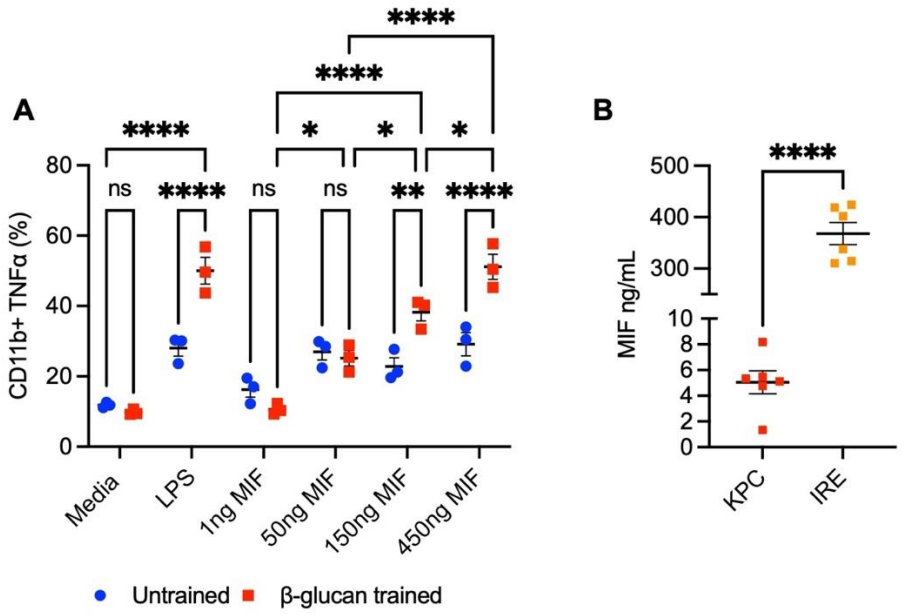


Figure 1.1: MIF elicits trained innate response in a dose dependent manner and is released from PC cells by IRE.

(A) Summarized pancreatic CD11b+TNF-α+ cells from untrained versus β-glucan trained mice upon *ex vivo* restimulation with LPS or increasing doses of rMIF assessed by flow cytometry, n=3 per group. (B) Concentration of MIF (ng/mL) in supernatants after culture of $1x10^6$ KPC cells for 24 hours or IRE ablation of KPC cells as measured by ELISA, n=6 per group. Data presented as mean ± SEM. Significance: ns= not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

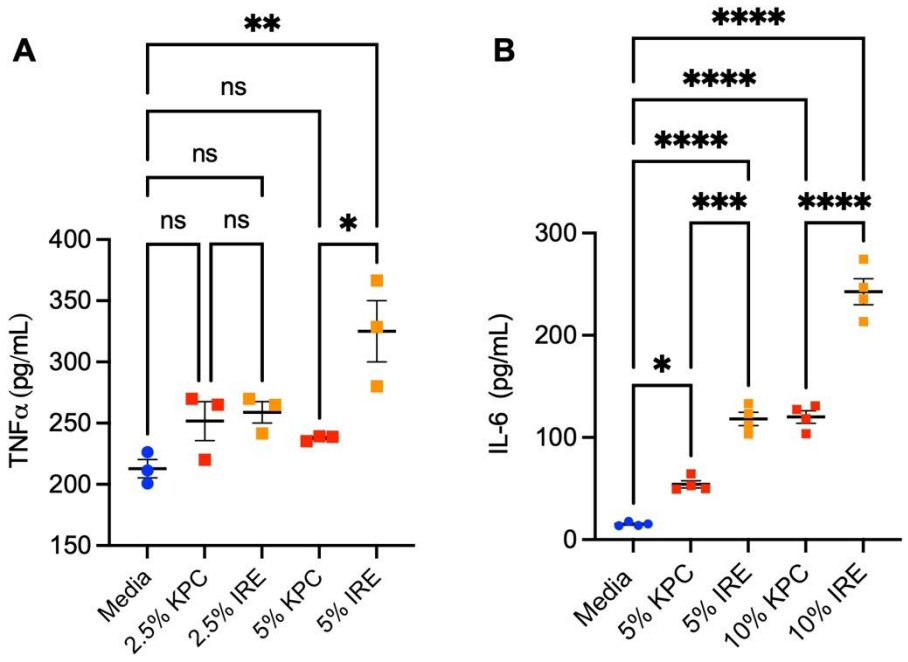


Figure 1.2: IRE potentiates the trained response *ex vivo* from pancreas derived myeloid cells.

(A) TNF-α and (B) IL-6 (pg/mL) levels measured by ELISA from β-glucan trained CD11b+ pancreatic cells restimulated *ex vivo* with KPC conditioned media or IRE conditioned media for 24 hours. Data presented as mean ± SEM. Significance: ns= not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

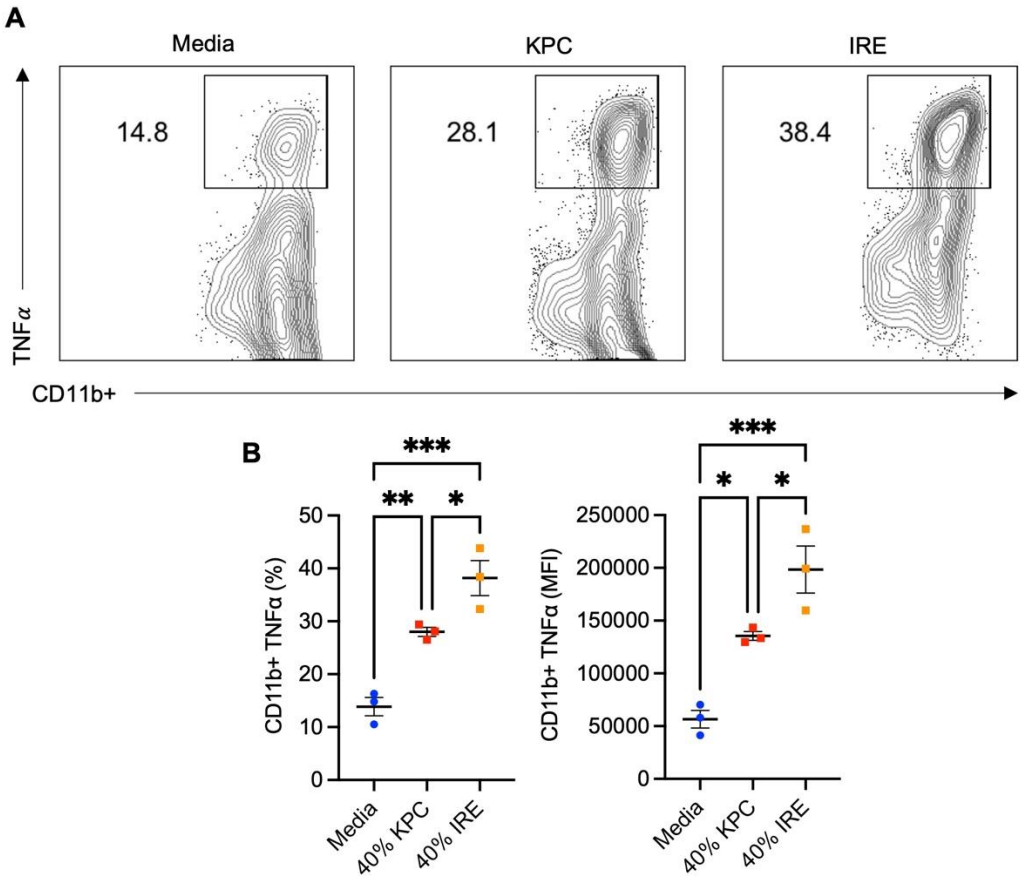


Figure 1.3: Validation of enhanced trained response of pancreas derived myeloid cells via flow cytometry after exposure to IRE conditioned media.

(A) Representative flow cytometry contour plots and (B) quantified percent and MFI of β-glucan trained CD11b+TNF-α+ cells restimulated with media control, KPC conditioned media or IRE conditioned media, n=3 per group. Data presented as mean ± SEM. Significance: ns= not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

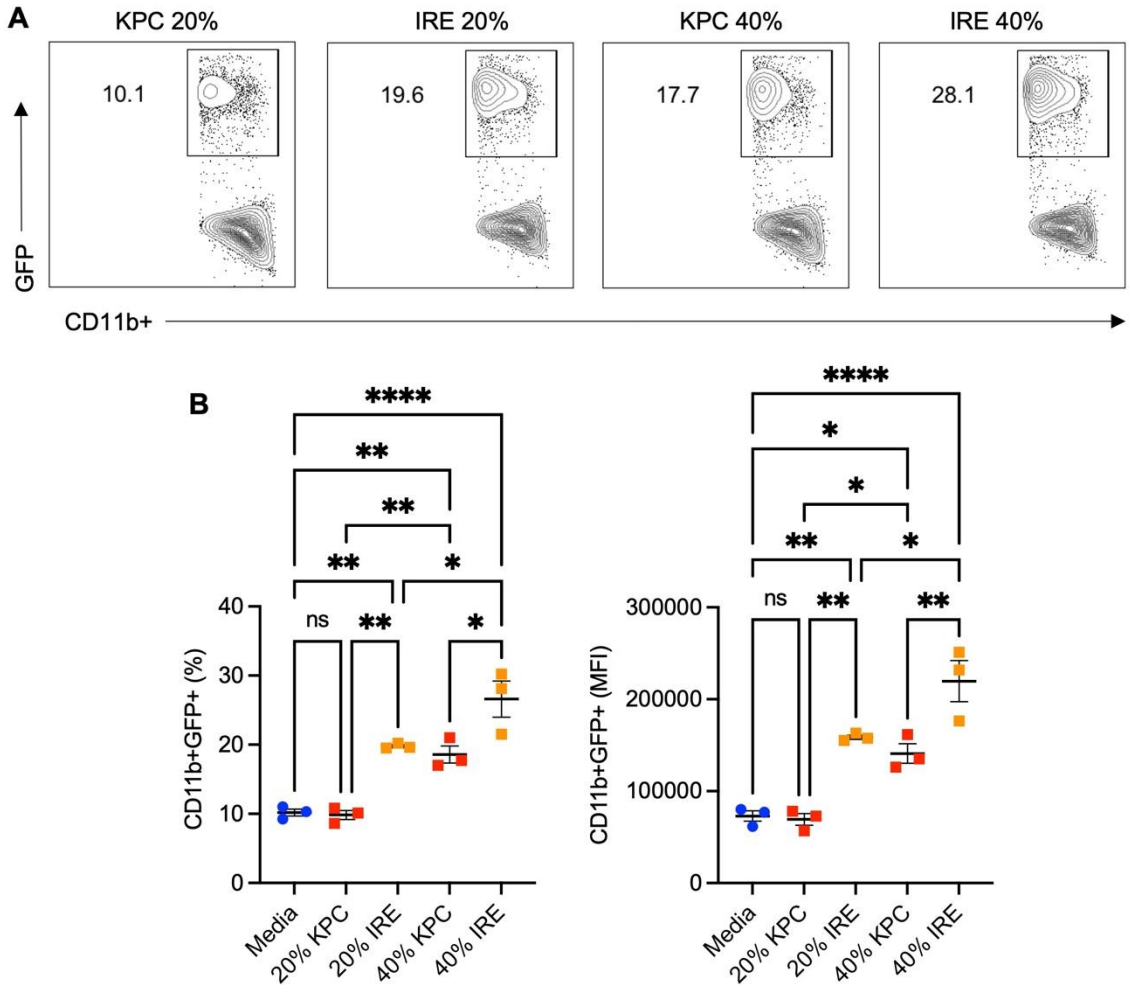


Figure 1.4: IRE increases phagocytotic activity of β-glucan trained myeloid cells.

β-glucan trained pancreatic CD11b+ cells were stimulated with media alone or KPC supernant or IRE KPC supernatant and then cocultured with KPC^{GFP+} tumor cells. (A) Representative flow plots and summarized data (B) are shown. Data presented as mean ± SEM. Significance: ns= not significant; *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

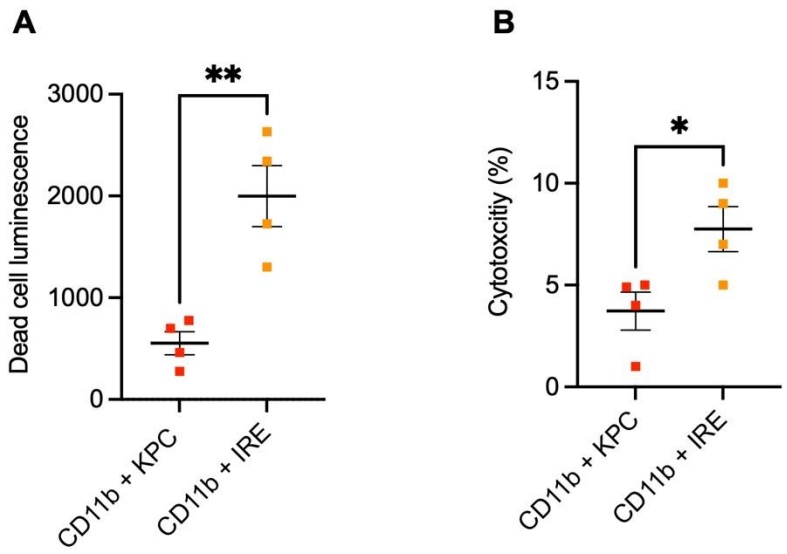


Figure 1.5: IRE augments cytotoxicity of β-glucan trained myeloid cells.

(A) Summarized dead cell luminescence and (B) percent cytotoxicity assay after CD11b+ cells from 7-day β-glucan trained mice were incubated at a ratio of 1:30 KPC^{Luc+} to CD11b+ cells for 24 hours, n=4 per group. Data presented as mean ± SEM. Significance: ns= not significant; *$p < 0.05$, **$p < 0.01$.

CHAPTER I DISCUSSION

Absence of immunologic memory has been generally accepted to be a distinguishing characteristic of the innate immune system for centuries and only recently was the evolution of an immunological memory first established in plants and invertebrates. A study by Kurtz *et al.,* in the early 2000s demonstrated that crustaceans were capable of defense against reinfection to tapeworms which was dependent on antigenic resemblance between consecutively encountered species.[138] Building upon these findings vertebrate species have portrayed similar responses suggesting innate immune memory to be an evolutionary conserved phenomenon.[139]

Heightened innate reactions to a secondary challenge is the hallmark of trained immunity, whereby host protection is inferred against an unrelated pathogen.[140] However, until now, no studies have discussed or demonstrated methods to provoke or augment the level of trained response despite secondary stimuli being one of the basic tenants of trained immunity. Studies have demonstrated exogenous pathogen associated molecular patterns (PAMPs) or DAMPs induce trained immunity, but fail to focus intently on the secondary response, which we feel to be particularly critical in the setting of cancer treatment or prevention. As stated above, epigenetic, metabolic, and transcriptomic reprogramming constitute the cellular mechanistic underpinnings of trained immunity. These changes lead

to classic cytokine signatures including increased IL-6, TNF-α, and IL-1β, which are considered surrogate markers of training.[141, 142]

The most important finding from part one of this study is that supernatant derived from IRE ablated cancer cells incited a more potent trained response from β-glucan trained myeloid cells compared to tumor conditioned media alone. We first reported that tumor conditioned media may specifically function as the secondary stimulus due to inherent release of DAMPs, such as MIF.[131] However, in the practical sense, this would require tumor progression or development to release soluble factors to act as the secondary stimulus toward trained innate cells—an undesirable outcome particularly in the setting of an aggressive malignancy such as PC. Considering IRE has previously been reported to release DAMPs and other pro-inflammatory factors, we were prompted to posit this ablation modality as a more proactive method to reactivate the trained innate cells while simultaneously controlling tumor growth. Indeed, here in part one we found a substantial release of MIF from cultured KPC cells by IRE compared to spontaneous release to the culture media. Further, re-exposure of β-glucan-trained pancreatic myeloid cells to IRE ablated media increased their level of trained response compared to tumor conditioned media alone. Aside from trained immunity, innate immune cells are well known to possess inherent anti-cancer functions such as direct cell mediated cytotoxicity and phagocytosis.[143] Upregulation of these functions can be achieved via β-glucan alone yet more notably, we demonstrate these functions can be augmented via the action of IRE ablation. These data are the first to provide a novel and clinically available secondary

stimulus capable of engaging and intensifying the level of trained response from myeloid cells.

CHAPTER II

IRE AND β-GLUCAN SYNERGIZE *IN VIVO* AGAINST PANCREATIC CANCER

INTRODUCTION

The *ex vivo* experiments above demonstrated that IRE may be utilized as a secondary stimulus to elicit trained responses from β-glucan exposed myeloid cells. Further, their response and innate anti-tumor functions were not only provoked but augmented by IRE compared to tumor conditioned media. This provided us with strong rationale to test this combination *in vivo* against an already established orthotopic PC tumor model.

Herbal supplements and natural compounds have long been used as immune modulators to fight against cancer, however anti-tumor immune effects of trained immunity agonists have only recently been described in both prevention and treatment.[144-146] β-Glucan has already been strongly linked to immunotherapy efficacy. Orally administered β-glucans enhanced anti-tumor effect of mAb against established murine xenograft tumors.[147] Early work out of our lab by Li *et al*., utilizing particulate β-glucan demonstrated trafficking to the spleen and lymph nodes, ultimately activating DCs and increased IFN-γ production by T cells.[129] Geller's study was the first to describe the

anti-PC effect of IP β-glucan, which also synergized with ICI therapy.[131] From this original study we postulated several complementary questions. First, is it possible to engage the trained response in the setting established PC tumors? How may we obtain meaningful prolongations in local control and progression free survival beyond statistically significant overall survival? Next, while mechanisms for epigenetic and metabolic remodeling are continuing to be recognized, the duration of training is a subject of intense investigation. Could the duration of training by β-glucan be prolonged by IRE? Adaptive immune cells are critical in anti-tumor immunity, however, the lack of evidence to support ICI in PC questions their roles in treating this particular solid tumor.[148] Furthermore, the anti-tumor effect of β-glucan has previously been shown to occur independent of the adaptive response.[131, 149] In considering prior IRE studies, adaptive anti-tumor immunity has been provoked by ablation.[150-152] How will this combination impact the adaptive immune phenotype?

IP β-Glucan's specific tropism to the murine pancreas accompanied by a robust influx of trained myeloid cells leads to reduced PC tumor burden with prolonged survival.[131] IRE alone has been partially effective in treatment of solid tumors, positively altering the immunosuppressive PC stroma, and has been cooperative in combination with immunotherapies.[150, 153, 154] Given these findings and our aforementioned *ex vivo* results, we hypothesized that the combination therapy of β-glucan and IRE would further reduce PC tumor burden compared to β-glucan or IRE alone and prolong overall survival.

CHAPTER II RESULTS

β-Glucan in combination with IRE reduces murine PC tumor burden and prolongs survival.

To address this hypothesis, wild-type (WT) C57Bl/6 mice were first challenged with 1 x10^5 KPC cells on day 0 via orthotopic injection into the tail of the pancreas near its bifurcation. The tumors were allowed to progress for 7 days before mice were treated/trained with IP β-glucan. An additional 7 days were allowed for sufficient training to occur before mice underwent IRE or sham placebo surgery on day 14 (Figure 2.1). Notably, β-glucan treated mice had reductions in maximum tumor diameter compared to PBS control treated mice at the time of IRE or Sham surgery (Figure 2.2). The mice then were monitored postoperatively for 10 days before humane euthanasia on study day 24 or were continually monitored for survival. On day 24, tumors were then grossly compared (Figure 2.3a). Analysis of tumor burden first confirmed efficacy of IP β-glucan and IRE treatments as monotherapy. In comparison, combination therapy achieved a significant reduction in tumor burden as demonstrated by measurements of tumor weight and maximum tumor diameter (Figure 2.3b). To determine whether the combination therapy would have beneficial long-term implications for PC in this aggressive tumor model, we then followed these mice observing for survival. The data depicted in Figure 2.4 demonstrate single IP treatment with β-glucan and IRE ablation alone significantly prolonged survival compared to PBS controls. The combination of IP β-glucan and IRE

demonstrated an 81% increase in survival (median 70.5 days) compared to PBS control (median 39 days).

IRE enhances myeloid cell epigenetic and transcriptomic reprogramming in β-glucan treated tumors.

Epigenetic and transcriptomic reprogramming are hallmarks of trained immunity, therefore we wanted to determine whether these tumors demonstrated such changes on the molecular level.[155] We thus measured histone modifications in innate myeloid cells from each treatment group and found significantly more H3K27Ac, H3K4Me3, and H3K27Me3 expression in CD11b+ cells derived from β-glucan plus IRE treated tumors (Figure 2.5) We then performed RT-PCR analysis and found that the combination therapy down regulated transcriptional expression of Arg-1 and IL-6 in CD11b+ cells (Figure 2.6a,b), which have been found to drive immunosuppression in PC.[156, 157] β-Glucan treated mice were also found to have increased expression of TNF-α, iNOS, IL-1β, and IL-12 in CD11b+ cells (Figure 2.7a-d). These findings support our hypothesis, suggesting that IRE sustains the training effect of β-glucan ultimately to decrease PC progression and results in metabolic and transcriptomic reprogramming in innate myeloid cells.

β-Glucan and IRE increase total immune cell infiltration in the PC TME and IRE potentiates the trained innate response from myeloid cells early within PC tumors.

Induction of peripheral trained immunity stimulates an increase of trained myeloid cells within the naïve murine pancreas. This effect is most prominent 7 days following single IP injection of β-glucan and wanes to normal naïve levels by thirty days post

training. Additionally, IP injection of β-glucan has previously been given prophylactically, prior to tumor exposure.[131] Therefore, there is a need to positively alter the PC TME in the setting of β-glucan training over time and to evaluate β-glucan given in a neoadjuvant setting. We next examined whether the combination of β-glucan and IRE modulated the intratumoral immune cell composition. Flow-cytometric analysis revealed comparable frequencies (gating strategy demonstrated in Figure 2.8) of lymphocytes, myeloid cells including macrophages, neutrophils, monocytes, and dendritic cells at day 24 post tumor challenge (Figure 2.9). However, absolute numbers of tumor infiltrating immune cells and myeloid cells per gram of tumor tissue were distinctively increased in the mice treated with combination therapy. These findings were consistent across all examined innate and adaptive cell populations (Figure 2.10 a-f). These data suggest that IRE allows for establishment of a prolonged influx of leucocytes to the PC TME in the setting of prior β-glucan exposure. Prior exposure to IP β-glucan increased CD11b+F4/80+ macrophage, and CD11b+Ly6C+ monocyte TNF-α when re-exposed to lipopolysaccharide (LPS).[131] Next, we wanted to evaluate whether the infiltrating innate cells exhibited a trained phenotype. To address this, tumors were harvested from the mice on day 24 post tumor challenge (Figure 2.1) and tumor single cell suspensions were stimulated with LPS. As demonstrated in Figure 2.11, mice treated with single IP injection of β-glucan followed by IRE exhibited a trained immunity phenotype expressing significantly more TNF-α from all CD11b+ myeloid cells, CD11b+F4/80+ macrophages, and CD11b+Ly6C+ monocytes, as compared to control PBS, β-glucan, and IRE alone groups. These data are consistent and supported by our prior findings in which exposure to β-glucan has demonstrated an influx of trained myeloid cells into the naïve (non-tumor bearing) murine pancreas.[131] These data also

suggest IRE is capable of potentiating the trained effect of β-glucan *in vivo* and support our hypothesis that IRE may enhance the anti-PC effect of β-glucan.

The combination of β-glucan and IRE reduces distant tumor burden late in disease progression.

Despite local tumor control or complete tumor resection, many patients diagnosed with PC will experience distant progression.[158] Therefore, we wanted to evaluate distant anatomical locations known to harbor PC metastasis to determine if our combination therapy impacted PC metastasis. To this end, we followed mice for survival and evaluated each respective group for lung metastases once control PBS mice demonstrated signs of advanced tumor burden. Strikingly, we observed no metastatic lung nodules in the mice that received dual therapy, whereas the control groups had significantly increased number and size of nodules (Figure 2.12). These data indicate that the combination therapy not only has local effect, but off target distant implications in reducing tumor burden as well. It is known that the immune influx to the naïve WT pancreas resulting from single dose IP β-glucan is transient, however it is unknown whether the trained response is sustained over time.[131] To answer this question, we analyzed the myeloid cells derived from the primary tumors of each respective group once PBS controls reached described physiologic endpoints. Importantly, the trained TNF-α responses to stimulation with LPS from the CD11b+Ly6C+ monocytes and CD11b+F4/80+ macrophages appear to be lost over time (Figure 2.13). These data demonstrate that these aggressive PC tumors escape the trained response provided by single IP β-glucan treatment even with tumor ablation. However, the prior study used LPS as a secondary stimulus to evaluate trained response. We reasoned

that the act of IRE itself may serve as the secondary stimulus engaging the trained myeloid cells to produce more TNF-α. To investigate this question, we then cultured single cell suspensions from the tumors of these long duration survival mice in the absence of LPS to evaluate for unprovoked TNF-α production from the infiltrating myeloid cell population. We found that the CD11b+ myeloid cells obtained from tumors in mice who received combination therapy expressed an increase in frequency of TNF-α production to control treated mice, with a similar trend in MFI (Figure 2.14). This data implicates that IRE alone may serve as the secondary stimulus eliciting the trained response from β-glucan trained myeloid cells synergizing to control PC progression and metastasis.

The antitumor effect of β-glucan in combination with IRE occurs independent of the adaptive immune response.

In this study we did observe an increase in absolute number of CD4+ and CD8+T cells (data not shown). To further investigate the T cell phenotype within this novel therapy, we repeated our experimental design in Figure 2a, and evaluated immune checkpoint molecules and activation markers on CD8+ and CD4+ T cells by flow cytometry. We found an overall decrease in PD-1 expression from CD8+ T cells (Figure 2.15 a, b) and increases in Granzyme B expression (Figure 2.16 a, b) in β-glucan and IRE treated mice, suggesting a more active effector CD8+ T cell phenotype. However, CD69 and TIM-3 expression was similar on CD8+ T cells among different groups (Figure 2.17). Comparable frequencies of PD-1, Granzyme B, CD69, and TIM-3, were also observed from CD4+ T cells (Figure 2.18). Finally, β-glucan treatment decreased LAG-3 expression in both CD8+ and CD4+ T cells although the overall expression levels were

low (Figure 2.17 and Figure 2.18). To definitively determine whether an adaptive immune response was involved in eradicating disease from our dual therapy we utilized $Rag^{-/-}$ mice (lacking T and B cells) within our orthotopic KPC model (Figure 2.19A). Treatment of $Rag^{-/-}$ mice with single dose IP β-glucan followed by IRE ablation similarly reduced tumor burden (Figure 2.19B), as measured by reduction in tumor weight and maximum tumor diameters. We also observed a median survival of 107 days in the combination group, a 189% increase in survival from the PBS control (median 37 days). Additionally, three mice (50%) achieved recurrence free survival (Figure 2.20). These data support a critical role for innate immune cells in the control and treatment of PC in the setting of treatment with β-glucan and IRE. Further, these anti-tumor innate immune responses occur without actively exhausting or suppressing adaptive T cells.

CHAPTER II FIGURES

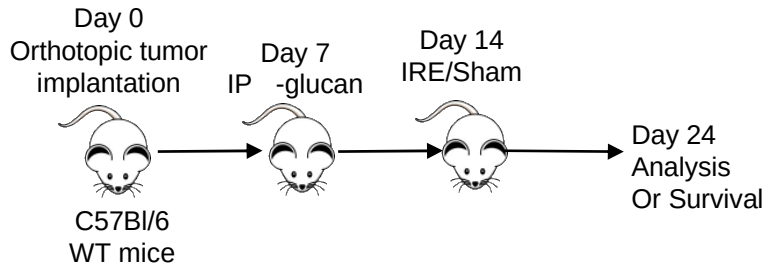


Figure 2.1: Experimental design scheme.

Murine KPC cells were orthotopically implanted into the pancreas at Day 0. Mice were treated with one IP β-glucan injection (1mg/mouse) or PBS control 7 days after tumor challenge. On day 14, sham surgery or IRE ablation was performed. Mice were then followed for survival or tumor immune analysis on day 24.

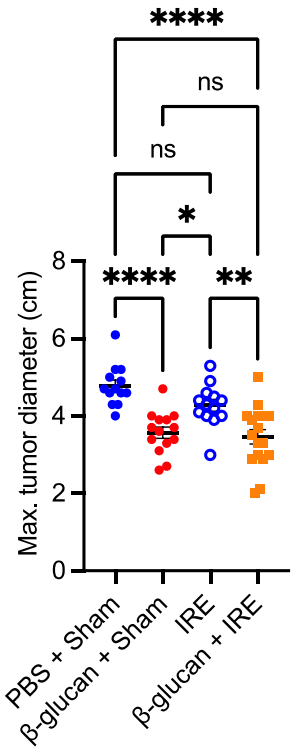
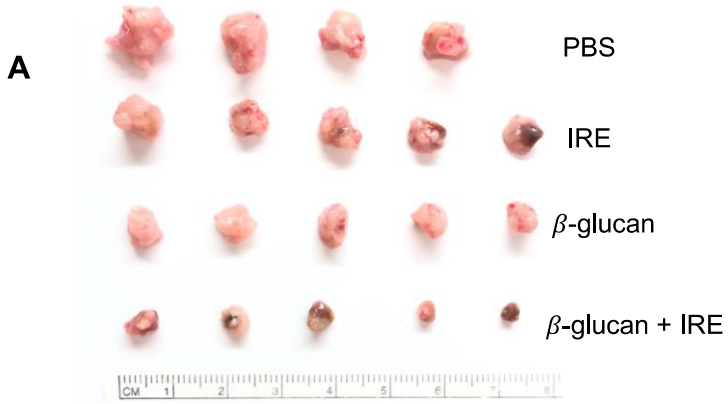


Figure 2.2: β-Glucan reduced maximum tumor diameter at time of IRE versus Sham. Maximum tumor diameter measured at the time of IRE or sham surgery. PBS + Sham n = 13, β-glucan + Sham n = 13, IRE n = 14, and β-glucan + IRE n = 17, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ****p<0.0001.



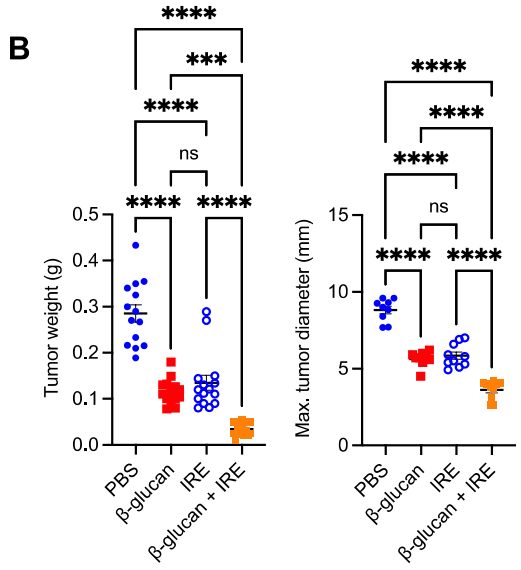


Figure 2.3: β-Glucan in combination with IRE reduces PC tumor burden.

(A) Representative image of orthotopic pancreatic KPC tumors on Day 24 post tumor challenge from PBS control and subsequent treatment groups. (B) Tumor weight (left) and maximum tumor diameter (right). PBS n=14, β-glucan n=14, IRE n=15, β-glucan + IRE n=15, respectively. Data are representative of two or three independent experiments and presented as mean ± SEM. Significance: ns= not significant; ***p<0.001, ****p<0.0001.

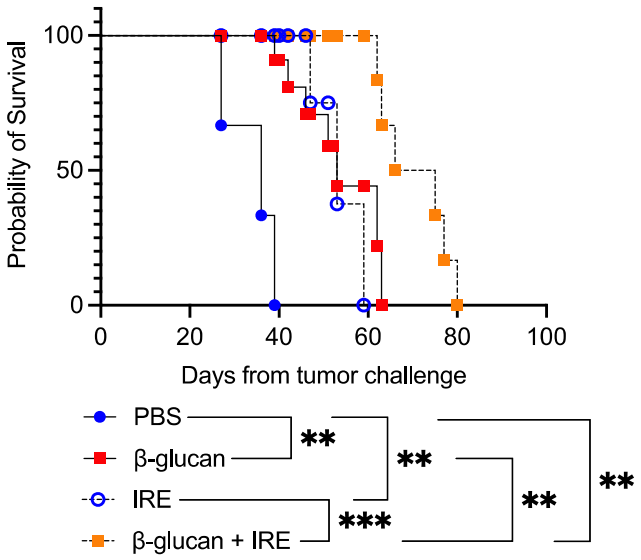


Figure 2.4: Treatment with β-Glucan and IRE prolongs overall survival in an aggressive orthotopic PC tumor model.

Overall survival in WT mice. PBS n=5, β-glucan n=7, IRE n=5, β-glucan + IRE n=7, respectively. Data are presented as mean ± SEM. Significance: $^{**}p<0.01$, $^{***}p<0.001$.

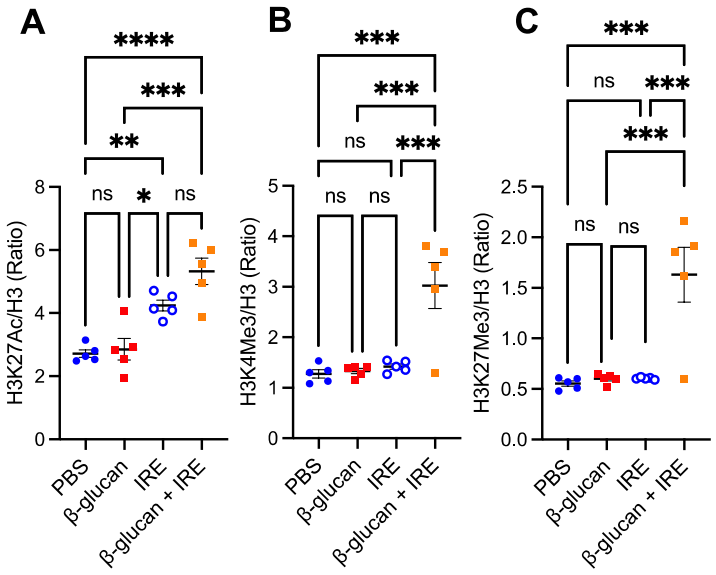


Figure 2.5: IRE augments epigenetic reprogramming within β-glucan trained myeloid cells derived from PC tumors.

Quantification of histone deacetylation markers H3K27Ac (A), H3K4Me3 (B), and H3K27Me3 (C) compared to total H3 in CD11b+ cells from tumor single cell suspension measured by ELISA. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

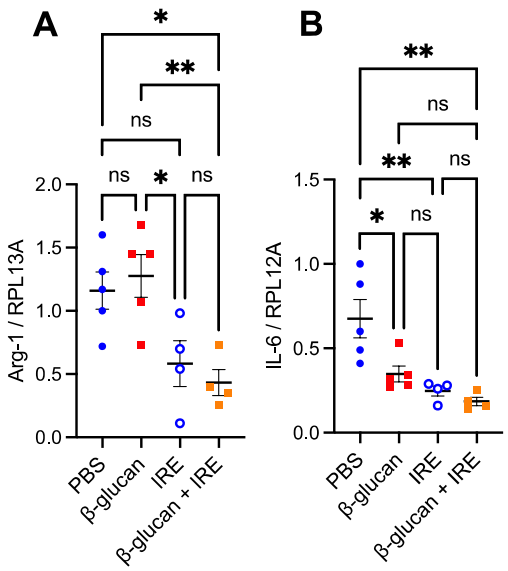


Figure 2.6: IRE and β-glucan downregulate gene expression of Arg-1 and IL-6.

(A) Arg-1 and (B) IL-6 mRNA expression in CD11b+ cells measured by RT-PCR. PBS n=5, β-glucan n=5, IRE n=5, β-glucan + IRE n=5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ****p<0.0001.

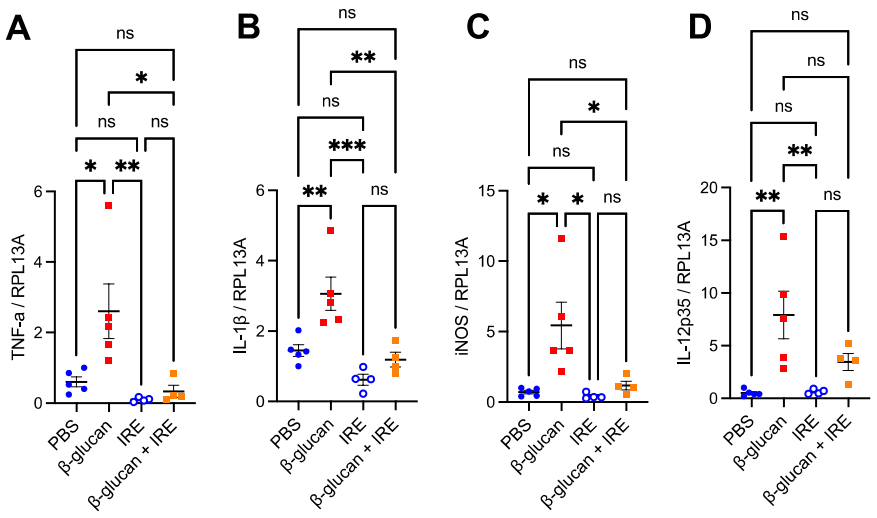


Figure 2.7: β-Glucan upregulated gene expressions in CD11b+ cells.

Quantified mRNA expression of (A) TNF-α, (B) IL-1β, (C) iNOS, and (D) IL-12p35 in CD11b+ cells measured by RT-PCR. PBS n=5, β-glucan n=5, IRE n=5, β-glucan + IRE n=5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; **p*<0.05, ***p*<0.01, ****p*<0.001.

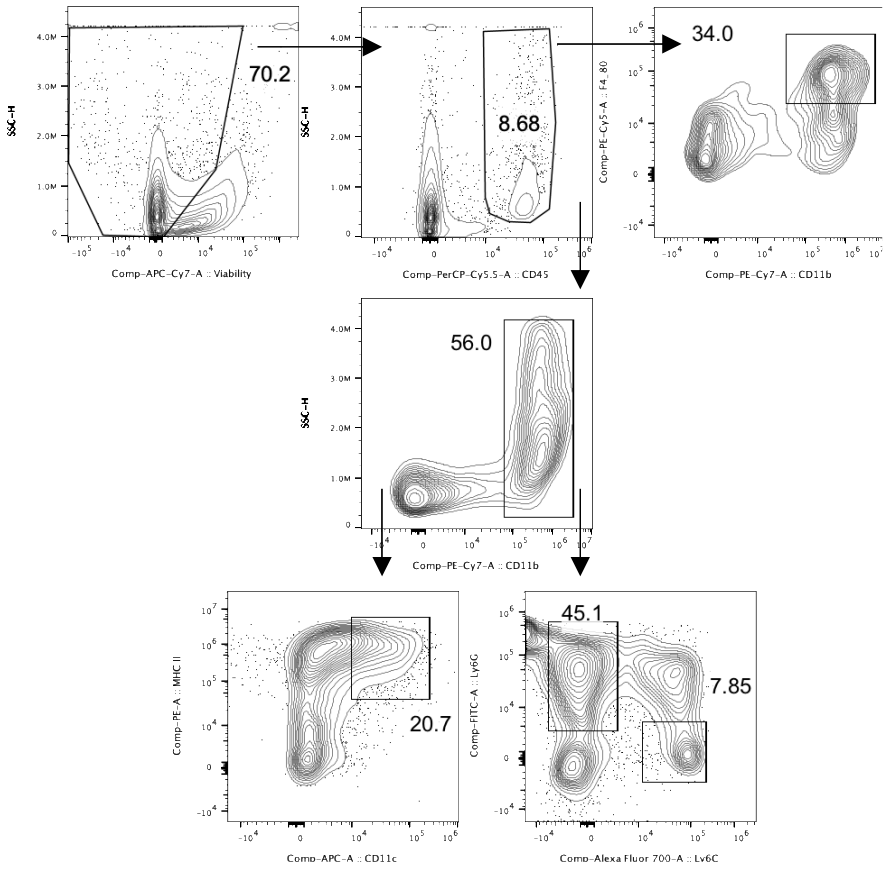


Figure 2.8: Myeloid cell gating strategy.

Total myeloid cell frequency (CD11b+) was considered out of live, CD45+ cells. Macrophages were gated out of CD45+ cell and considered as CD11b+F4/80+. From CD45+CD11b+, dendritic cells were thenCD11c+MHCII+, monocytes Ly6C+Ly6G- and finally neutrophils were Ly6C-Ly6G+.

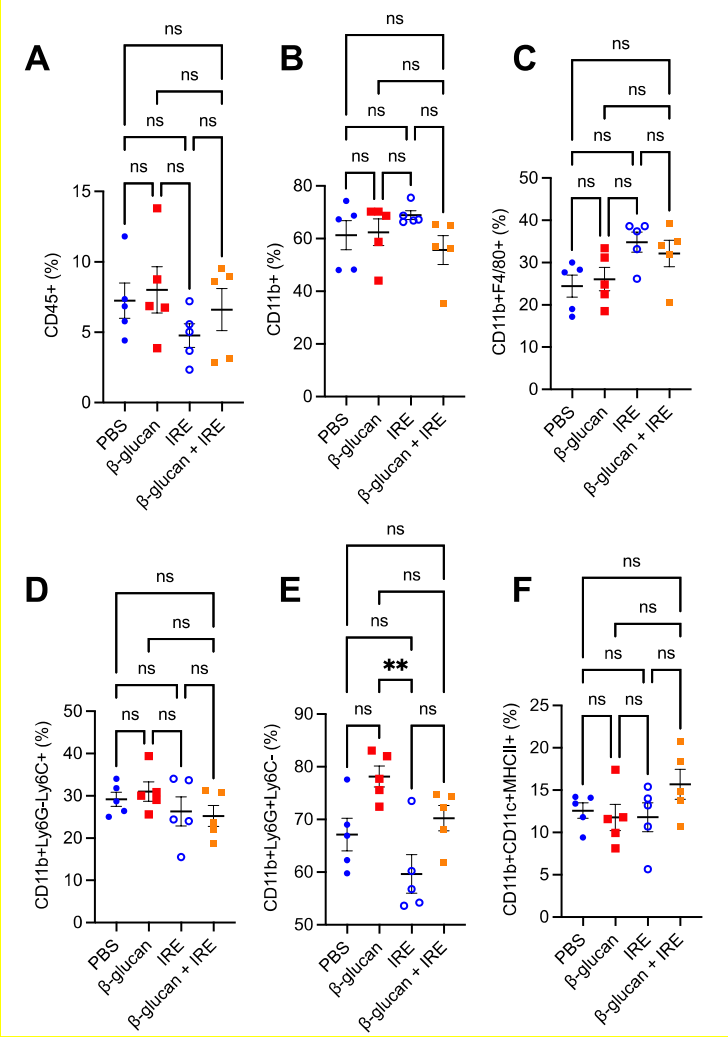


Figure 2.9: Comparison of innate cell frequencies within KPC tumors.

Frequency of (A) CD45+ leucocytes, (B) CD11b+ myeloid cells, (C) CD11b+F4/80+ macrophages, (D) CD11b+Ly6G-Ly6C+ monocytes, (E) CD11b+Ly6G+Ly6C- neutrophils, and CD11b+CD11c+MHCII+ DCs within KPC tumors treated by PBS, β-glucan, IRE, or β-glucan + IRE. n = 5 per group. Data are presented as mean ± SEM. Significance: ns= not significant; $**p<0.01$.

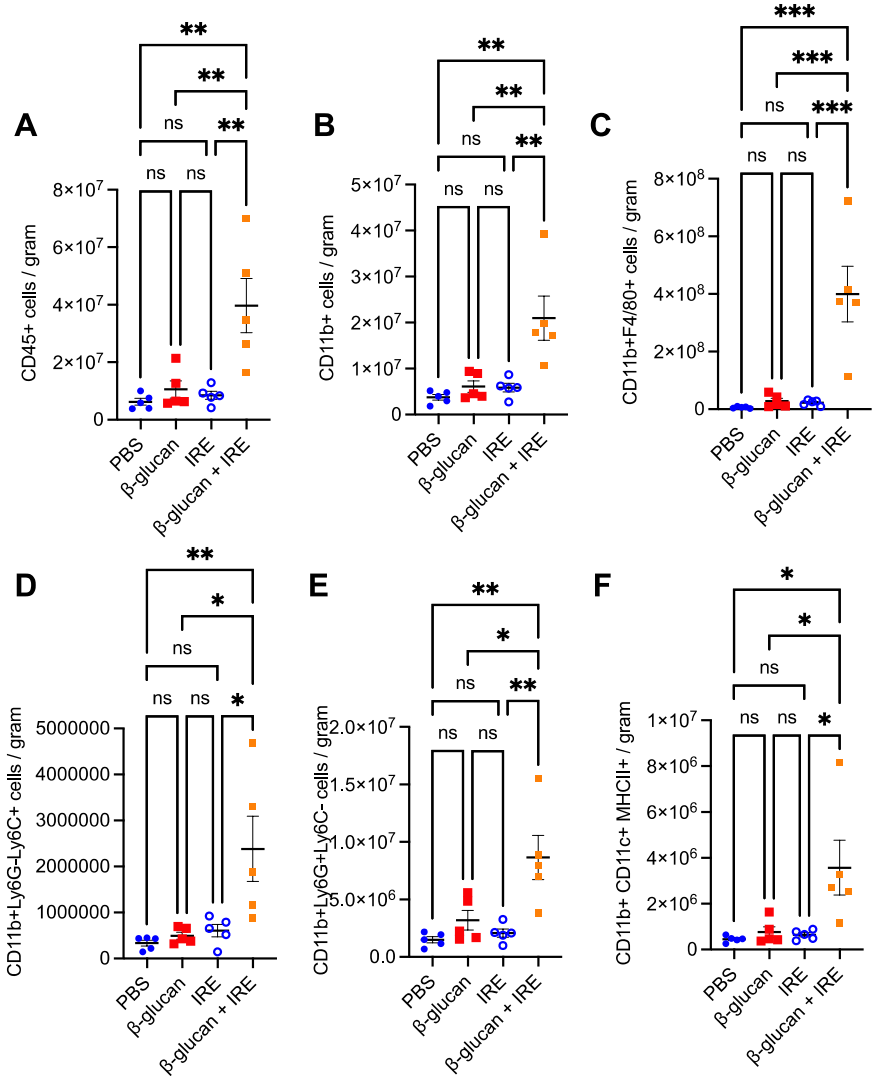


Figure 2.10: Combined β-glucan and IRE treatment increases total immune cell infiltration in the PC TME.

Absolute number of live CD45+ (A), CD11b+ (B), CD11b+F4/80+ macrophages (C), CD11b+Ly6G-Ly6C+ monocytes (D), CD11b+Ly6G+Ly6C- neutrophils (E), CD11b+CD11c+MHCII+ dendritic cells (F) per gram of tumor tissue as quantified by flow cytometry. Data are representative of two or three independent experiments and

presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

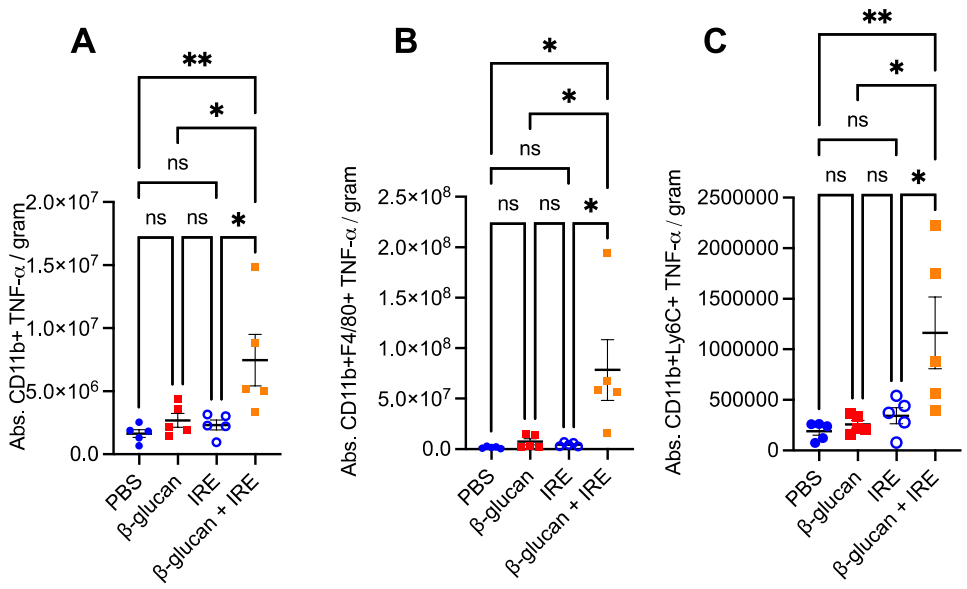


Figure 2.11: IRE induces a trained innate response in monocytes and macrophages in early tumor progression.

Absolute TNF-α from overall (A) CD11b+, (B) CD11b+F4/80+ macrophages, and (C) CD11b+Ly6C+ monocytes per gram of tumor tissue as quantified by flow cytometry. Data are representative of two or three independent experiments and presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

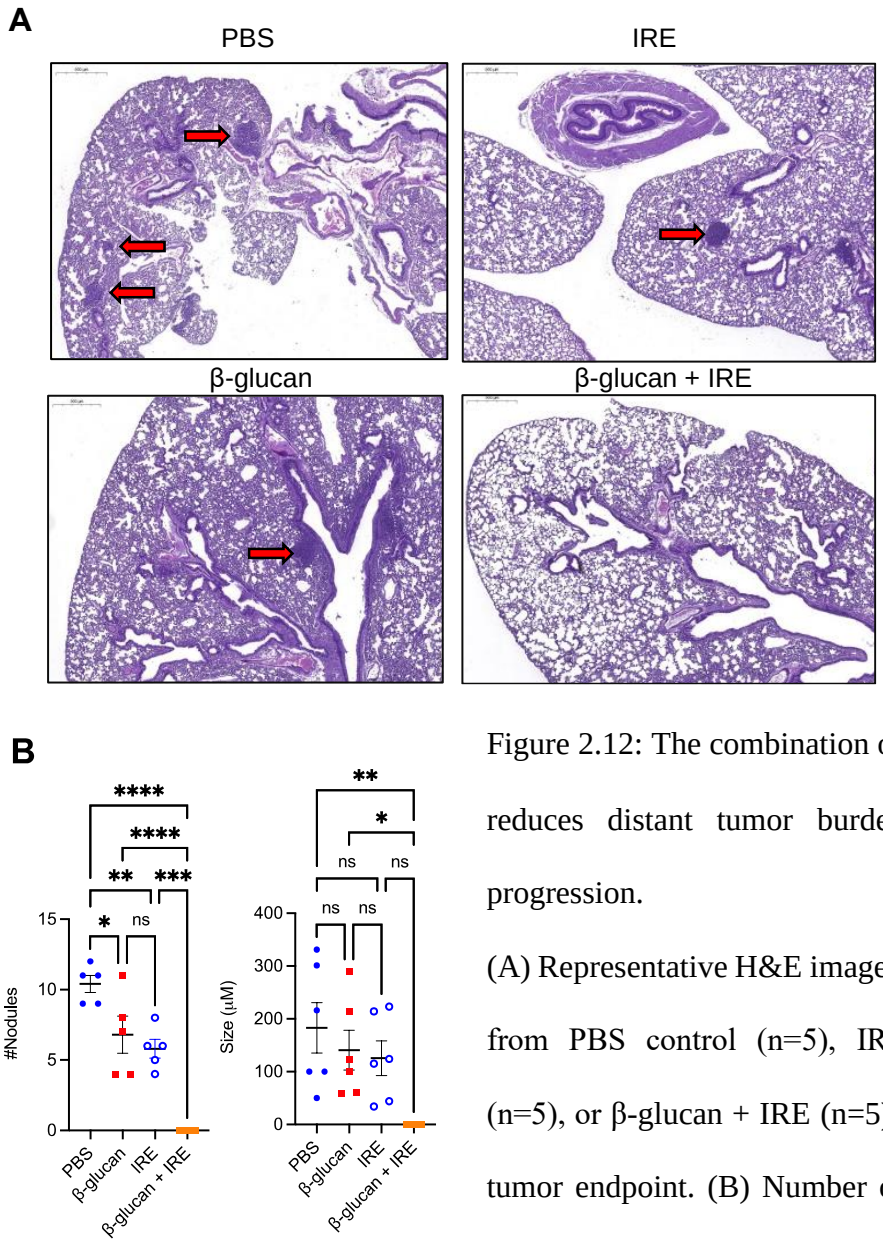


Figure 2.12: The combination of β-glucan and IRE reduces distant tumor burden late in disease progression.

(A) Representative H&E images of lungs harvested from PBS control (n=5), IRE (n=5), β-glucan (n=5), or β-glucan + IRE (n=5) at the time of PBS tumor endpoint. (B) Number of lung nodules per histologic section per group and average measured size (μm) of nodules (right). Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

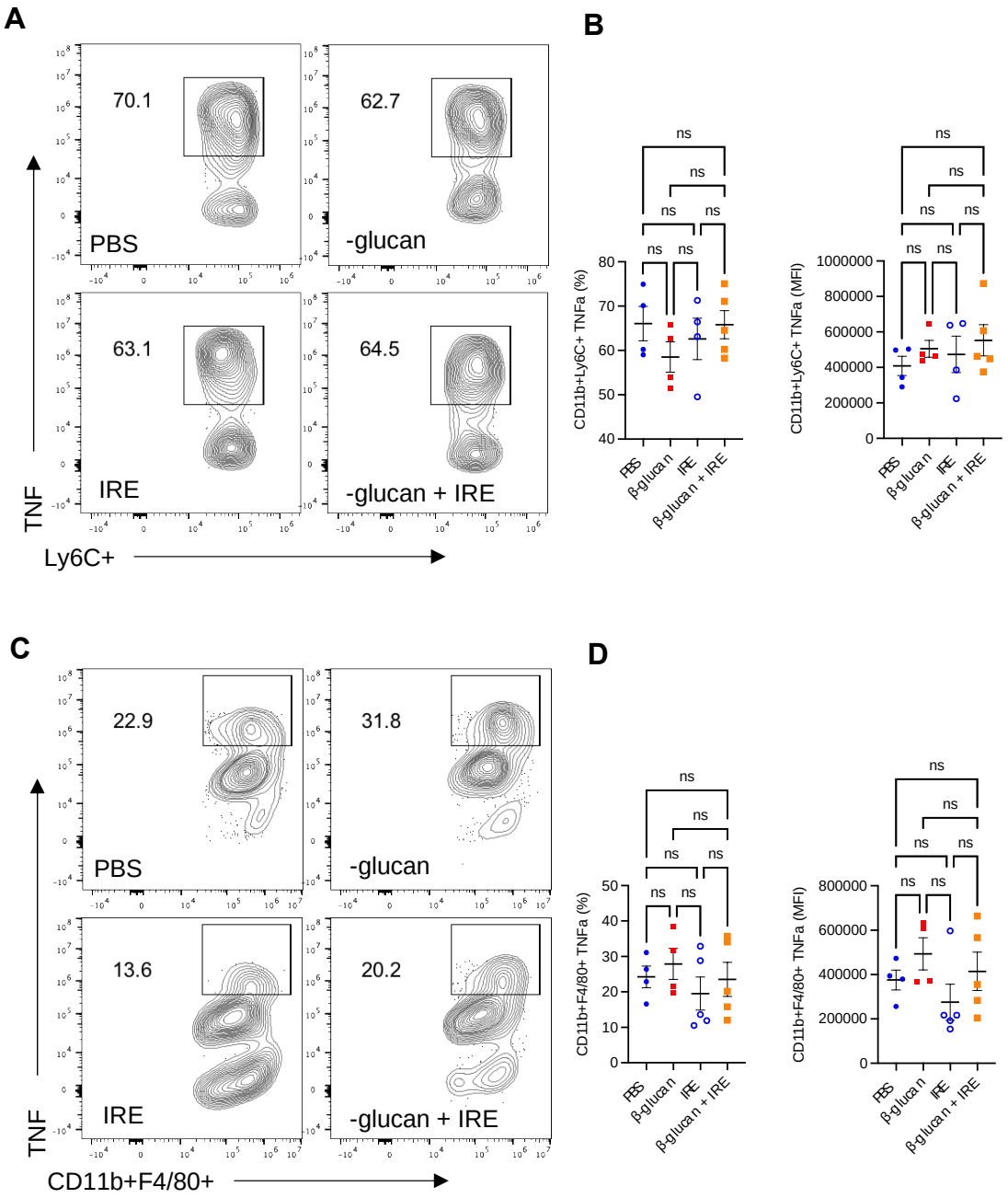

A
70.1
62.7
PBS
-glucan
63.1
64.5
IRE
-glucan + IRE
TNF
Ly6C+
B
CD11b+Ly6C+ TNFa (%)
CD11b+Ly6C+ TNFa (MFI)
ns
PBS
β-glucan
IRE
β-glucan + IRE
C
22.9
31.8
PBS
-glucan
13.6
20.2
IRE
-glucan + IRE
TNF
CD11b+F4/80+
D
CD11b+F4/80+ TNFa (%)
CD11b+F4/80+ TNFa (MFI)

Figure 2.13: Trained responses from monocytes and macrophages are lost over time.

(A) Representative flow contour plots of Ly6C+TNF-α+ cells late in disease progression from KPC tumors within PBS, β-glucan, IRE, and β-glucan + IRE treated mice. (B) Summarized CD11b+Ly6C+TNF-α+ percent and MFI. PBS n = 4, IRE n = 4, β-glucan n = 4, β-glucan + IRE n = 5. (C) Representative flow contour plots of CD11b+F4/80+TNF-α+ cells late in disease progression in KPC tumors from PBS, β-glucan, IRE, and β-glucan + IRE treated mice. (D) Summarized CD11b+F4/80+TNF-α+ percent and MFI. PBS n = 4, IRE n = 4, β-glucan n = 5, β-glucan + IRE n = 5 Data are presented as mean ± SEM. Significance: ns= not significant

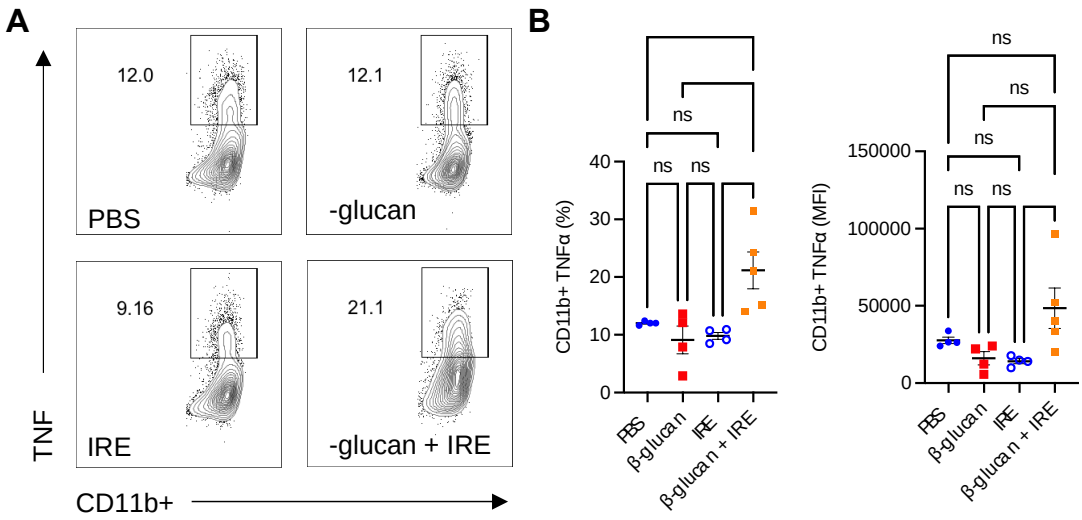


Figure 2.14: IRE alone increased trained response from myeloid cells.

(A) Representative flow cytometry contour plots of CD11b+ TNF-α from PBS control (n=4), IRE (n=4), β-glucan (n=4), or β-glucan + IRE (n=5) cultured in the presence of golgi plug without LPS stimulation. (B) Quantified flow cytometry data in percent CD11b+TNF-a and MFI (right). Data presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

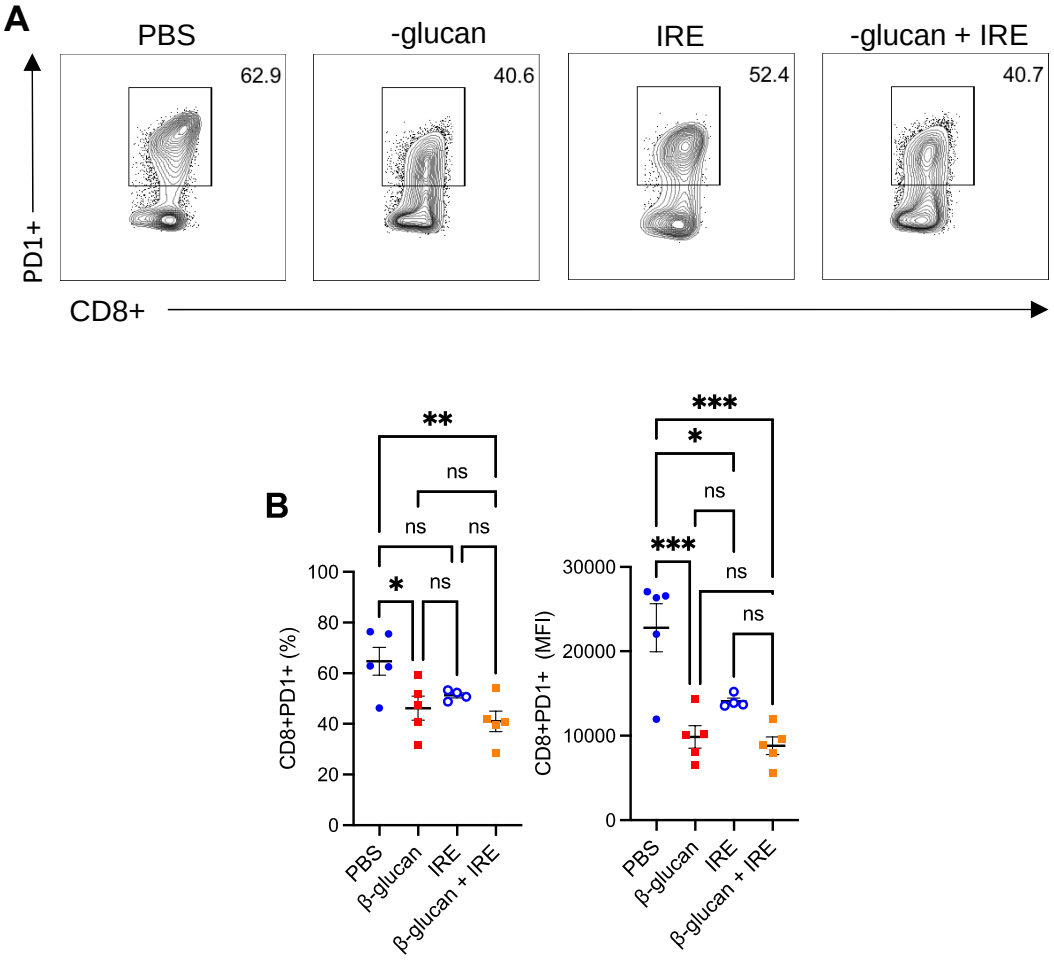


Figure 2.15: Combination of β-glucan and IRE decrease levels of CD8+ T cell PD-1. **(**A) Representative flow cytometry contour plots of CD8+PD1+ T cells. Cells were first gated on viability and CD45+. (B) Quantified percent and MFI of CD8+PD-1+ T cells. PBS n=5, β-glucan n=5, IRE n = 4, β-glucan + IRE n=5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

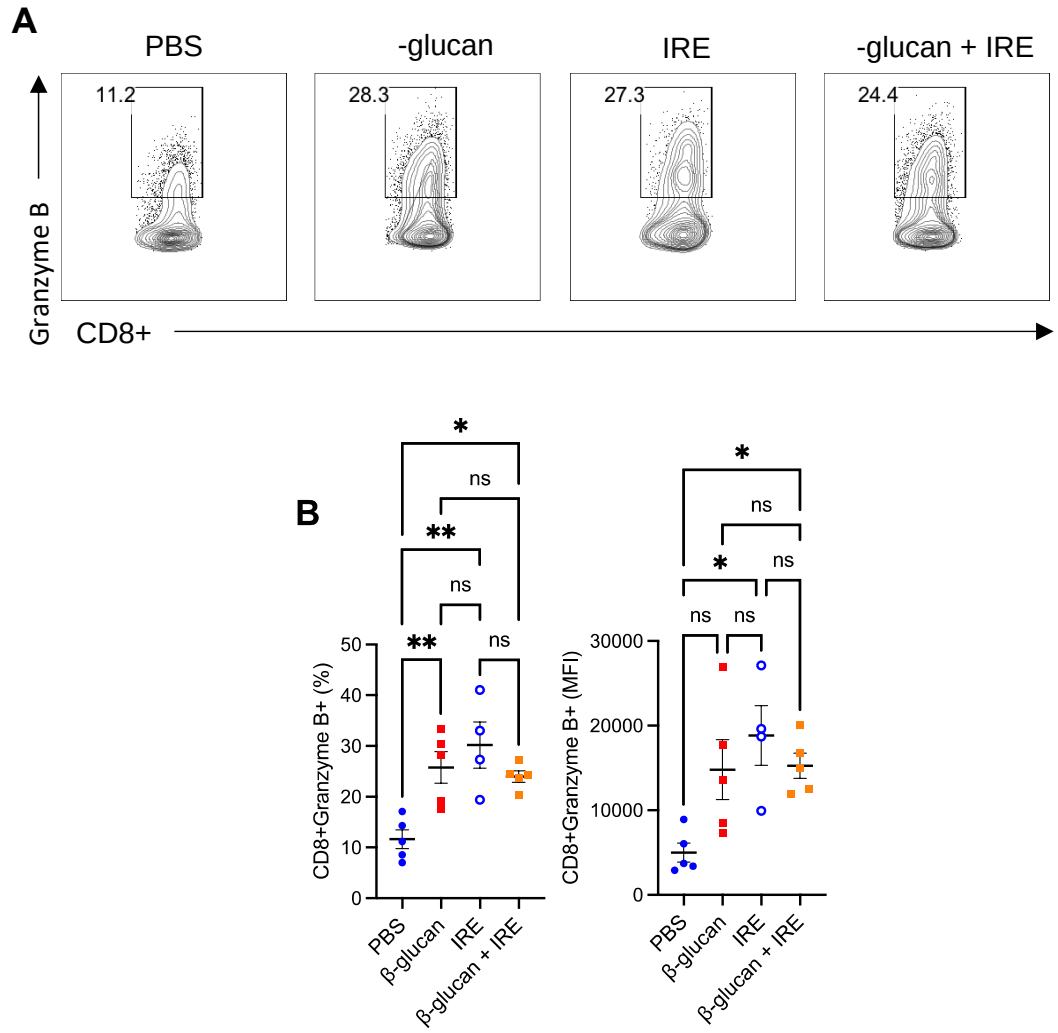


Figure 2.16: Combination of β-glucan and IRE increase Granzyme B expression in CD8+ T cells.

(A) Representative flow cytometry contour plots of CD8+Granzyme B+ T cells. (B) Summarized percent and MFI of CD8+Granzyme B+ expression. PBS n=5, β-glucan n=5, IRE n = 4, β-glucan + IRE n=5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

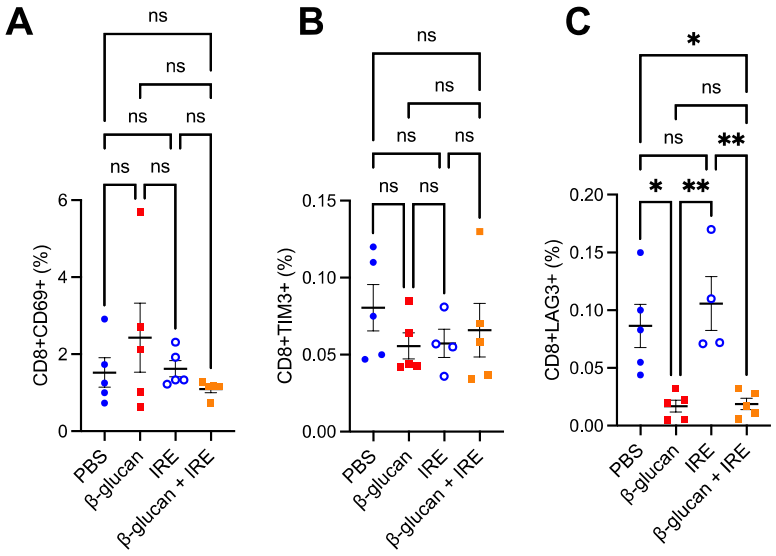


Figure 2.17: Further characterization of immune checkpoint and activation makers on CD8+ T cells.

Quantified frequency of (A) CD69, (B) TIM-3, and (C) LAG-3 expression on CD8+ T cells within PBS, β-glucan, IRE, or β-glucan + IRE treated KPC tumors. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p <0.01.

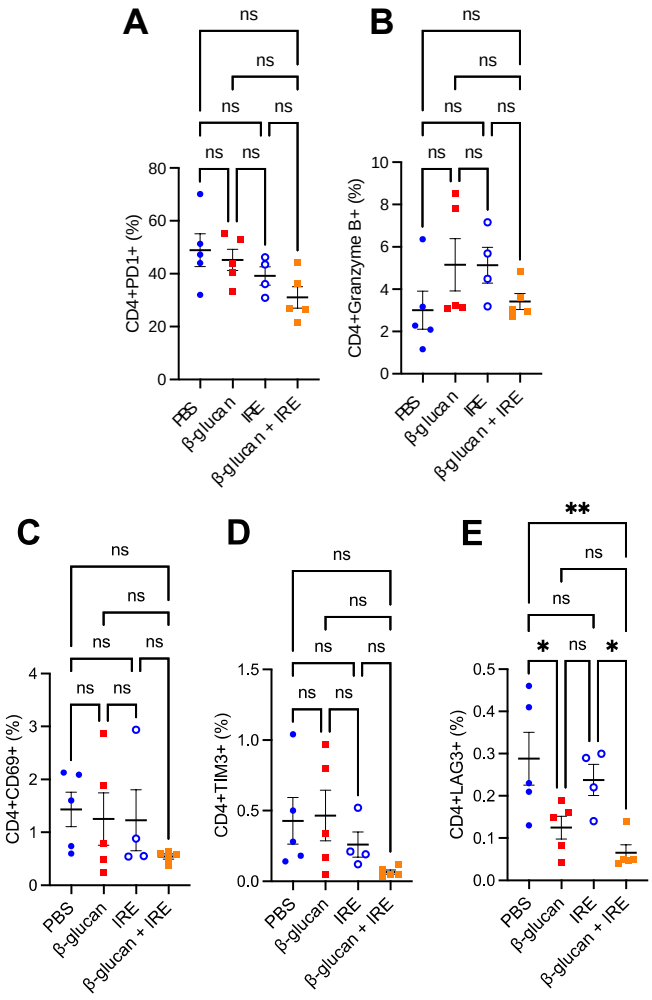


Figure 2.18: Frequency of immune checkpoint and activation markers on CD4+ T cells. Quantified frequency of (A) PD-1, (B) Granzyme B, (C) CD69, (D) TIM-3, and (E) LAG-4 expression on CD4+ T cells within each group. PBS n = 5, β-glucan n = 5, IRE n = 4, and β-glucan + IRE n = 5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01.

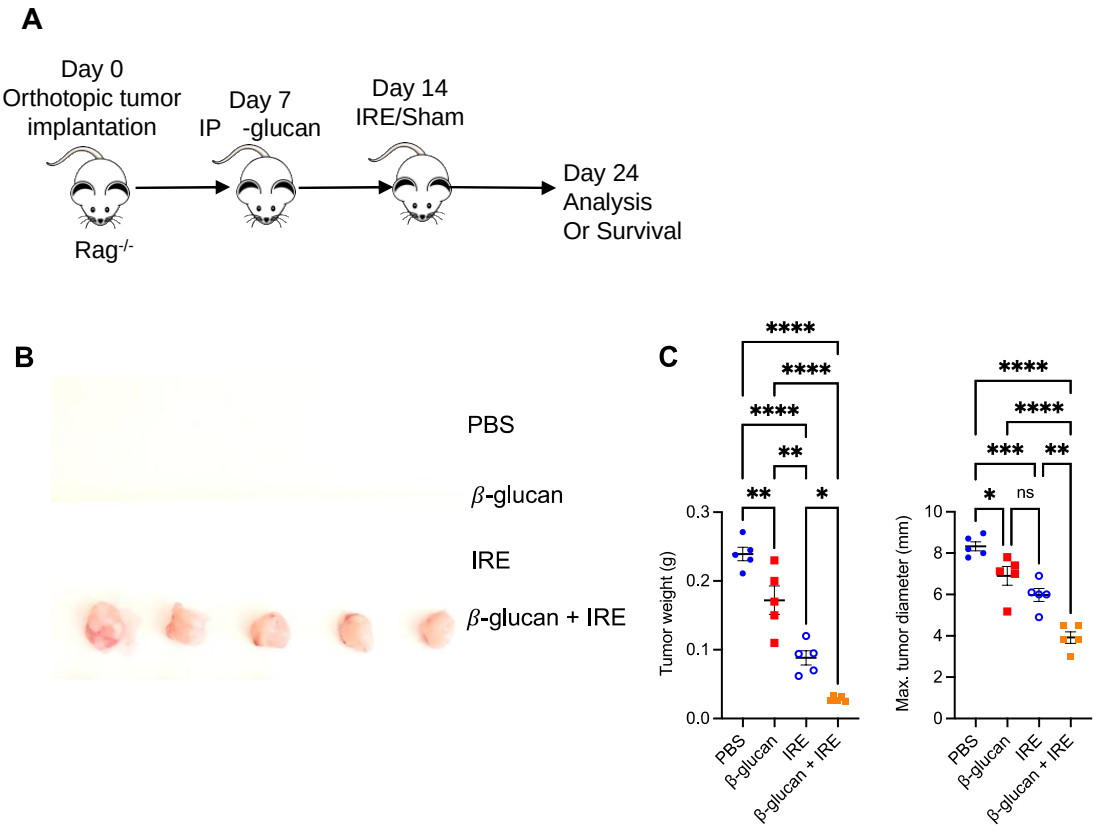


Figure 2.19: The anti-tumor effect of β-glucan and IRE occurs independent of the adaptive immune cells.

(A) Schematic representation of experimental design utilizing $Rag^{-/-}$ mice. (B) Gross depiction of orthotopic tumors harvested from $Rag^{-/-}$ mice 24 days after tumor challenge. (C) Tumor weight (left) and maximum tumor diameter (right) of tumors depicted above. PBS n=5, IRE n=5 β-glucan n=5 β-glucan + IRE n=5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p <0.01, ***p<0.001, ****p<0.0001.

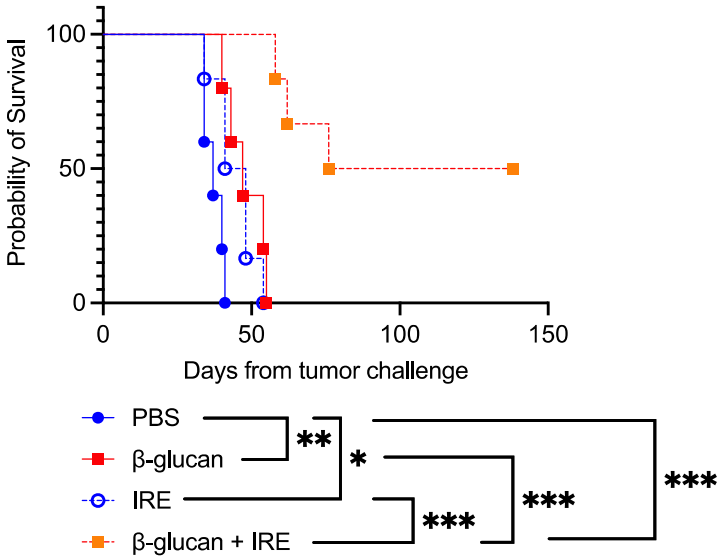


Figure 2.20: Treatment with β-glucan and IRE prolongs survival in the absence of T and B cells.

Overall survival of $Rag^{-/-}$ KPC-bearing mice treated with different regimens. PBS n=5, IRE n=5 β-glucan n=5 β-glucan + IRE n=6, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; *p < 0.05, **p <0.01, ***p < 0.001, **** p<0.0001.

CHAPTER II DISCUSSION

The previously described decrease in PC tumor burden and prolongation in survival provided by β-glucan alone is encouraging yet modest. Further, the trained effect from increasing myeloid cells within the pancreas 7 days following single IP β-glucan injection declines overtime.[131] These findings are characteristic of PC, who's immunosuppressive TME has easily resisted prior trials of mono and dual ICI.[159] Therefore, we find it incumbent to provide combinatorial approaches that may prolong the anti-tumor trained immunity effect. Our findings that β-glucan and IRE independently reduce PC tumor burden is consistent with prior animal studies.[98, 131, 160] Consequently, we found an enhanced therapeutic outcome when β-glucan and IRE were used sequentially. Our data also described changes in epigenetic and transcriptional profile in innate myeloid cells clearly supporting an augmented trained immune phenotype within β-glucan IRE treated tumors that is sustained over time. Immunologically, increases were observed in absolute number across all cell types suggesting that IRE allowed for a prolonged influx of leucocytes after β-glucan training. Prior utilization of CCR2-/- mice has demonstrated complete inhibition of trained myeloid cells to the murine pancreas and critically implicated monocyte derived macrophages as the main effector cell in the pancreas after IP β-glucan administration. Our data finding prominent cytokine responses from monocytes and macrophages from the tumors of β-glucan trained mice also agrees with prior literature.[161, 162] However, this phenotype was lost overtime as tumor burden

progressed suggesting PC tumors eventually escape the trained effect provided by single dose of IP β-glucan even in combination with IRE. More studies are needed to evaluate the exact duration of training to better understand long term benefit of trained immunity in the setting of cancer. Evaluation of long-term trained immunity in this combination therapy may best be addressed via cell subset deletion or adoptive transfer studies, which is a future direction of this work. Despite the loss of trained effect from monocytes and macrophages in mice with advanced disease, they demonstrated reduced metastatic lung nodules and increased TNF-a cytokine production from primary tumors without LPS re-stimulation. These data indicate IRE may act as the secondary stimulus to trained myeloid cells within the PC TME and the proposed combination therapy can be used limit metastasis in the adjuvant setting.

IRE has provoked immunologic changes within the TME of subcutaneous and orthotopic PC tumor models. He *et al.*, reported increased effector and memory T cell frequency in mice treated with IRE, which subsequently rejected secondary tumor challenges.[163] Clinical studies also have documented transient decreases in systemic Tregs after IRE, supporting combined use of IRE with immunotherapies to enhance T cell activation.[164, 165] In our orthotopic model, increased absolute CD4+ and CD8+ T cell numbers were observed in tumors following combination therapy, however no direct effect on cytokine production occurred. Additionally, these infiltrating CD8+ T cells in IRE plus β-glucan treated tumors expressed lower levels of PD-1 and LAG3 and higher levels of Granzyme B indicating they are not exhausted in this therapy. These findings are consistent with our prior findings that β-glucan cooperates with anti-PDL-1 Ab therapy to prolong

survival in this KPC orthotopic model.[131] These findings suggest a potential avenue to successfully implement ICI therapy in a PC model. In this study, a similar or even better therapeutic efficacy of β-glucan and IRE was observed in $Rag^{-/-}$ mice reducing tumor burden and prolonging survival. Although histologic analysis of the $Rag^{-/-}$ was not provided, we would expect these mice to have reduced lung metastatic burden as we observed long term tumor free survival. Further, the better therapeutic efficacy of this combination therapy in $Rag^{-/-}$ mice may imply critical roles of Tregs in this KPC model.[166] Reduction of tumor burden in the absence of adaptive immunity questions the ability to provide a long-lasting anti-tumor protection. At present trained immunity is generally accepted to be shorter lived than classical immune memory, yet its duration is not well defined. Developing novel approaches such as this combination therapy to establish a "sustained" trained immune response may hold the key for lasting innate immune memory.

This data agrees with our prior work where β-glucan significantly reduced tumor burden in NSG mice (lacking mature T, B, and NK cells) orthotopically implanted with KPC cells.[131] While IRE is known to induce an immunogenic cell death releasing specific neoantigens that could contribute to T cell activation, regulation of innate cell responses via release of DAMPs may be a greater consequence in the setting β-glucan training in PC. Regardless of T cell activation/status, this data supports more effective anti-tumor therapy of trained immunity when paired with an active process of tumor killing such as IRE.

NK cells have been described in the context of trained immunity, particularly in the setting of murine and human cytomegalovirus (CMV) infection. [167] Upon CMV

reinfection, NK cells clonally expand and more rapidly degranulate providing enhanced immune protection to their host. These so called "memory" NK cells more closely resemble adaptive T cell responses.[107, 168] Adoptive transfer of MCMV exposed NK cells increased survival to MCMV challenged mice. BCG vaccination in humans has also induced a long last memory in NK cells, which demonstrate enhanced IFN-γ production even one year after vaccination.[169] However, NK cells remain unimplicated in the setting of β-glucan training. β-Glucan trained mice induced lung interstitial macrophage training and reduced lung metastasis in NSG mice.[170] Moreover, we have also previously observed myeloid cell training in the murine pancreas after monoclonal antibody depletion of NK cells as well as in NSG mice.[131] Collectively, these data suggest that NK cells may not play an essential role in this combination therapy.

IP β-glucan holds promise in targeting pancreatic cancer via its unique trafficking and induction of trained immunity in the murine pancreas. Yet, there are substantial limitations to consider in translating this approach to patients. First, access and delivery of β-glucan to the peritoneal cavity is limited in humans. Direct peritoneal access is achieved at the time of diagnostic laparoscopy, definitive resection, or IRE in eligible patients, however timing for optimal administration of β-glucan remains in question. It is also likely that multiple doses would be needed or if subsequent doses would provide any further benefit. In addition, the murine pancreas resides in an intra-peritoneal anatomical location as compared to retro-peritoneal of the human pancreas.[171] Influx of CD45+ immune cells to the pancreas also poses a theoretical threat of inflammation and subsequent pancreatitis. However, based on our prior data, WT mice treated with IP β-glucan have normal levels

of serum amylase and histologic architecture of pancreatic acini and islet cells in comparison to PBS controls.[131] Thus, we believe the phenomenon β-glucan trafficking to the pancreas to be safe, although more translational studies are needed to prove this point.

CHAPTER III

ORAL β-GLUCAN AND IRE: FROM MOUSE TO HUMAN

INTRODUCTION

The preclinical data above have clearly demonstrated benefit in the treatment of PC *in vivo* with reduction in local and distant tumor burden. Further we observed augmented epigenetic and transcriptomic changes within tumors treated in combination with IRE and β-glucan—strongly supporting our original hypothesis. Characterization of the immune landscape increased total innate and adaptive cell infiltrations with prolongations in trained response that were lost over time and with disease progression. This data provides a critical understanding of how trained immunity may be elicited within established tumor setting and that provoking trained responses is associated with therapeutic efficacy. Again, this phenotype was independent of the adaptive response, however, occurs without exhausting local effector T cells. This is an important finding highlighting a potential avenue to translate ICI therapy to patients with PC.

Thus far we have utilized IP delivered β-glucan to induce trained immunity within the murine PC TME. All previous studies utilizing β-glucan have also employed the IP approach to induce trained immunity in various settings.[131, 134, 172-174] However, this administration route of training poses several unique clinical and physiologic barriers considering obvious differences in the anatomy and localization between the human and

mouse pancreas, limiting its translatability to humans.[175] Therefore, we wanted to first explore an alternative delivery method for induction of trained immunity by β-glucan. Until now, it has remained unknown whether orally delivered β-glucan may induce trained immunity in the pancreas. Next, Induction of trained immunity via β-glucan has been previously demonstrated in healthy human myeloid cells, however it has yet to be evaluated in the context of pancreatic cancer or with IRE. Based on these findings, we also sought to explore whether inducing trained immunity in stage III LAPC patients was possible and if it could be done in conjunction with IRE therapy.

CHAPTER III RESULTS

Orally administered β-glucan traffics to the murine pancreas, increases myeloid cell frequency, induces trained immunity, and prolongs PC survival in combination with IRE.

We first investigated the optimal dose of oral β-glucan which may induce trained immunity and determined 6 doses of 1mg/mouse to produce peak trained response from pancreatic CD11b+ myeloid cells re-exposed to LPS (Figure 3.1). We also fed WT mice orally with DTAF labeled β-glucan for six consecutive days and evaluated for β-glucan trafficking within the pancreas on day 7. A single dose of IP DTAF-β-glucan was given 24 hours prior as a positive control for comparison. Oral β-glucan was found to traffic into the murine pancreas at low levels as quantified by the frequency and absolute numbers of CD11b+DTAF+ myeloid cells and it is less potent compared to IP injected β-glucan (Figure 3.2). Importantly, oral β-glucan also stimulated a distinct influx of CD11b+ myeloid cells in the pancreas compared to PBS control (Figure 3.3). In addition, these myeloid cells were also more responsive to secondary exposure to LPS expressing more TNF-α, indicating training had occurred by oral β-glucan (Figure 3.4). We also evaluated the trained response from other innate cell subtypes and found the CD11b+CD11c+ DCs to significantly increase TNF-α (Figure 3.5), implying an alternative underlying cellular mechanism compared to IP administered β-glucan. We next sought to determine if these trained cells would also respond to tumor conditioned or IRE conditioned media. Figure 3.6a exhibits representative data of CD11b+ myeloid cells derived from the pancreas of mice fed β-glucan for 6 days and then re-exposed to normal media, KPC conditioned media, or IRE treated media. Myeloid cells cultured in the presence of the IRE ablated

media produced significantly more TNF-α in both frequency and MFI (Figure 3.6b). These data support that the intensity of the trained response can be amplified, and that IRE ablation augments the level of trained response. Next, we determined if orally administered β-glucan may also provide long-term tumor benefit in prolongation of survival and if its combination with IRE would further increase its hypothesized effect. WT mice were treated with daily neoadjuvant oral β-glucan beginning on day 7 post tumor implantation. This was also continued in a neoadjuvant manner for the combination group receiving IRE. After recovery from IRE or placebo surgery, oral β-glucan was again initiated in an adjuvant setting and continued daily for 4 weeks as mice were observed for survival (Figure 3.7a). Importantly, mice who received oral β-glucan lived significantly longer than control PBS treated mice (median survival 52 vs 37 days, Figure 3.7b). Moreover, improved overall survival was observed when oral β-glucan trained mice were treated in combination with IRE ablation (median survival 93 days) a 151% increase in survival compared to PBS control. Together, these data suggest that oral administration of β-glucan can also induce trained immunity in the murine pancreas, similarly prolonging PC survival, and works in treatment combination with IRE.

Effect of β-glucan on healthy donor and PC patient peripheral blood monocytes.

As a validation of β-glucan training in human monocytes we repeated the *in vitro* training assay first described by Cheng *et al.*[115] Healthy donor human CD14+ monocytes were sorted from the peripheral blood of five individual subjects, cultured in the presence of β-glucan for 24 hours, and then re-exposed to secondary stimulus (LPS) 7 days later. Consistent with prior findings, Figure 3.8a, demonstrates that CD14+ monocytes trained

with β-glucan showed increase TNF-α production upon re-exposure to LPS. Next, we trained CD14+ monocytes with β-glucan, and then rechallenged them with either tumor conditioned or IRE ablated media from the human S2013 PC cell line. The trained CD14+ cells demonstrated significantly elevated levels of TNF-α after exposure to S2013 IRE conditioned media (Figure 3.8b). PC is known to cause a systemic immune cell exhaustion.[176] Therefore, we wanted to determine if CD14+ monocytes could be trained from the peripheral blood of newly diagnosed treatment naïve PC patients. To test this, we repeated this experiment with CD14+ monocytes obtained from PC patients. We observed that CD14+ monocytes derived from treatment naïve PC patients were also trained by β-glucan as demonstrated by an increase in TNF-α production following LPS restimulation (Figure 3.8c). Similarly, IRE-ablated tumor conditioned media elicited more potent trained response compared to regular tumor culture supernatants as revealed by increased TNF-a production (Figure 3.8d). Given these findings and our oral β-glucan data presented above, we next wanted to evaluate the potential trained immunity effect of oral β-glucan from peripheral blood of stage III PC patients who were scheduled to receive IRE. To do so, baseline blood was collected from five patients prior to undergoing surgical IRE (baseline). After undergoing IRE and recovering to meet acceptable discharge standards the patients began taking 1000mg oral β-glucan daily. Blood was then collected after 3 months of oral β-glucan therapy post IRE. To perform a comprehensive evaluation of the peripheral immune landscape, mass cytometry (CyTOF) was performed comparing baseline and 3-month samples. Twenty unique cell populations from the peripheral blood were identified and compared between the time points (Figure 3.9). Summary of cell surface markers and heatmap profile provided in Figure 3.10. Examination of t-SNE plots revealed there to be

consistent observable differences in the peripheral immune landscape before and after oral β-glucan of all five patients (Figure 3.11). Notably, consistent increases in frequency of CD86+ monocytes, cluster 0, (Figure 3.12a) were observed for all five patients whereas the frequency of Tregs, cluster 13, significantly decreased overtime (Figure 3.12b). Encouragingly, 4 of five patients demonstrate heightened TNF-α production from CD86+ monocytes 3 months post IRE after daily oral β-glucan (Figure 3.12c,d). Although preliminary, these data support the ability of oral β-glucan to positively alter the immune landscape of advanced stage PC patients providing rationale for further clinical investigation.

CHAPTER III FIGURES

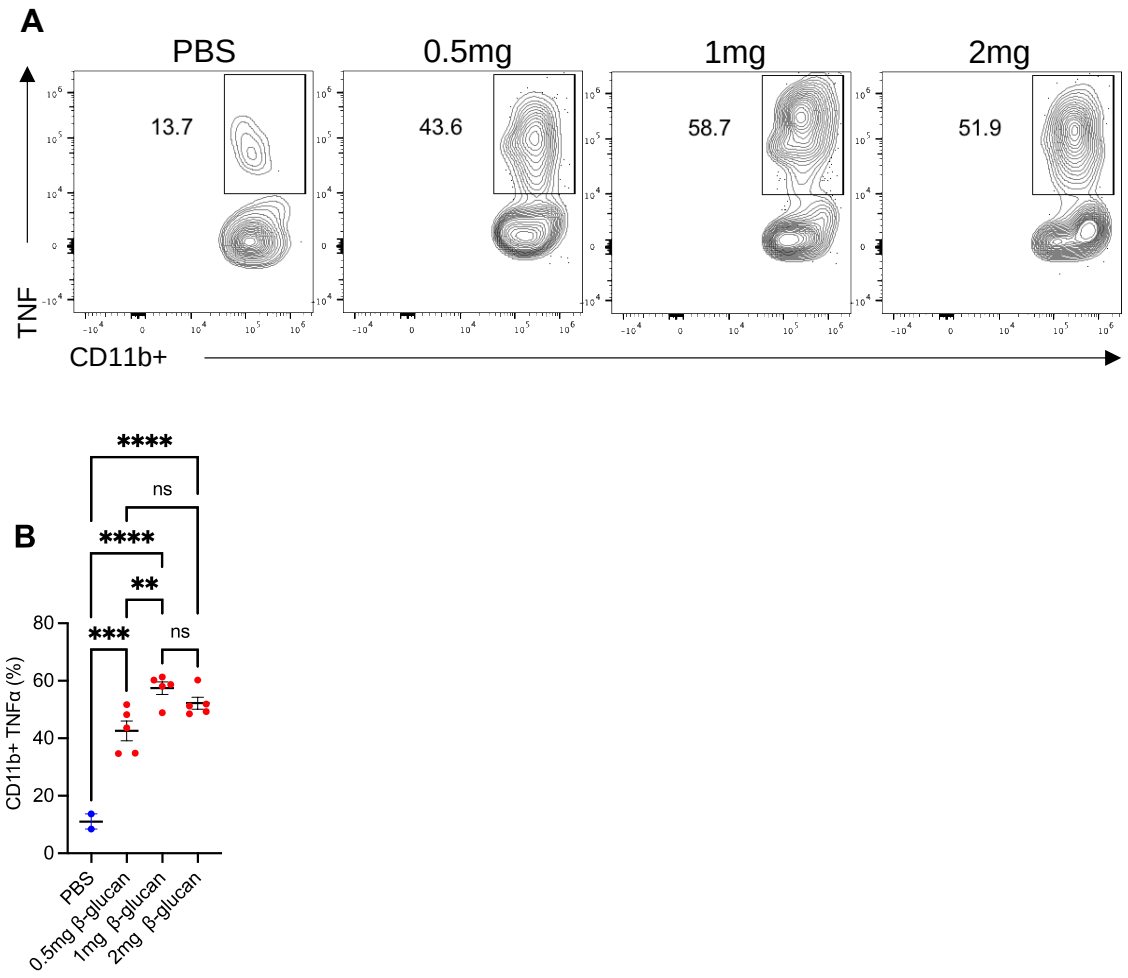


Figure 3.1: Oral β-glucan induces trained response within murine pancreas.

(A) Representative flow contour plots of CD11b+TNF-α+ cells from the pancreas of WT mice fed PBS or increasing doses of oral β-glucan (0.5mg, 1mg, 2mg) daily for one week. (B) Quantified percent CD11b+TNF-α+ cells are shown, PBS n =2, 0.5mg β-glucan n = 5, 1mg β-glucan n = 5, 2mg β-glucan n= 5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; **p<0.01, ***p<0.001, ****p<0.0001.

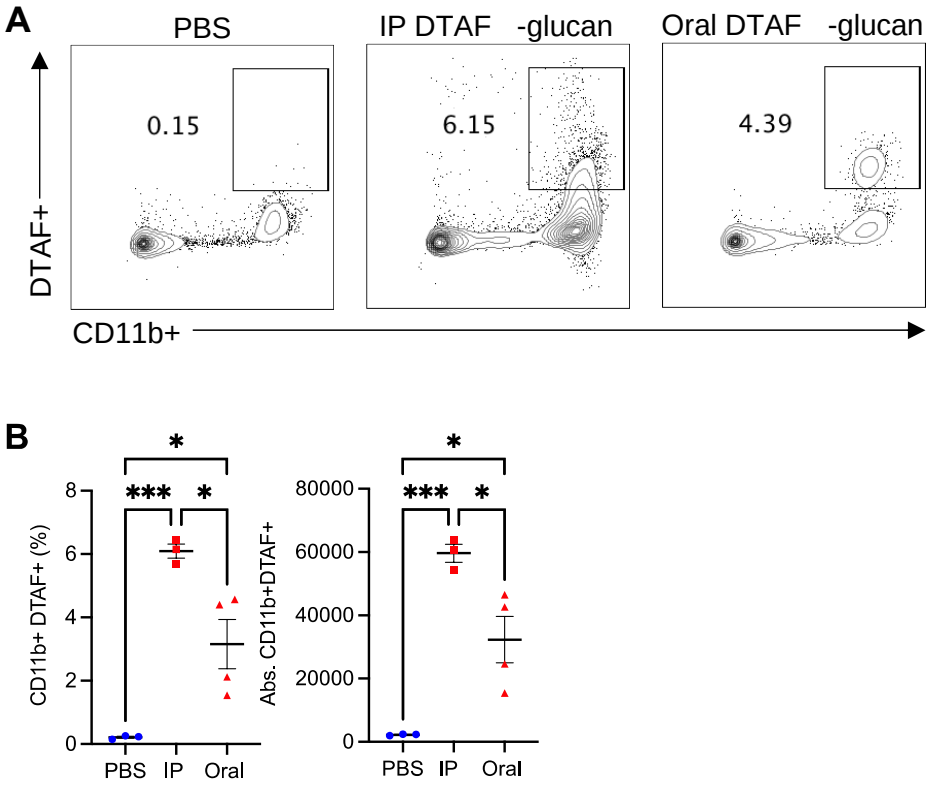


Figure 3.2: Oral β-glucan traffics into the murine pancreas.

(A) WT mice were treated with one dose of IP DTAF-β-glucan or oral DTAF-β-glucan for 6 days. On day 7, mice were euthanized, and pancreatic myeloid cells were assessed for β-glucan uptake by flow cytometry. Representative flow contour plots are shown. Cells were first gated on viable, CD45+CD11b+ cells. (B) Summarized percent CD11b+DTAF+ cells and absolute number of CD11b+DTAF+ cells (right) are shown. PBS n=3, IP DTAF-β-glucan n=3, oral DTAF-β-glucan n=4. Data are representative of two independent experiments and presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

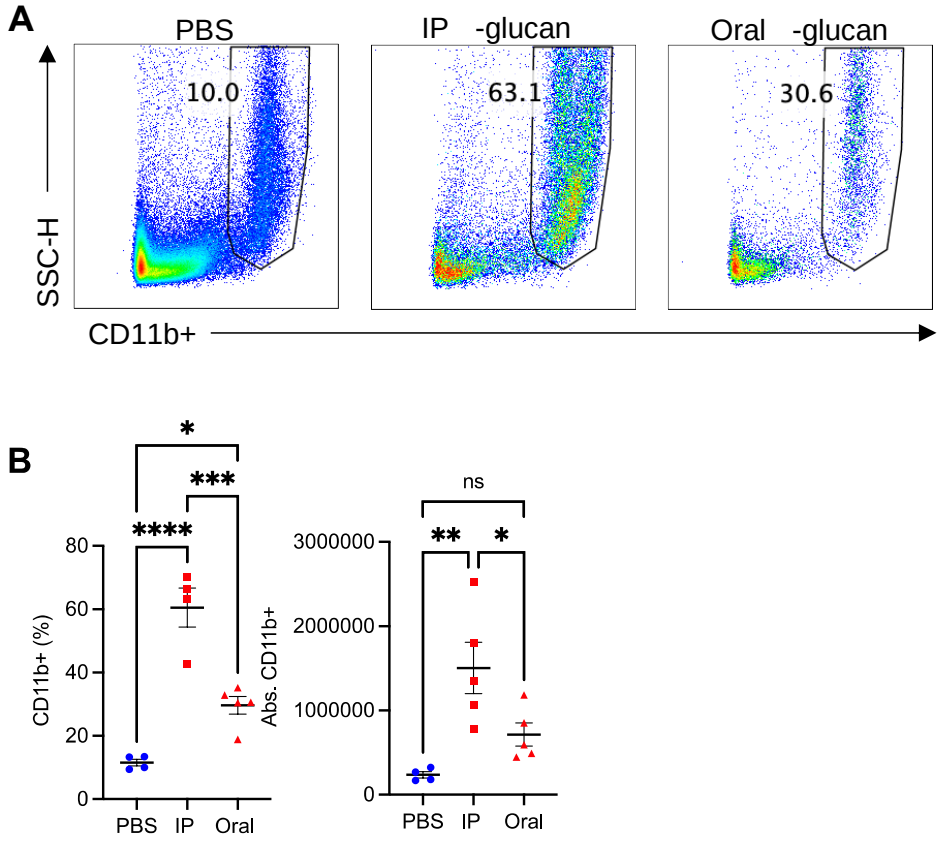


Figure 3.3: Oral β-glucan promotes influx of CD11b+ myeloid cells in the pancreas.

(A) Representative flow dot plots of viable, CD45+CD11b+ cells from the pancreas of PBS, IP β-glucan, or oral β-glucan treated mice. (B) Summarized percent CD11b+ cells and absolute number of CD11b+ cells (right) are shown. PBS n=4, IP β-glucan n=5, oral β-glucan n=5. Data are representative of two independent experiments and presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

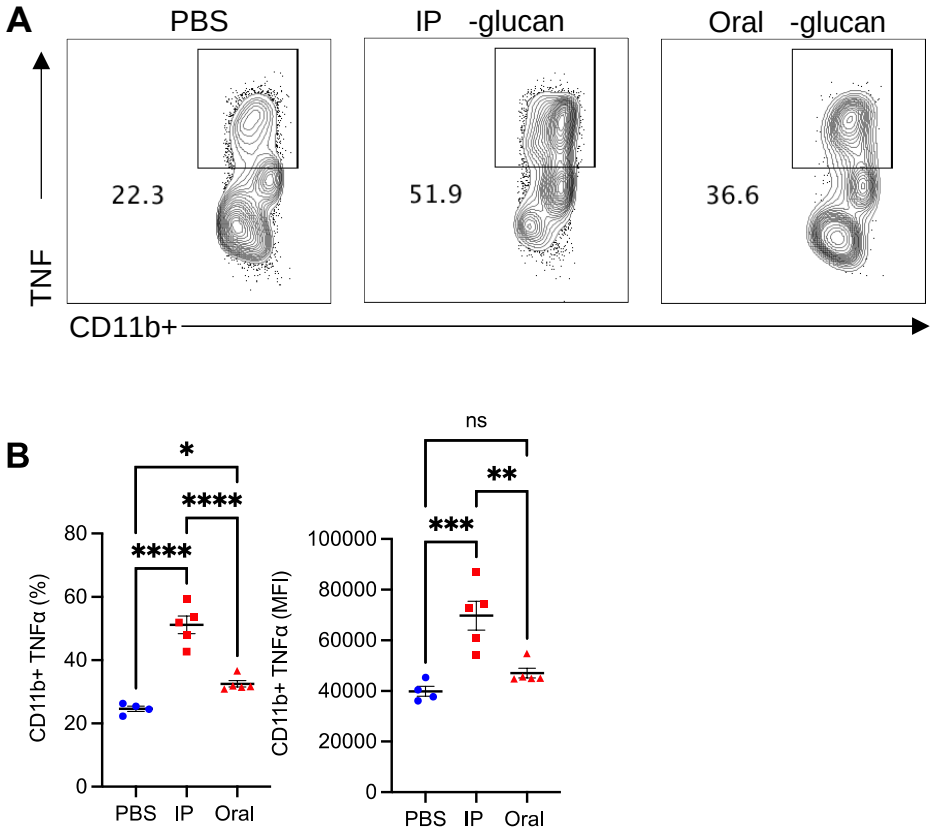


Figure 3.4: Comparison of myeloid trained response by IP and oral β-glucan.

(A) Representative flow contour plots of TNF-a producing CD11b+ cells from PBS, IP β-glucan, or oral β-glucan treated mice. (B) Quantified percent CD11b+TNF-a+ cells and MFI (right) are shown. PBS n=4, IP β-glucan n=5, oral β-glucan n=5. Data are representative of two independent experiments and presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

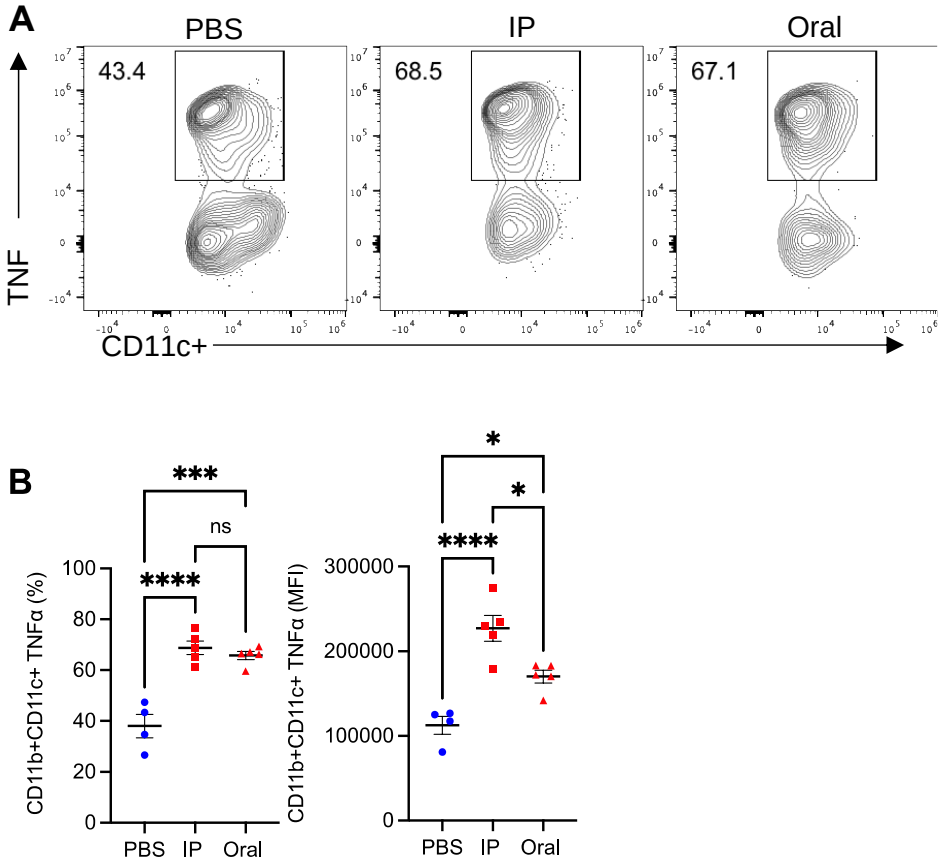


Figure 3.5: Oral β-glucan induces trained response in DCs within the pancreas.

(A) Representative flow contour plots comparing CD11b+CD11c+ TNF-α expression. (B) Quantified percent and MFI of CD11c+TNF-α+ cells are shown. PBS n = 4, IP β-glucan n = 5, and oral β-glucan n = 5, respectively. Data are presented as mean ± SEM. Significance: ns= not significant; **p*<0.05, ****p*<0.001, *****p*<0.0001.

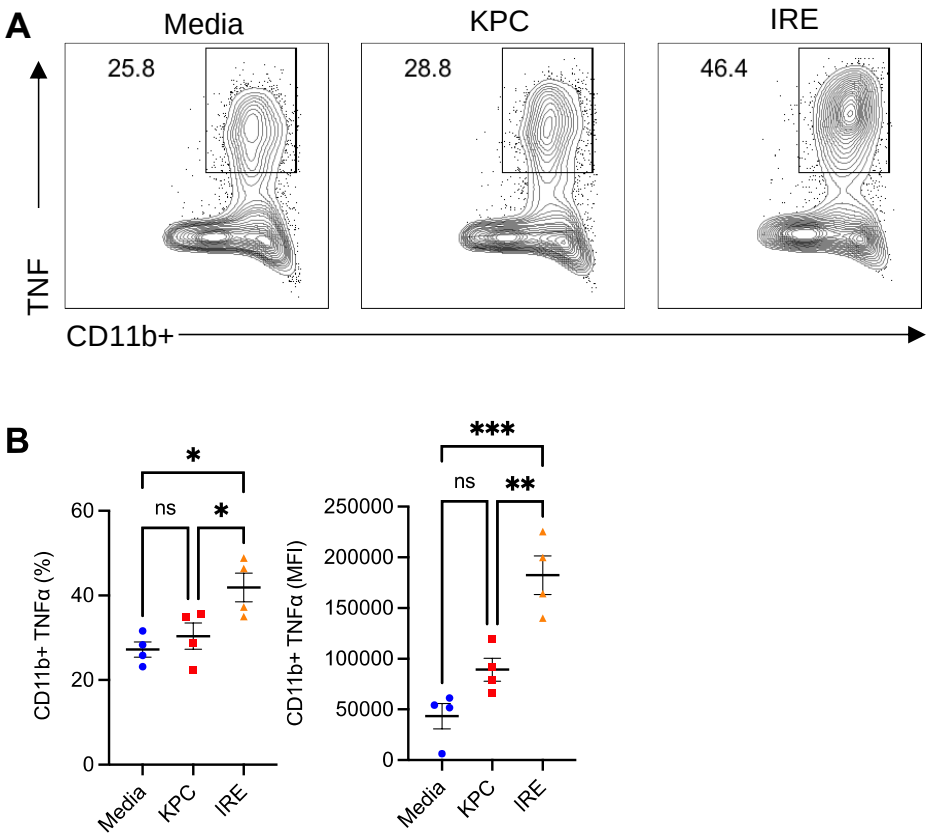


Figure 3.6: IRE conditioned media augments trained response from myeloid cells after oral β-glucan.

(A) Representative flow contour plots of TNF-a producing CD11b+ cells from the pancreas of mice fed 6 doses of oral β-glucan and exposed to media, KPC, or IRE conditioned media. (B) Summarized percent and MFI of CD11b+TNF-a+ cells are shown. Media n=4, IP β-glucan n=4, oral β-glucan n=4. Data are representative of two independent experiments and presented as mean ± SEM. Significance: ns= not significant; *p<0.05, **p<0.01, ***p<0.001.

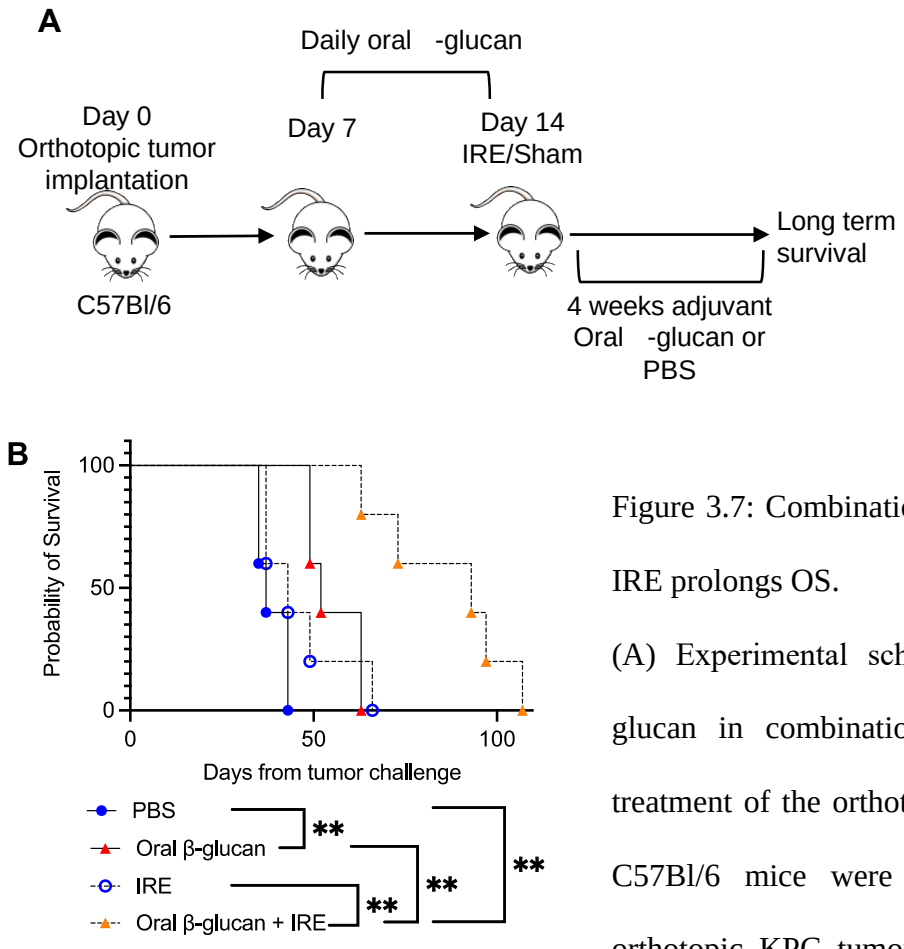


Figure 3.7: Combination of oral β- with IRE prolongs OS.

(A) Experimental scheme for oral β-glucan in combination with IRE for treatment of the orthotopic KPC model. C57Bl/6 mice were challenged with orthotopic KPC tumors on Day 0 and allowed to recover for 1 week before beginning daily neoadjuvant oral β-glucan administration. After 1 week of neoadjuvant oral β-glucan, mice underwent IRE or sham surgery. Oral β-glucan was then continued daily for 4 weeks postoperatively. (B) Overall survival. PBS n=5, IRE n=5, β-glucan n=5, β-glucan + IRE n=5, respectively. Data are representative of two independent experiments and presented as mean ± SEM. Significance: $^{**}p<0.01$.

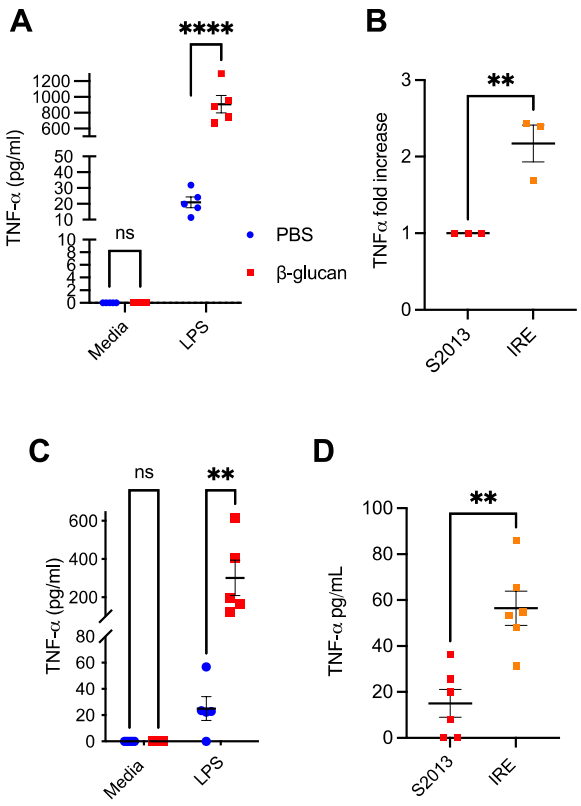


Figure 3.8: Effect of β-glucan on healthy donor and PC patient peripheral blood mononuclear cells *in vitro.*

(A) TNF-a production after *in vitro* β-glucan trained or untrained healthy donor CD14+ monocytes (n=5) obtained via cell sorting and restimulated with LPS measured by ELISA. (B) Fold change in TNF-a production by *in vitro* β-glucan -trained healthy donor CD14+ monocytes (n=3) upon co-culture with S2013 or IRE ablated media. (C) TNF-a production after *in vitro* β-glucan trained or untrained PC treatment naïve CD14+ monocytes (n=5) obtained via cell sorting and restimulated with LPS measured by ELISA. (D) TNF-a production by *in vitro* β-glucan -trained PC treatment naïve CD14+ monocytes (n=3) upon co-culture with S2013 or IRE ablated

media. Data are presented as mean ± SEM. Significance: ns= not significant; **p<0.01, ****p<0.0001.

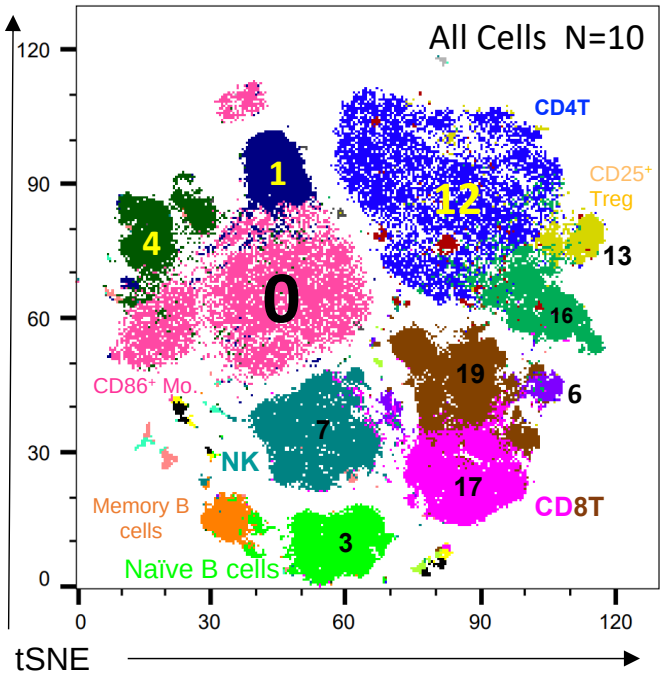


Figure 3.9: Characterization of peripheral blood by mass cytometry in LAPC post IRE and oral β-glucan.

Representative t-SNE plot of the 10 experimental samples identifying 20 distinct cell clusters.

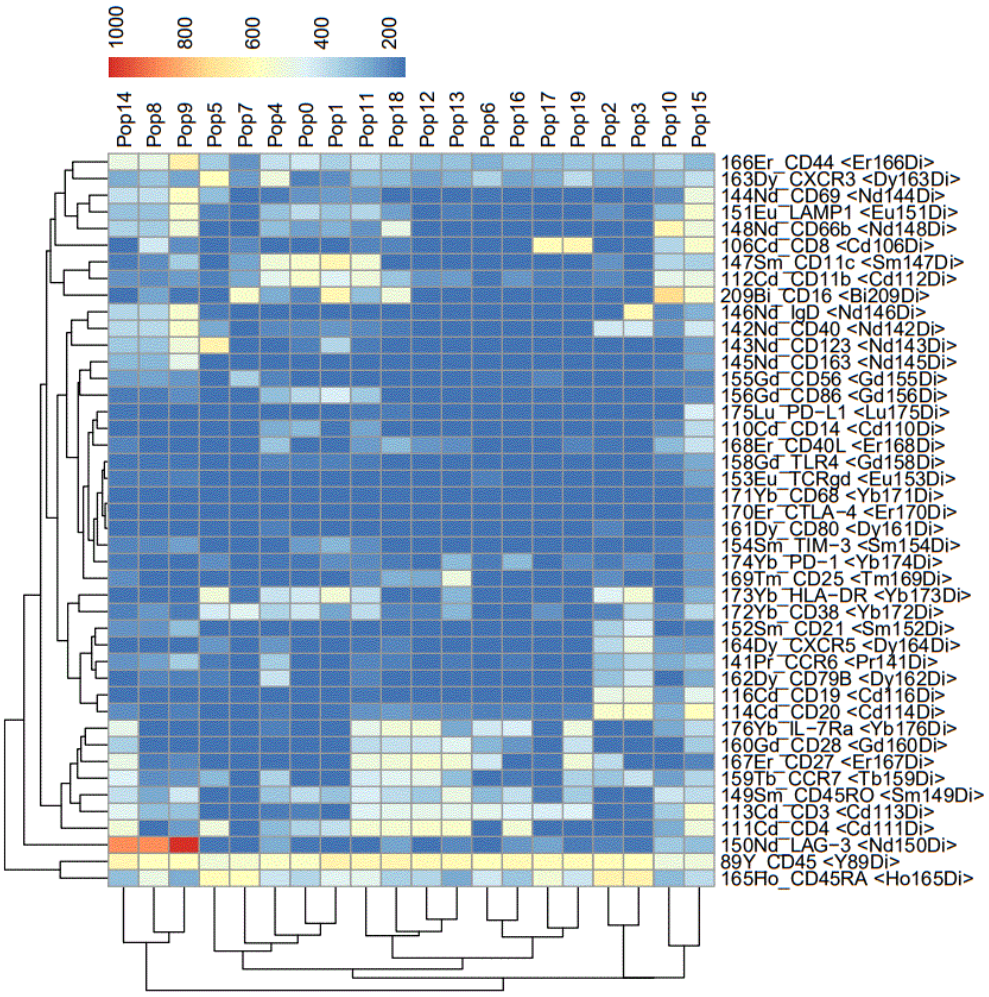


Figure 3.10: CyTOF heat map and summary of cell surface markers.

Heatmap scaled expression of cell surface markers within each cell cluster as determined by CyTOF workflow analysis.

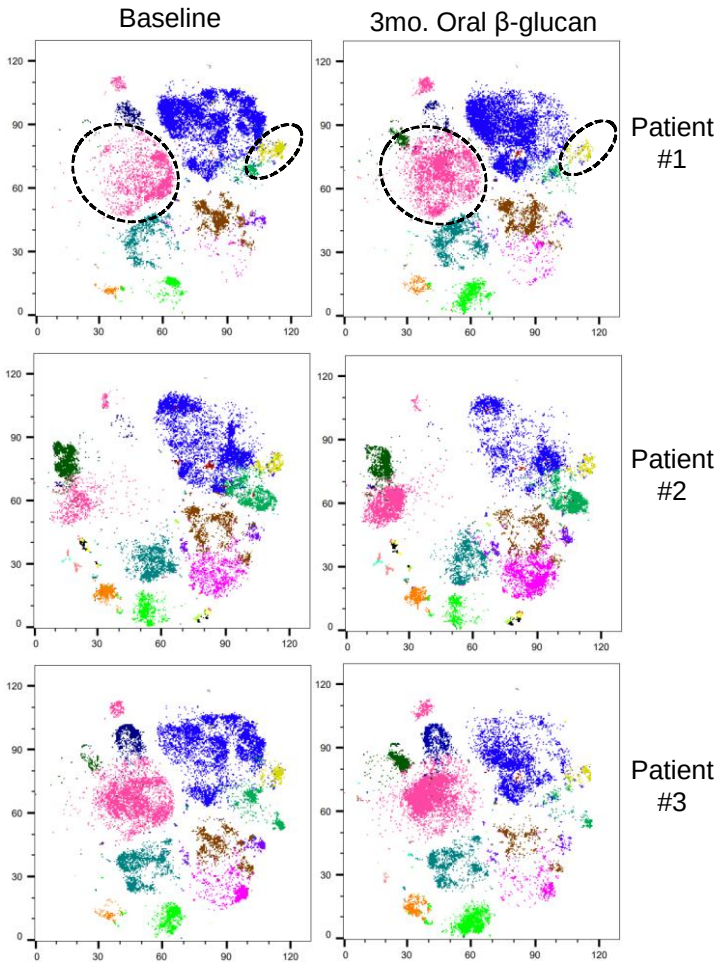


Figure 3.11: Oral β-glucan positively alters peripheral immune landscape in advanced PC.

Individual t-SNE plots of PBMCs from three representative patients before (baseline) and after IRE and 3months of oral β-glucan.

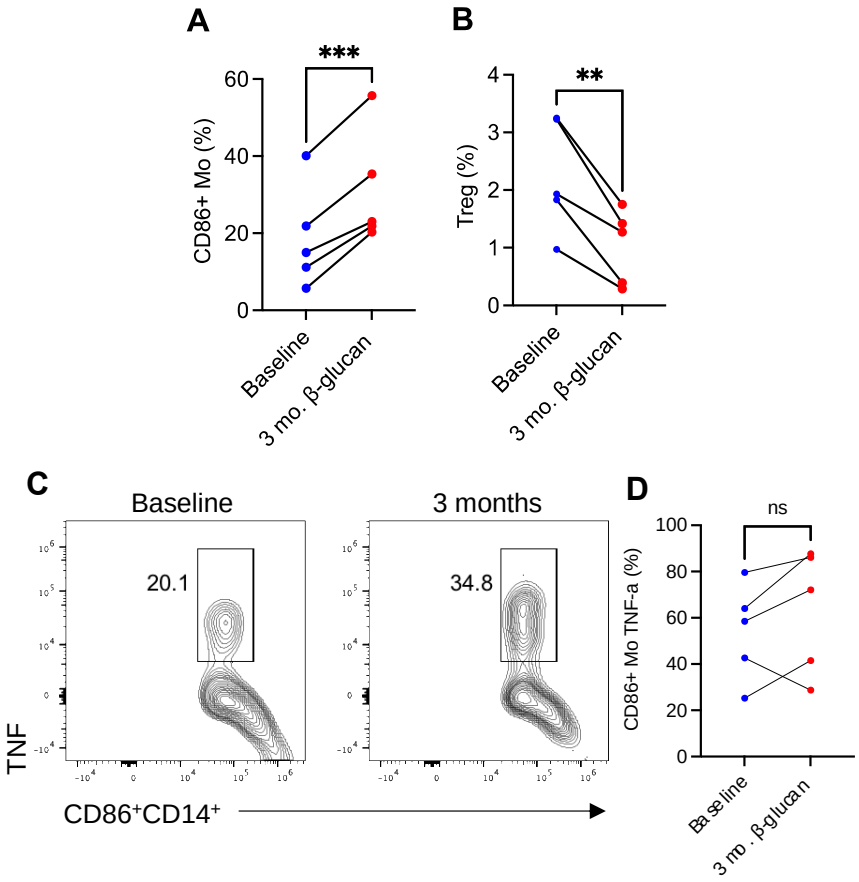


Figure 3.12: Effect of oral β-glucan on LAPC patient peripheral blood mononuclear cells after IRE.

(A) Quantified percent CD86+ monocytes and Tregs (B) at baseline and after IRE and 3 months of oral β-glucan. (C) Representative flow contour plots and (D) quantified percent of TNF-α production from CD86+ monocytes at baseline and 3 months post IRE and oral β-glucan. PC monocytes were restimulated with LPS (1ng/mL). Data are presented as mean ± SEM. Significance: ns= not significant; ***p*<0.01, ****p*<0.001.

CHAPTER III DISCUSSION

Oral administration of β-glucan offers many advantages over IP β-glucan injection such as ease of access and better patient compliance. Prior studies utilizing oral β-glucan demonstrated effective anti-cancer properties.[177] Human and animal studies have reported anti-viral properties of oral β-glucan, yet no studies have used oral β-glucan as an agonist of trained immunity in the setting of cancer.[178, 179] We found that oral β-glucan traffics to the pancreas in small quantities inciting a phenotype of peripheral trained immunity. Oral β-glucans are known to directly interact with intestinal mucosa, to be taken up by M cells, and interact with resident macrophages and dendritic cells (DCs) being distributed throughout the general circulation.[180, 181] This may explain our finding that pancreatic CD11b+CD11c+ DCs to have increased cytokine production after oral β-glucan intake. However, the specific biodistribution of oral β-glucan, its overall tropism to the pancreas, and its effector cell targets remain to be better understood. Notably, systemic administration of IP β-glucan had more pronounced effects, which calls for more studies comparing antitumor immunity versus ease and safety of β-glucan administration routes in the PC setting. With these findings it is reasonable to hypothesize that IP β-glucan to have more pronounced training effects within the pancreas including enhanced epigenetic and transcriptional modifications. Myeloid cells isolated from pancreases of mice who received 7 days of oral β-glucan were also more responsive to IRE ablated media. With this understanding, we also observed dramatic improvement in overall survival when oral β-glucan was given in combination with IRE. Together, these findings provide further credence to our above conclusions combining β-glucan with IRE in PC and call for further studies to address signaling pathways between IRE and trained myeloid cells.

Prior studies investigating induction of trained immunity in human subjects as a cancer therapeutic primarily focus on BCG in non-muscle invasive bladder cancer.[182, 183] While the anti-cancer effects and mechanisms of β-glucan continue to be appreciated, oral β-glucan is known to be well tolerated in humans and can augment innate immune cells similar to BCG.[180] To date, few quality data exists evaluating use of β-glucan in cancer patients. One study found oral β-glucan reduced adverse effects of mucositis from systemic chemotherapy in colorectal cancer patients.[184] In 23 advanced stage breast cancer patients, oral β-glucan increased proliferation and activation of PBMCs measured by increased CD95 and CD45RA on CD14+ monocytes fifteen days after treatment.[185] Our previous study showed that oral β-glucan modulates peripheral monocyte composition and function in human non-small cell lung carcinoma patients.[186] The current study is the first report indicating monocytes obtained from PC patients can be trained *in vitro* and to describe alternations in the peripheral immune phenotype in PC patients after oral ingestion of β-glucan. Interestingly, we found increased frequency of CD86+CD11b+CD11c+ monocytes after three months of adjuvant oral β-glucan following IRE and decreased Treg frequency in five stage III PC patients. In addition, CD86+ monocytes from 4 of 5 patients showed trained immunity phenotype. These data demonstrate that human monocytes from PC patients can be trained through oral β-glucan, providing a potential avenue to modulate immunosuppressive myeloid cells within the PC TME. However, a more thorough assessment of the functional state of these cells, their influence on the TME, and their clinical effects and outcomes is needed.

OVERALL SUMMARY AND FUTURE DIRECTIONS

This dissertation began with an appreciation for the complexity PC as a disease process as well for the difficulty in its treatment. PC portends a grim prognosis, and many patients continue to rely on non-specific, toxic, and palliative treatments. Currently available ICI therapy has failed to be translated to patients with PC despite remarkable success in other solid tumors. The PC TME is nearly impenetrable and is intrinsically immunosuppressive thus promoting a vicious cycle of tumor growth and immune evasion. IRE has recently emerged as an investigative ablation modality in the treatment of LAPC with promising results as a consolidative treatment following induction chemotherapy. Moreover, it appears to facilitate the action of various immunotherapies, positively alters the dense heterogenous stoma, and is a physical modality for active tumor neoantigen and DAMP release. Our lab first described the unique trafficking pattern of IP β-glucan to the murine pancreas, which drives an influx of trained myeloid cells primed to respond to secondary unrelated stimuli. Remarkably, β-glucan's tropism for the murine pancreas reprograms the myeloid compartment and has anti-tumor properties. In recognizing a desperate need for innovative treatment alternatives in PC, the inspiration of this thesis was derived from a keen interest in improving β-glucan's effect via surgical ablation.

In summary, the work presented within this dissertation has largely supported the original hypothesis that IRE would augment β-glucan induced training within innate myeloid cells and ultimately enhance its efficacy in the treatment of PC. In part 1, we observed the ability of IRE to release DAMPs (i.e., MIF) in large quantity from cultured KPC tumor cells, which elicits a potent trained response in a dose dependent manner. We

then witnessed the superiority of IRE conditioned media in eliciting a trained response *ex vivo*. Furthermore, exposure of β-glucan trained myeloid cells to IRE potentiated their anti-tumor functionality as demonstrated by enhanced phagocytosis and cytotoxicity. This data is important because it is the first study that has aimed to describe a clinically available mechanism to not only provoke but strengthen the trained response.

In Part 2, we proceeded to test the combination of IRE and β-glucan *in vivo* against an aggressive orthotopic PC tumor model. We reported a striking decrease tumor burden and prolongation of overall survival for WT mice treated with our novel combination. The tumors not only were reduced in size but had evidence for epigenetic and transcriptomic reprogramming—pathognomonic signs of trained immunity. As a surrogate quantification of training, we also report augmented innate cell responses in the TME including increased absolute myeloid cells and cytokine production with combination β-glucan and IRE therapy. Surprisingly, we also observed decreased tumor burden within the lungs of mice treated in combination. This dissertation also sheds light onto the timing and duration of trained immunity. Based on current knowledge in the field of trained immunity, it is generally accepted that this form of immunologic memory is relatively short term and is an area of active research and debate. In this study we demonstrated the trained response from β-glucan plus IRE treated KPC tumors 24 days after tumor challenge, as well as increased frequency of TNF-α from β-glucan and IRE treated tumors near physiologic endpoint nearly 40 days post tumor challenge. This is a marked improvement upon our prior data utilizing single dose IP injection β-glucan, which peaks in secondary response to LPS within 7-10 days. Developing novel approaches such as this to establish "sustained"

trained immunity may hold the key for providing a lasting response. We then proceeded to demonstrate reduction in tumor burden and prolongations in OS that occurred despite the presence of adaptive immune T and B cells. This data agrees with prior investigations that demonstrate trained immunity occurs independent of adaptive immunity.[187] More encouraging, however, is that the infiltrating T cells were not suppressed or exhausted in our combination therapy. Unexpectedly, we found overall lower frequency of CD8+PD-1+ and more CD8+Granzyme B+, suggesting a more capable effector T cell phenotype This highlights a potential avenue to successfully utilize current ICI in a setting where prior attempts have failed.

In part 3, we then investigated oral β-glucan as an alternative route of administration for induction of trained immunity within the pancreas. This was prompted by several practical limitations of translating IP β-glucan to PC patients. The data above established that oral β-glucan is capable of provoking trained immunity in the murine pancreas. Further, oral β-glucan was observed to traffic to the murine pancreas and incite enhanced TNF-α production when restimulated with IRE conditioned media. CD11b+CD11c+ DCs may be the underlying effector cells as they were found to have increased cytokine production after oral β-glucan intake. This finding agrees with Li *et al.*, who found orally administered β-glucan to activate tumor capturing DCs which ultimately expanded antigen specific CD4 and CD8 T cells.[188] However, more specific cell depletion studies are needed to make this conclusion. Although direct comparisons of IP and oral administration were not our primary objective, IP β-glucan did have significantly increased trafficking, TNF-α production, and percent myeloid cell infiltration to the pancreas. More

studies are needed to compare their therapeutic efficacy against PC. Incredibly, we were also able to demonstrate prolongation in overall survival for mice treated with IRE and continued oral β-glucan providing support for utilizing β-glucan in an adjuvant setting. Additionally, whereas, prior investigations have utilized β-glucan in a prophylactic setting prior to tumor challenge this study has entirely focused on induction of training in a treatment setting with established tumors. This dissertation then concluded with exciting human data comprehensively characterizing the peripheral blood of LAPC patients taking adjuvant oral β-glucan after being treated with open *in situ* IRE. All five patients demonstrated increased frequency of peripheral CD14+CD86+ monocytes as well as decrease in Treg frequency. Four of those patients also had heightened CD86+ secondary responses (i.e. increased TNF-α expression) to LPS restimulation suggesting training to have occurred within this cell population. While this data is exciting immunologically, the clinical value and implication remain to be seen.

This dissertation prompts many important questions and future experimental directions. First, the direct mechanism and cellular pathways ascertaining how IRE amplifies β-glucan for the development of trained immunity need to be explored. For example, neutralization of MIF *in vitro* or utilization of KPC tumor bearing MIF knockout mouse models would provide clarity on whether the release of MIF by IRE is essential for eliciting the trained response. While these experiments are ongoing, we hypothesize that complete inhibition of MIF or its knockout would demonstrate only partial reduction in trained response due to IRE's ability to nonspecifically release extensive tumor neoantigen. Other studies have well described the release of other DAMPs such as high motility group

box protein-1 (HMGB1), ATP, and calreticulin via IRE.[189] Recent work in investigating allogeneic heart transplants have demonstrated upregulation Dectin-1 and TLR4 receptors on monocytes via HMGB-1 and subsequent production of cytokines TNF-α and IL-6.[190] This data support that IRE alone may stimulate these ligands to illicit features of trained immunity and further support a synergism with β-glucan, which is well known to stimulate Dectin-1 and TLR signaling.[191, 192] IRE's ability to increase the anti-tumor functions (phagocytosis and direct cytotoxicity) by β-glucan provokes further drives hypotheses that addition of anti-CD47 antibody may augment macrophage mediated phagocytosis via blocking "don't eat me" CD47 signals abundant on tumor cells.[193] Next, while the data herein provide convincing evidence for an upregulated and prolonged tumor infiltrating myeloid cells compared to our prior studies, further work is needed to delineate the primary effector cell within this phenotype. Adoptive transfer studies as well as select myeloid cell depletions would provide direct evidence. As our oral data may suggest, we hypothesize that DCs may be key to the response to IRE within the murine pancreas. Interestingly, recent combination of IRE and CD40 antibody improved survival and decreased metastatic burden in an orthotopic KPC model.[194] Gemcitabine plus nab-paclitaxel and CD40 activation allowed for clonal T cell expansion and durable remission of a PC model. These data directly agree with the author's belief that IRE releases tumor neoantigen providing antigen presenting cells with abundance of material available for cross talk to the adaptive immune system. Although the anti-tumor effect of the combination therapy presented within this dissertation does not depend on the adaptive response, we have seen favorable effector T cell markers including downregulated PD-1 and increased Granzyme-B. This in consideration with our prior findings of β-glucan's synergistic effect with anti-PDL-1

and Zhao's finding that IRE reverses resistance to anti-PD-1 checkpoint blockade also beg the question whether triple combination therapy of β-glucan, IRE, and PD-1 may further treatment efficacies. Delineating the role and alterations to the gut microbiome in β-glucan training and its relation to this anti-PC phenotype is another area of research that will need clarification. PC tumor responses to chemotherapy and immunotherapy have been linked to immunosuppressive microbes.[195] Whether IP and oral β-glucan induced trained immunity is dependent on the gut microbiome remains unexplored. The pancreas was once thought to be a sterile organ. However, increasing data support the ability of the gut microbiome to modulate PC tumorigenesis, TME, and treatment responses. The cancerous pancreas has been found to harbor more microbes than the naïve pancreas in both mice and humans. Interestingly, ablation of the gut microbiome with oral antibiotics has been found protective against PC disease progression and fecal transfer from tumor bearing mice reversed tumor protection.[196] Rationally, we believe oral administration of β-glucan is more likely to positively alter the gut to pancreas microbial axis via direct interactions at the gut epithelial barriers. Whereas IP β-glucan, given via a sterile approach, may fore go alterations in the gut microbiome. Utilization of germ free and gnotobiotic mouse models will be able to directly answer these questions. Evaluation of the primary TME and off target metastases or organs are also needed. While this dissertation demonstrated a decrease in lung metastases, the immune phenotype and evaluations of trained immunity were lacking. Similarly, IRE has previously demonstrated an abscopal effect—whereby non ablated regions demonstrate phenotypic differences after treatment.[100] Would addition of adjuvant β-glucan to IRE therapy decrease MDSCs or increase M1/M2 ratios in metastatic lesions? Subsequent experiments will focus on answering these questions.

Finally, appropriate translation of this combination therapy beyond the present data will necessitate several requirements. First, the recruiting of patients should be done under strict inclusion and exclusion criteria. For example, consistency among pre and post operative chemotherapy is critical in determining the exact immunologic effects of β-glucan on these immunosuppressed patients. Similarly, utilization of IRE in a standardized way will help ensure accurate assessments of its ability to augment the trained response and vice versa. Evaluations of tumor tissue and bone marrow in PC patients taking β-glucan are needed to determine its effect on granulopoiesis and the TME. Certainly, the effect of β-glucan should be tested through all stages of disease to determine whether disease burden has a negative impact on trained immunity. Furthermore, a trial of β-glucan given preoperatively should be explored to better mimic the preclinical conditions we describe above. While these studies are forthcoming, we predict trained immunity to play an increasing role in bettering the applicability of immunotherapy in PC.

In conclusion, these findings demonstrate the antitumor potential of yeast-derived β-glucan-induced trained immunity within the pancreas by two routes of administration whose effect can be augmented by IRE, providing a novel modality to augment trained response for treatment of PC. This combination significantly reduced local and distant tumor burden in mice bearing orthotopic PC tumors, establishing solid proof of concept for future clinical trials. We also provide insight to the duration of trained effect demonstrated within the PC TME. This work builds upon our previous findings first describing induction of peripheral trained immunity in the pancreas via β-glucan and is the first to describe changes within the peripheral immune landscape by oral β-glucan in a high-risk LAPC patient population. Although our patient data are pre-liminary, these results provide

optimism for future work investigating the combination of IRE and trained immunity, which we hypothesize will favorably alter the TME in human subjects.

METHODS

Mice

Wild-type (WT) C57Bl/6 female mice six to eight weeks old, were purchased from Charles River Laboratories (Wilmington, MA, USA). B6.129S7-Rag^{tm1Mom}/J (Rag-/-) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed at the University of Louisville in a specific pathogen-free (SPF) animal facility. All experiments were conducted in accordance with the relevant laws and institutional guidelines provided by the Rodent Rearing Facility (RRF) and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Louisville (Louisville, KY, USA).

β-glucan preparation

Saccharomyces cerevisiae derived particulate β-glucan (Biothera), was dissolved in 1x PBS and sonicated at 20 pulses twice for 10 seconds each on ice using a Qsonica Q55-110 Q55 Sonicator (Cole-Parmer) before treatment or injection.

DTAF labeled β-glucan preparation and administration

5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF, 2 mg/ml, Sigma Aldrich) was incubated with continuous shaking at room temperature for 8 hours in 20mg/mL β-glucan in borate buffer (pH 10.8). The mixture was then washed and centrifuged in cold sterile endotoxin-free Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich) to remove any visible traces of DTAF in the supernatant. The DTAF- β-

glucan was maintained and stored at 10mg/L concentration in 4°C. Mice were given once daily 1mg oral DTAF- β-glucan for six days or IP injected with 1mg of DTAF- β-glucan 24 hours prior to euthanasia and harvest of the pancreas for trafficking studies.

Pancreas and tumor digestions/ preparation of single cell suspensions

Weighted and measured tumors or normal pancreases were mechanically minced and digested in a mixture of complete RPMI 1640 and [1x] digestion buffer containing 300 U/ml collagenase I, 60 U/ml Hyaluronidase, and 80 U/ml DNase (Sigma) at 37°C with 5% CO2 30 minutes with continuous rotation. The digestion reaction was stopped with cold complete RPMI 1640 then the cells were passed through a sterile nylon 40 μm basket filter. Undigested tissue was smashed through the filter using a sterile syringe stopper. RBC lysis was performed by adding 0.5 mL of sterile ACK buffer (Thermo Fisher Scientific).

Ex vivo restimulation assays

Single cell suspensions from the pancreas were plated in a 24-well plate (2×10^6 cells/well) and stimulated with LPS (10 ng/ml), rMIF, tumor conditioned media, or IRE conditioned media and incubated at 37°C with 5% CO_2 for 4 hours in the presence of 1X brefeldin A (Biolegend). The cells were then harvested using a mini-cell scrapper (United Biosystems), pelleted, and stained for surface markers followed by intracellular TNF-α staining.

In vitro ablations

Tumor cells were removed from culture with 0.25% trypsin, washed with complete DMEM, and resuspended in tumor conditioned media at a concentration of 2×10^6

cells/mL. Cells were electroporated in 4mm gap sterile electroporation cuvettes (Universal Medical) using the Safety Stand 630B adaptor for the BTX 830 square wave electroporation system, Harvard apparatus. The cells were subjected to electroporation at room temperature with the following parameters: Voltage 500-1000V, pulses 10-20, 0.1ms duration, and 1s pulse interval.

Phagocytosis assay

WT mice were IP injected with β-glucan, the pancreas was harvested 7 days later, and processed to single cell suspensions. Positive selection using CD11b+ microbeads (Miltenyi Biotec) was performed utilizing the autoMACS Pro Separator. Next, 0.75 x 10^6 of the resulting CD11b+ cells were washed with complete RPMI 1640 and incubated for 24 hours with either KPC tumor conditioned media or IRE conditioned media. Next, GFP^+ KPC tumor cells were added to the activated pancreatic CD11b+ cells at a ratio of 4:1 and incubated for 1.5 hours at 37 °C. Samples were gently vortexed every 15 minutes. The reaction was stopped by adding 1 mL of cold PBS. Samples were incubated with Fc Block for 10 minutes at 4 °C, stained for viability, anti-CD11b (APC, Biolegend) for 30 minutes at 4 °C, and then analyzed using CyTEK. For analysis, after gating on live cells, CD11b+ cells that also co-expressed GFP were determined to be phagocytic.

Cytotoxicity assay

Pancreases from β-glucan IP treated mice were harvested and processed to single cell suspensions. Positive selection using the autoMACS Pro Separator and CD11b+ Microbeads (Miltenyi Biotec) was performed to isolate myeloid cells. CD11b+ cells were then co-cultured with luciferase-expressing KPC pancreatic tumor cells or IRE ablated

(500V, 20 pulses) luciferase-expressing KPC pancreatic tumor cells in a 96 well plate at 37°C for 24 hours. The plates were centrifuged at ambient temperature and 20 μL of the supernatant was mixed with 100 μL of the Luciferase Assay Reagent (Promega). Luciferase activity measured in the supernatant and whole cell lysates correlated with tumor cells that had been killed by the effector cells and was measured using a luminometer (Femtomaster FB 12, Zylux Corporation). Spontaneous luciferase signal from plated tumor cells was subtracted from the measurement of the supernatant. Luciferase values are represented as Relative Light Units (RLUs).

In vivo orthotopic tumor model

Orthotopic pancreatic tumors were established by surgical implantation of $0.1x10^6$ KPC cells into the pancreatic parenchyma of 6- to 8-week-old female C57Bl/6 or Rag-/- mice. The KPC cell line was derived from the *LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx1-Cre* (KPC) mouse model and purchased from Ximbio, as previously described.[131] Briefly, mice were anesthetized with 2% inhaled isoflurane, given intramuscular buprenorphine analgesia (0.2mg/kg), and supplemental oxygen. The abdomen was prepped and draped sterile fashion. A 2cm left upper quadrant laparotomy was performed. The tail of the pancreas was identified and eviscerated. A 30-gauge needle was inserted into the tail of the pancreas at its bifurcation. A 1:1 ratio of KPC tumor cells suspended in cold 1x PBS and Matrigel Matrix (Discovery Labware, Inc., Corning REF 35623) was infused into the pancreas using a 30-gauge needle creating a wheal. The pancreas was placed back into anatomical position. The abdomen was closed in two layers using inner absorbable 5-0

vicryl suture and skin closed with 4-0 Nylon suture (CP medical). All mice were monitored for 48 h following surgery and were administered buprenorphine for analgesia.

IRE and sham surgery

Fourteen days following tumor injection the mice were again anesthetized and prepped as described above. A separate 2cm midline laparotomy was performed. The tumor was then identified within the tail of the pancreas, externalized, and measured using calipers. IRE was performed when the tumors reached 3-4mm in maximum diameter using the BTX 830 square wave electroporation system, Harvard apparatus. Platinum tweezer trodes (BTX) were used to deliver 100 pulses at 1000V/cm, 0.1ms duration, and 0.1s pulse interval. Sham/placebo surgery was performed for PBS control and β-glucan control groups, omitting only IRE.

ELISA

The supernatants of cultured and ablated KPC and S2013 were analyzed using murine and human MIF ELISA kits (R&D Systems). Supernatant from *in vivo* trained *ex vivo* stimulated CD11b+ cells were analyzed using murine TNF-α and IL-6 ELISA kits (Biolegend). Histones were extracted from CD11b+ cells, which were positively selected from PBS, β-glucan, IRE, and β-glucan plus IRE treated KPC tumors utilizing the Histone Extraction Kit (Active Motif Inc., Catalong #40028). Histone modifications were quantified using EpiQuik Total Histone H3, Acetyl histone H3K27, Tri-Methyl histone H3K4, and Tri-methyl histone H3K27 Quantification Kits (Colorimetric). Supernatants from *in vitro* trained human CD14+ cells were analyzed using human TNF-α ELISA kits

(Biolegend). All assays were performed using the provided manufacturer instructions and all conditions were performed in duplicates.

qRT-PCR

RNA was extracted using TRIzol (Thermo Fisher) from CD11b+ cells isolated from PBS, β-glucan, IRE, and β-glucan plus IRE treated KPC tumors using CD11b microbeads separation. RNA was then reverse transcriped to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). mRNA expression analysis was carried out using iQ™ SYBR® Green Supermix and CFX Connect PCR Machine (Bio-Rad).

Surface staining for flow cytometry

Pancreas and pancreatic tumor single cell suspensions were washed first with PBS and then Fc receptors were blocked for 10 minutes at 4°C. For pancreatic myeloid cells, single cell suspensions were stained with viability dye-APC eFluor-780 (eBioscience), anti-CD45-PerCP-Cy5.5, anti-CD11b-PE-Cy7, anti-F4/80-FITC, anti-Ly6C-AlexaFluor 700, and anti-Ly6G-APC (Biolegend). For T cells, cells were stained with viability dye-APC eFluor 780, anti-CD3-PE-Cy5, anti-CD4-APC, anti-CD8-PerCp-Cy5.5, anti-CD69-AlexaFluor 700, anti-TIM-3-PE-Cy7, anti-LAG-3-PE-Dazzle 594, and anti-PD-1-APC (Biolegend). Cells were incubated at 4°C for 30 min, washed with cold PBS, filtered, and collected using CyTEK Aurora Northern lights 2 laser flow cytometer (CyTEKbio). All flow cytometry data were analyzed using FlowJo software (BD).

Intracellular staining

Mononuclear single cell suspensions were stimulated in culture for 4-6 hours with LPS (10ng/mL) or 1x PMA/I then stained for surface markers as previously described above after being washed with cold PBS. The cells were then fixed using 500mL of fixation buffer (Biolegend) for 20 minutes at room temperature. Next, the cells were centrifuged at 1600rpm for 5 minutes at 4°C, supernatants discarded, and then washed twice with 1x permeabilization buffer (Biolegend). Cells were then stained with anti-TNF-α-PE, anti-IFN-γ-PE, or anti-Granzyme B-PE (Biolegend) along with respective isotype controls and incubated at 4°C overnight. The samples were washed with 1mL of 1X permeabilization buffer, filtered and suspended in 250mL of 1X permeabilization buffer for acquisition by the CyTEK flow cytometer.

Histology

Mice followed for survival were humanely euthanized once showing signs of disease progression. The lungs were harvested and placed in 4% formalin followed by paraffin embedding. Tissues were cut in 5mm sections and then stained with hematoxylin and eosin (H&E).

Human subject study

Patients with pathologically confirmed and surgically unresectable stage III LA pancreatic adenocarcinoma were recruited after informed consent under IRB #16.110 to undergo IRE followed by adjuvant oral β-glucan. All patients were provided a highly purified 500mg β-glucan supplement (Wellmune, Immune Health Basics). Oral β-glucan administration was

initiated at the time of post IRE hospital discharge and continued twice daily. Baseline blood was obtained preoperatively prior to IRE and at their three month post operative office visit.

Isolation of human PBMC and CD14+ monocytes

Human blood was obtained via venipuncture after informed consent into sterile EDTA collection tubes. Whole blood was mixed with RPMI 1640 in a 1:1 ratio and then gently layered over 5mL of lymphocyte cell separation medium and then centrifuged at 2000rpm for 20 minutes at room temperature. The resulting mononuclear cell layer was carefully removed and then washed in RPMI 1640. The cells were then stained for viability dye and anti-CD14 (FITC, Biolegend) and then sorted using the BD FACSAria.

In vitro training assay with β-glucan

Human CD14+ monocyte cells were cultured in complete RMPI 1640 at a concentration of $0.2x10^6$ cells per 48 well plate in 500µL total. The CD14+ cells were stimulated with 10ng/mL of β-glucan for 24 hours at 37°C and 5% CO_2. The cells were then washed and allowed to rest for 6 days. On day 7, the cells were stimulated with either LPS (10ng/mL) or tumor conditioned supernatant or IRE treated supernatants for 24 hours. The supernatants were then collected for quantification of TNF-α using ELISA.

CyTOF mass cytometry sample preparation

Mass cytometry antibodies were either purchased from Fluidigm or were conjugated from commercially available purified antibodies to the appropriate metal isotope using the

MaxPar X8 Polymer or MCP9 Polymer kits (Fluidigm). Peripheral blood from five stage III LA-PDAC patients was acquired at baseline (prior to IRE or β-glucan) and 3 months post IRE while taking 1,000mg daily oral adjuvant β-glucan. Blood was processed into PBMCs as described above and were kept frozen at –140°C prior to use. About 2×10^6 cells per sample were used. Cells were first stained for viability with 5 uM cisplatin (Fluidigm) in serum-free RPMI 1640 for 5 minutes at RT. Cells were then washed with RPMI 1640 containing 10% FBS for 5 min at 300 × *g*. Cells were stained with the surface antibodies for 30 minutes at RT and washed twice with Maxpar Cell staining buffer (Fluidigm). The cytoplasmic/secreted antibody cocktail was then added and incubated with the cells for 30 minutes at RT. Following incubation, cells were washed with 1mL of 1X Maxpar Perm-S buffer for 5 minutes at 800 × *g* and gently blotted to remove all liquid from the tube. In order to stain for nuclear antigens, cells were then suspended in 1mL of 1X Maxpar nuclear antigen staining buffer for 30 minutes at RT. The nuclear antigen antibody cocktail was then added and incubated for 30 minutes at RT. Cells were washed twice for 5 minutes at 800 × g with 2mL of Nuclear Antigen Staining Permeability buffer. Finally, cells were fixed with 1.6% formaldehyde for 10 minutes at RT, then incubated overnight in 125nM of Intercalator-Iridium (Fluidigm) at 4 °C.

CyTOF data acquisition and analysis

Prior to acquisition, the cell samples were washed twice with Cell Staining Buffer (Fluidigm) and kept on ice. Directly prior to the acquisition, cells were suspended in a 1:9 solution of Cell Acquisition Solution: EQ 4 element calibration beads (Fluidigm). Using the CyTOF software, FCS files were normalized into .fcs files for data analysis. CyTOF

data was analyzed using FlowJo, CytoBank software package, and the CyTOF workflow which includes a suite of packages available in R (r-project.org). For analysis conducted within the CyTOF workflow, FlowJo workspace files exported from flow Workspace and CytoML were used.

Statistical analysis

All results are presented as mean ± Standard Error of the Mean. Flow cytometry data was analyzed using Flow Jo version 10.7.1 for Mac OSx. Statistical significance was determined by ordinary one-way ANOVA with post-hoc multiple comparison or by independent or paired samples T test when appropriate using GraphPad Prism version 9.2 for Mac OSx. All P values of < 0.05 were considered significant.

REFERENCES

1. Hu, J.X., et al., *Pancreatic cancer: A review of epidemiology, trend, and risk factors.* World J Gastroenterol, 2021. **27**(27): p. 4298-4321.
2. Mahadevan, V., *Anatomy of the pancreas and spleen.* Surgery (Oxford), 2019. **37**(6): p. 297-301.
3. Grant, T.J., K. Hua, and A. Singh, *Molecular Pathogenesis of Pancreatic Cancer.* Prog Mol Biol Transl Sci, 2016. **144**: p. 241-275.
4. Aier, I., et al., *A systematic assessment of statistics, risk factors, and underlying features involved in pancreatic cancer.* Cancer epidemiology, 2019. **58**: p. 104-110.
5. Society, A.C., *Key Statistics for Pancreatic Cancer.*
6. Medicine, U.o.P.P.S.o., *About Pancreatic Cancer*
7. Klein, A.P., *Pancreatic cancer epidemiology: understanding the role of lifestyle and inherited risk factors.* Nat Rev Gastroenterol Hepatol, 2021. **18**(7): p. 493-502.
8. McGuigan, A., et al., *Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes.* World J Gastroenterol, 2018. **24**(43): p. 4846-4861.
9. Huang, J., et al., *Worldwide Burden of, Risk Factors for, and Trends in Pancreatic Cancer.* Gastroenterology, 2021. **160**(3): p. 744-754.

10. *The global, regional, and national burden of pancreatic cancer and its attributable risk factors in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017.* Lancet Gastroenterol Hepatol, 2019. **4**(12): p. 934-947.

11. Distler, M., et al., *Precursor Lesions for Sporadic Pancreatic Cancer: PanIN, IPMN, and MCN.* BioMed Research International, 2014. **2014**: p. 474905.

12. Peters, M.L.B., et al., *Progression to pancreatic ductal adenocarcinoma from pancreatic intraepithelial neoplasia: Results of a simulation model.* Pancreatology, 2018. **18**(8): p. 928-934.

13. Lynch, S.M., et al., *Cigarette Smoking and Pancreatic Cancer: A Pooled Analysis From the Pancreatic Cancer Cohort Consortium.* American Journal of Epidemiology, 2009. **170**(4): p. 403-413.

14. Bosetti, C., et al., *Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4).* Annals of oncology, 2012. **23**(7): p. 1880-1888.

15. Wang, Y.-T., et al., *Association between alcohol intake and the risk of pancreatic cancer: a dose–response meta-analysis of cohort studies.* BMC Cancer, 2016. **16**(1): p. 212.

16. Lucenteforte, E., et al., *Alcohol consumption and pancreatic cancer: a pooled analysis in the International Pancreatic Cancer Case–Control Consortium (PanC4).* Annals of Oncology, 2012. **23**(2): p. 374-382.

17. U.S. Cancer Statistics Working Group. U.S. Cancer Statistics Data Visualizations Tool, b.o.s.d.-U.S.D.o.H.a.H.S.

18. Larsson, S.C., N. Orsini, and A. Wolk, *Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies.* International Journal of Cancer, 2007. **120**(9): p. 1993-1998.

19. Permert, J., et al., *Pancreatic cancer is associated with impaired glucose metabolism.* The European journal of surgery= Acta chirurgica, 1993. **159**(2): p. 101-107.

20. Huxley, R., et al., *Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies.* British journal of cancer, 2005. **92**(11): p. 2076-2083.

21. Bosetti, C., et al., *Diabetes, antidiabetic medications, and pancreatic cancer risk: an analysis from the International Pancreatic Cancer Case-Control Consortium.* Annals of oncology, 2014. **25**(10): p. 2065-2072.

22. De Souza, A., et al., *Diabetes Type 2 and Pancreatic Cancer: A History Unfolding.* Jop, 2016. **17**(2): p. 144-148.

23. Midha, S., S. Chawla, and P.K. Garg, *Modifiable and non-modifiable risk factors for pancreatic cancer: A review.* Cancer Letters, 2016. **381**(1): p. 269-277.

24. Kirkegård, J., et al., *Acute Pancreatitis and Pancreatic Cancer Risk: A Nationwide Matched-Cohort Study in Denmark.* Gastroenterology, 2018. **154**(6): p. 1729-1736.

25. Vujasinovic, M., et al., *Risk of developing pancreatic cancer in patients with chronic pancreatitis.* Journal of Clinical Medicine, 2020. **9**(11): p. 3720.

26. Han, M., T.P.T. Tran, and J.-K. Oh, *Chronic pancreatitis and cancer risk in a matched cohort study using national claims data in South Korea.* Scientific Reports, 2022. **12**(1): p. 5545.

27. Brand, R.E., et al., *Advances in counselling and surveillance of patients at risk for pancreatic cancer.* Gut, 2007. **56**(10): p. 1460-1469.

28. Habbe, N., et al., *Familial Pancreatic Cancer Syndromes.* Endocrinology and Metabolism Clinics, 2006. **35**(2): p. 417-430.

29. Reimer, R., et al., *Pancreatic cancer in father and son.* The Lancet, 1977. **309**(8017): p. 911.

30. Hruban, R.H., et al., *Familial pancreatic cancer.* Annals of Oncology, 1999. **10**: p. S69-S73.

31. Pereira, S.P., et al., *Early detection of pancreatic cancer.* The Lancet Gastroenterology & Hepatology, 2020. **5**(7): p. 698-710.

32. Kalyan, A., et al., *Ipilimumab and gemcitabine for advanced pancreas cancer: A phase Ib study.* Journal of Clinical Oncology, 2016. **34**(15_suppl): p. e15747-e15747.

33. Mizrahi, J.D., et al., *Pancreatic cancer.* The Lancet, 2020. **395**(10242): p. 2008-2020.

34. Ryan, D.P., T.S. Hong, and N. Bardeesy, *Pancreatic adenocarcinoma.* N Engl J Med, 2014. **371**(11): p. 1039-49.

35. Gillen, S., et al., *Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages.* PLoS medicine, 2010. **7**(4): p. e1000267.

36. Kamisawa, T., et al., *Pancreatic cancer.* The Lancet, 2016. **388**(10039): p. 73-85.

37. Conroy, T., et al., *FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer.* New England Journal of Medicine, 2018. **379**(25): p. 2395-2406.

38. Ryan, D.P., T.S. Hong, and N. Bardeesy, *Pancreatic Adenocarcinoma.* New England Journal of Medicine, 2014. **371**(11): p. 1039-1049.

39. Klaiber, U., A. Mihaljevic, and T. Hackert, *Radical pancreatic cancer surgery-with arterial resection.* Transl Gastroenterol Hepatol, 2019. **4**: p. 8.

40. Oba, A., et al., *Neoadjuvant Treatment in Pancreatic Cancer.* Front Oncol, 2020. **10**: p. 245.

41. Conroy, T., et al., *FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer.* New England Journal of Medicine, 2011. **364**(19): p. 1817-1825.

42. Von Hoff, D.D., et al., *Increased Survival in Pancreatic Cancer with nab-Paclitaxel plus Gemcitabine.* New England Journal of Medicine, 2013. **369**(18): p. 1691-1703.

43. Suker, M., et al., *FOLFIRINOX for locally advanced pancreatic cancer: a systematic review and patient-level meta-analysis.* The Lancet Oncology, 2016. **17**(6): p. 801-810.

44. Hammel, P., et al., *Effect of Chemoradiotherapy vs Chemotherapy on Survival in Patients With Locally Advanced Pancreatic Cancer Controlled After 4 Months of*

Gemcitabine With or Without Erlotinib: The LAP07 Randomized Clinical Trial. JAMA, 2016. **315**(17): p. 1844-1853.

45. Iacobuzio-Donahue, C.A., et al., *DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer.* Journal of clinical oncology, 2009. **27**(11): p. 1806.

46. Waldman, A.D., J.M. Fritz, and M.J. Lenardo, *A guide to cancer immunotherapy: from T cell basic science to clinical practice.* Nature Reviews Immunology, 2020. **20**(11): p. 651-668.

47. Oiseth, S.J. and M.S. Aziz, *Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead.* Journal of cancer metastasis and treatment, 2017. **3**: p. 250-261.

48. Decker, W.K., et al., *Cancer immunotherapy: historical perspective of a clinical revolution and emerging preclinical animal models.* Frontiers in immunology, 2017. **8**: p. 829.

49. Hester, R., P.K. Mazur, and F. McAllister, *Immunotherapy in Pancreatic Adenocarcinoma: Beyond "Copy/Paste".* Clin Cancer Res, 2021. **27**(23): p. 6287-6297.

50. Safdar, N., A. Said, and M.R. Lucey, *The role of selective digestive decontamination for reducing infection in patients undergoing liver transplantation: a systematic review and meta-analysis.* Liver Transpl, 2004. **10**(7): p. 817-27.

51. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* New England Journal of Medicine, 2010. **363**(8): p. 711-723.

52. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade.* Science, 1996. **271**(5256): p. 1734-6.

53. Robert, C., et al., *Pembrolizumab versus Ipilimumab in Advanced Melanoma.* N Engl J Med, 2015. **372**(26): p. 2521-32.

54. Taube, J.M., et al., *Association of PD-1, PD-1 Ligands, and Other Features of the Tumor Immune Microenvironment with Response to Anti–PD-1 Therapy.* Clinical Cancer Research, 2014. **20**(19): p. 5064-5074.

55. Michielin, O., et al., *Evolving impact of long-term survival results on metastatic melanoma treatment.* Journal for ImmunoTherapy of Cancer, 2020. **8**(2): p. e000948.

56. Royal, R.E., et al., *Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma.* J Immunother, 2010. **33**(8): p. 828-33.

57. Brahmer, J.R., et al., *Safety and activity of anti–PD-L1 antibody in patients with advanced cancer.* New England Journal of Medicine, 2012. **366**(26): p. 2455-2465.

58. Duffy, A.G., et al., *A pilot study of immune checkpoint inhibition in combination with radiation therapy in patients with metastatic pancreatic cancer.* Journal of Clinical Oncology, 2017. **35**(15_suppl): p. e15786-e15786.

59. Aglietta, M., et al., *A phase I dose escalation trial of tremelimumab (CP-675,206) in combination with gemcitabine in chemotherapy-naive patients with metastatic pancreatic cancer.* Annals of Oncology, 2014. **25**(9): p. 1750-1755.

60. Le, D.T., et al., *Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade.* Science, 2017. **357**(6349): p. 409-413.

61. Marabelle, A., et al., *Efficacy of Pembrolizumab in Patients With Noncolorectal High Microsatellite Instability/Mismatch Repair-Deficient Cancer: Results From the Phase II KEYNOTE-158 Study.* J Clin Oncol, 2020. **38**(1): p. 1-10.

62. Ullman, N.A., et al., *Immunologic Strategies in Pancreatic Cancer: Making Cold Tumors Hot.* Journal of Clinical Oncology, 2022. **40**(24): p. 2789-2805.

63. Wu, K., et al., *Emerging Trends and Research Foci in Tumor Microenvironment of Pancreatic Cancer: A Bibliometric and Visualized Study.* Front Oncol, 2022. **12**: p. 810774.

64. Hosein, A.N., R.A. Brekken, and A. Maitra, *Pancreatic cancer stroma: an update on therapeutic targeting strategies.* Nat Rev Gastroenterol Hepatol, 2020. **17**(8): p. 487-505.

65. Erkan, M., et al., *The Activated Stroma Index Is a Novel and Independent Prognostic Marker in Pancreatic Ductal Adenocarcinoma.* Clinical Gastroenterology and Hepatology, 2008. **6**(10): p. 1155-1161.

66. Feig, C., et al., *The pancreas cancer microenvironment.* Clin Cancer Res, 2012. **18**(16): p. 4266-76.

67. Vonlaufen, A., et al., *Pancreatic stellate cells: partners in crime with pancreatic cancer cells.* Cancer Res, 2008. **68**(7): p. 2085-93.

68. Martinez-Bosch, N., J. Vinaixa, and P. Navarro, *Immune Evasion in Pancreatic Cancer: From Mechanisms to Therapy.* Cancers (Basel), 2018. **10**(1).

69. Huang, B., et al., *Gr-1+ CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer research, 2006. **66**(2): p. 1123-1131.

70. Rodriguez, P.C., et al., *Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma.* The Journal of experimental medicine, 2005. **202**(7): p. 931-939.

71. Corzo, C.A., et al., *Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells.* The Journal of Immunology, 2009. **182**(9): p. 5693-5701.

72. Porembka, M.R., et al., *Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth.* Cancer Immunology, Immunotherapy, 2012. **61**(9): p. 1373-1385.

73. Porembka, M.R., et al., *Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth.* Cancer Immunology, Immunotherapy, 2012. **61**: p. 1373-1385.

74. Diaz-Montero, C.M., et al., *Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin–*

cyclophosphamide chemotherapy. Cancer immunology, immunotherapy, 2009. **58**: p. 49-59.

75. Bayne, L.J., et al., *Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer.* Cancer cell, 2012. **21**(6): p. 822-835.

76. Pylayeva-Gupta, Y., et al., *Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia.* Cancer cell, 2012. **21**(6): p. 836-847.

77. Kurahara, H., et al., *Significance of M2-polarized tumor-associated macrophage in pancreatic cancer.* Journal of Surgical Research, 2011. **167**(2): p. e211-e219.

78. Ben-Baruch, A. *Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators*. in *Seminars in cancer biology*. 2006. Elsevier.

79. Kurte, M., et al., *A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and transporter associated with antigen processing 1/2 in human melanoma cells.* The Journal of Immunology, 2004. **173**(3): p. 1731-1737.

80. Mantovani, A. and A. Sica, *Macrophages, innate immunity and cancer: balance, tolerance, and diversity.* Current opinion in immunology, 2010. **22**(2): p. 231-237.

81. Corzo, C.A., et al., *HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment.* Journal of Experimental Medicine, 2010. **207**(11): p. 2439-2453.

82. Chen, H., et al., *Neoantigen-based immunotherapy in pancreatic ductal adenocarcinoma (PDAC).* Cancer Letters, 2020. **490**: p. 12-19.

83. Schumacher, T.N. and R.D. Schreiber, *Neoantigens in cancer immunotherapy.* Science, 2015. **348**(6230): p. 69-74.

84. Balachandran, V.P., et al., *Identification of unique neoantigen qualities in long-term survivors of pancreatic cancer.* Nature, 2017. **551**(7681): p. 512-516.

85. Bailey, P., et al., *Exploiting the neoantigen landscape for immunotherapy of pancreatic ductal adenocarcinoma.* Sci Rep, 2016. **6**: p. 35848.

86. Davalos, R.V., L. Mir, and B. Rubinsky, *Tissue ablation with irreversible electroporation.* Annals of biomedical engineering, 2005. **33**: p. 223-231.

87. Maor, E., et al., *The effect of irreversible electroporation on blood vessels.* Technology in cancer research & treatment, 2007. **6**(4): p. 307-312.

88. Campana, L.G., et al., *Electrochemotherapy for disseminated superficial metastases from malignant melanoma.* Journal of British Surgery, 2012. **99**(6): p. 821-830.

89. Matthiessen, L.W., et al., *Electrochemotherapy for large cutaneous recurrence of breast cancer: a phase II clinical trial.* Acta Oncologica, 2012. **51**(6): p. 713-721.

90. Sersa, G., et al., *Electrochemotherapy of chest wall breast cancer recurrence.* Cancer Treat Rev, 2012. **38**(5): p. 379-86.

91. Edhemovic, I., et al., *Electrochemotherapy: a new technological approach in treatment of metastases in the liver.* Technology in cancer research & treatment, 2011. **10**(5): p. 475-485.

92. Martin 2nd, R., et al., *Irreversible electroporation therapy in the management of locally advanced pancreatic adenocarcinoma. JAm Coll Surg. 2012; 215 (3): 361–9. Narayanan G, et al. Percutaneous irreversible electroporation for downstaging and control of unresectable pancreatic adenocarcinoma.* Ann Surg Oncol, 2013. **20**(Suppl 3): p. S443-9.

93. Martin, R.C.G., et al., *Irreversible Electroporation in Locally Advanced Pancreatic Cancer: Potential Improved Overall Survival.* Annals of Surgical Oncology, 2013. **20**(3): p. 443-449.

94. Flak, R.V., et al., *Treatment of locally advanced pancreatic cancer with irreversible electroporation–a Danish single center study of safety and feasibility.* Scandinavian Journal of Gastroenterology, 2019. **54**(2): p. 252-258.

95. Holland, M.M., et al., *A prospective, multi-institution assessment of irreversible electroporation for treatment of locally advanced pancreatic adenocarcinoma: initial outcomes from the AHPBA pancreatic registry.* HPB, 2019. **21**(8): p. 1024-1031.

96. Martin II, R.C.G., et al., *Irreversible electroporation in locally advanced pancreatic cancer: A call for standardization of energy delivery.* Journal of Surgical Oncology, 2016. **114**(7): p. 865-871.

97. Bhutiani, N., et al., *Irreversible electroporation enhances delivery of gemcitabine to pancreatic adenocarcinoma.* Journal of Surgical Oncology, 2016. **114**(2): p. 181-186.

98. Zhao, J., et al., *Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer.* Nature Communications, 2019. **10**(1): p. 899.

99. Yang, J., et al., *Irreversible electroporation ablation overcomes tumor-associated immunosuppression to improve the efficacy of DC vaccination in a mice model of pancreatic cancer.* OncoImmunology, 2021. **10**(1): p. 1875638.

100. Narayanan, J.S.S., et al., *Irreversible Electroporation Combined with Checkpoint Blockade and TLR7 Stimulation Induces Antitumor Immunity in a Murine Pancreatic Cancer Model.* Cancer Immunology Research, 2019. **7**(10): p. 1714-1726.

101. Pandit, H., et al., *Evaluating the Regulatory Immunomodulation Effect of Irreversible Electroporation (IRE) in Pancreatic Adenocarcinoma.* Annals of Surgical Oncology, 2019. **26**(3): p. 800-806.

102. Scheffer, H.J., et al., *Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation.* Oncoimmunology, 2019. **8**(11): p. 1652532.

103. Johansson, H., et al., *Immune checkpoint therapy for pancreatic cancer.* World journal of gastroenterology, 2016. **22**(43): p. 9457.

104. Naeslund, C., *Expérience de vaccination par le BCG dans la province du Norrbotten (Suède).* Revue de la Tuberculose, 1931. **12**(5): p. 617-636.

105. Aaby, P., et al., *Randomized Trial of BCG Vaccination at Birth to Low-Birth-Weight Children: Beneficial Nonspecific Effects in the Neonatal Period?* The Journal of Infectious Diseases, 2011. **204**(2): p. 245-252.

106. Benn, C.S., et al., *A small jab - a big effect: nonspecific immunomodulation by vaccines.* Trends Immunol, 2013. **34**(9): p. 431-9.

107. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020. **20**(6): p. 375-388.

108. Netea, M.G., et al., *Trained immunity: a program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

109. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

110. Jensen, K.J., et al., *Heterologous Immunological Effects of Early BCG Vaccination in Low-Birth-Weight Infants in Guinea-Bissau: A Randomized-controlled Trial.* The Journal of Infectious Diseases, 2014. **211**(6): p. 956-967.

111. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell Host Microbe, 2012. **12**(2): p. 223-32.

112. Kleinnijenhuis, J., et al., *Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proceedings of the National Academy of Sciences, 2012. **109**(43): p. 17537-17542.

113. Netea, M.G. and J.W.M. van der Meer, *Trained Immunity: An Ancient Way of Remembering.* Cell Host & Microbe, 2017. **21**(3): p. 297-300.

114. Quintin, J., et al., *Candida albicans Infection Affords Protection against Reinfection via Functional Reprogramming of Monocytes.* Cell Host & Microbe, 2012. **12**(2): p. 223-232.

115. Cheng, S.-C., et al., *mTOR-and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity.* science, 2014. **345**(6204): p. 1250684.

116. Arts, R.J., et al., *Immunometabolic pathways in BCG-induced trained immunity.* Cell reports, 2016. **17**(10): p. 2562-2571.

117. Chavakis, T., I. Mitroulis, and G. Hajishengallis, *Hematopoietic progenitor cells as integrative hubs for adaptation to and fine-tuning of inflammation.* Nature immunology, 2019. **20**(7): p. 802-811.

118. Mitroulis, I., et al., *Modulation of myelopoiesis progenitors is an integral component of trained immunity.* Cell, 2018. **172**(1-2): p. 147-161. e12.

119. Christ, A., et al., *Western diet triggers NLRP3-dependent innate immune reprogramming.* Cell, 2018. **172**(1-2): p. 162-175. e14.

120. Alexander, M.P., et al., *Beta-glucan-induced inflammatory monocytes mediate antitumor efficacy in the murine lung.* Cancer Immunology, Immunotherapy, 2018. **67**(11): p. 1731-1742.

121. E M Hersh, a. J U Gutterman, and G.M. Mavligit, *BCG as Adjuvant Immunotherapy for Neoplasia.* Annual Review of Medicine, 1977. **28**(1): p. 489-515.

122. Hersh, E.M., J.U. Gutterman, and G.M. Mavligit, *BCG as adjuvant immunotherapy for neoplasia.* Annual review of medicine, 1977. **28**(1): p. 489-515.

123. Grossman, H.B., et al., *Bacillus calmette-guérin failures and beyond: contemporary management of non-muscle-invasive bladder cancer.* Rev Urol, 2008. **10**(4): p. 281-9.

124. Vetvicka, V., et al., *Effects of Medicinal Fungi-Derived β-Glucan on Tumor Progression.* J Fungi (Basel), 2021. **7**(4).

125. Hong, F., et al., *Mechanism by which orally administered β-1, 3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models.* The Journal of Immunology, 2004. **173**(2): p. 797-806.

126. Vetvicka, V., et al., *Targeting of natural killer cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and beta-glucan-primed CR3 (CD11b/CD18).* Journal of immunology (Baltimore, Md.: 1950), 1997. **159**(2): p. 599-605.

127. Liu, M., et al., *Dectin-1 activation by a natural product β-glucan converts immunosuppressive macrophages into an M1-like phenotype.* The Journal of Immunology, 2015. **195**(10): p. 5055-5065.

128. Tian, J., et al., *β-Glucan enhances antitumor immune responses by regulating differentiation and function of monocytic myeloid-derived suppressor cells.* European journal of immunology, 2013. **43**(5): p. 1220-1230.

129. Li, B., et al., *Orally Administered Particulate β-Glucan Modulates Tumor-Capturing Dendritic Cells and Improves Antitumor T-Cell Responses in Cancer.* Clinical Cancer Research, 2010. **16**(21): p. 5153-5164.

130. Osorio, F., et al., *DC activated via dectin-1 convert Treg into IL-17 producers.* European journal of immunology, 2008. **38**(12): p. 3274-3281.

131. Geller, A.E., et al., *The induction of peripheral trained immunity in the pancreas incites anti-tumor activity to control pancreatic cancer progression.* Nature Communications, 2022. **13**(1): p. 759.

132. Woeste, M.R., et al., *Optimizing patient selection for irreversible electroporation of locally advanced pancreatic cancer: analyses of survival.* Frontiers in Oncology, 2022. **11**: p. 5701.

133. Netea, M.G., L.A. Joosten, and J.W. van der Meer, *Hypothesis: stimulation of trained immunity as adjunctive immunotherapy in cancer.* Journal of leukocyte biology, 2017. **102**(6): p. 1323-1332.

134. Kalafati, L., et al., *Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity.* Cell, 2020. **183**(3): p. 771-785. e12.

135. Netea, M.G., L.A.B. Joosten, and J.W.M. van der Meer, *Hypothesis: stimulation of trained immunity as adjunctive immunotherapy in cancer.* Journal of Leukocyte Biology, 2017. **102**(6): p. 1323-1332.

136. Zhang, N., et al., *Irreversible Electroporation: An Emerging Immunomodulatory Therapy on Solid Tumors.* Frontiers in Immunology, 2022. **12**.

137. He, C., et al., *The role of irreversible electroporation in promoting M1 macrophage polarization via regulating the HMGB1-RAGE-MAPK axis in pancreatic cancer.* Oncoimmunology, 2021. **10**(1): p. 1897295.

138. Kurtz, J. and K. Franz, *Evidence for memory in invertebrate immunity.* Nature, 2003. **425**(6953): p. 37-38.

139. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nature Reviews Immunology, 2020. **20**(6): p. 375-388.

140. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

141. Kaufmann, E., et al., *Lack of evidence for intergenerational inheritance of immune resistance to infections.* Nat Immunol, 2022. **23**(2): p. 203-207.

142. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nature immunology, 2015. **16**(4): p. 343-353.

143. Koliaraki, V., et al., *Unfolding innate mechanisms in the cancer microenvironment: The emerging role of the mesenchyme.* The Journal of experimental medicine, 2020. **217**(4): p. e20190457.

144. Redelman-Sidi, G., M.S. Glickman, and B.H. Bochner, *The mechanism of action of BCG therapy for bladder cancer—a current perspective.* Nature Reviews Urology, 2014. **11**(3): p. 153-162.

145. Stewart, J.H. and E.A. Levine, *Role of bacillus Calmette–Guerin in the treatment of advanced melanoma.* Expert review of anticancer therapy, 2011. **11**(11): p. 1671-1676.

146. Ding, C., et al., *Inducing trained immunity in pro-metastatic macrophages to control tumor metastasis.* Nat Immunol, 2023. **24**(2): p. 239-254.

147. Cheung, N.-K.V., et al., *Orally administered β-glucans enhance anti-tumor effects of monoclonal antibodies.* Cancer Immunology, Immunotherapy, 2002. **51**: p. 557-564.

148. Chen, D.S. and I. Mellman, *Elements of cancer immunity and the cancer–immune set point.* Nature, 2017. **541**(7637): p. 321-330.

149. Kalafati, L., et al., *Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity.* Cell, 2020. **183**(3): p. 771-785.e12.

150. Burbach, B.J., et al., *Irreversible electroporation augments checkpoint immunotherapy in prostate cancer and promotes tumor antigen-specific tissue-resident memory CD8+ T cells.* Nature Communications, 2021. **12**(1): p. 3862.

151. Dai, Z., et al., *Irreversible electroporation induces CD8+ T cell immune response against post-ablation hepatocellular carcinoma growth.* Cancer Letters, 2021. **503**: p. 1-10.

152. Guo, X., et al., *Immunological effect of irreversible electroporation on hepatocellular carcinoma.* BMC Cancer, 2021. **21**(1): p. 443.

153. Bhutiani, N., et al., *Irreversible electroporation enhances delivery of gemcitabine to pancreatic adenocarcinoma.* J Surg Oncol, 2016. **114**(2): p. 181-6.

154. Tian, G., et al., *Immunomodulatory Effect of Irreversible Electroporation Alone and Its Cooperating With Immunotherapy in Pancreatic Cancer.* Frontiers in Oncology, 2021. **11**.

155. Bekkering, S., et al., *Trained immunity: reprogramming innate immunity in health and disease.* Annual review of immunology, 2021. **39**: p. 667-693.

156. Menjivar, R.E., et al., *Arginase 1 is a key driver of immune suppression in pancreatic cancer.* bioRxiv, 2022: p. 2022.06.21.497084.

157. Zhang, Y., et al., *Interleukin-6 Is Required for Pancreatic Cancer Progression by Promoting MAPK Signaling Activation and Oxidative Stress Resistance.* Cancer Research, 2013. **73**(20): p. 6359-6374.

158. Rembielak, A.I., et al., *Phase II trial of cetuximab and conformal radiotherapy only in locally advanced pancreatic cancer with concurrent tissue sampling feasibility study.* Translational Oncology, 2014. **7**(1): p. 55-64.

159. Henriksen, A., et al., *Checkpoint inhibitors in pancreatic cancer.* Cancer treatment reviews, 2019. **78**: p. 17-30.

160. Narayanan, J.S.S., et al., *Irreversible Electroporation Combined with Checkpoint Blockade and TLR7 Stimulation Induces Antitumor Immunity in a Murine Pancreatic Cancer Model.* Cancer Immunol Res, 2019. **7**(10): p. 1714-1726.

161. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell host & microbe, 2012. **12**(2): p. 223-232.

162. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* science, 2014. **345**(6204): p. 1251086.

163. He, C., et al., *T-cell activation and immune memory enhancement induced by irreversible electroporation in pancreatic cancer.* Clinical and Translational Medicine, 2020. **10**(2): p. e39.

164. Scheffer, H.J., et al., *Irreversible electroporation of locally advanced pancreatic cancer transiently alleviates immune suppression and creates a window for antitumor T cell activation.* OncoImmunology, 2019. **8**(11): p. 1652532.

165. Beitel-White, N., et al., *Real-time prediction of patient immune cell modulation during irreversible electroporation therapy.* Scientific Reports, 2019. **9**(1): p. 1-8.

166. Keenan, B.P., et al., *A Listeria vaccine and depletion of T-regulatory cells activate immunity against early stage pancreatic intraepithelial neoplasms and prolong survival of mice.* Gastroenterology, 2014. **146**(7): p. 1784-94.e6.

167. Luetke-Eversloh, M., et al., *Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells.* PLoS pathogens, 2014. **10**(10): p. e1004441.

168. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-561.

169. Kleinnijenhuis, J., et al., *BCG-induced trained immunity in NK cells: role for non-specific protection to infection.* Clinical immunology, 2014. **155**(2): p. 213-219.

170. Ding, C., et al., *Inducing trained immunity in pro-metastatic macrophages to control tumor metastasis.* Nature Immunology, 2023: p. 1-16.

171. Dolenšek, J., M.S. Rupnik, and A. Stožer, *Structural similarities and differences between the human and the mouse pancreas.* Islets, 2015. **7**(1): p. e1024405-e1024405.

172. Zheng, X., et al., *Extended chain conformation of β-glucan and its effect on antitumor activity.* Journal of Materials Chemistry B, 2017. **5**(28): p. 5623-5631.

173. Zheng, X., et al., *Uptake of intraperitoneally administrated triple helical β-glucan for antitumor activity in murine tumor models.* Journal of Materials Chemistry B, 2017. **5**(47): p. 9337-9345.

174. Geller, A., R. Shrestha, and J. Yan, *Yeast-Derived β-Glucan in Cancer: Novel Uses of a Traditional Therapeutic.* International Journal of Molecular Sciences, 2019. **20**(15): p. 3618.

175. Bella, Á., et al., *Mouse Models of Peritoneal Carcinomatosis to Develop Clinical Applications.* Cancers (Basel), 2021. **13**(5).

176. Chang, J.H., Y. Jiang, and V.G. Pillarisetty, *Role of immune cells in pancreatic cancer from bench to clinical application: An updated review.* Medicine, 2016. **95**(49): p. e5541-e5541.

177. Zhang, M., J.A. Kim, and A.Y.-C. Huang, *Optimizing Tumor Microenvironment for Cancer Immunotherapy: β-Glucan-Based Nanoparticles.* Frontiers in Immunology, 2018. **9**.

178. Vetvicka, V. and J. Vetvickova, *Glucan supplementation enhances the immune response against an influenza challenge in mice.* Annals of Translational Medicine, 2015. **3**(2).

179. Dharsono, T., et al., *Effects of yeast (1, 3)-(1, 6)-beta-glucan on severity of upper respiratory tract infections: a double-blind, randomized, placebo-controlled study in healthy subjects.* Journal of the American College of Nutrition, 2019. **38**(1): p. 40-50.

180. Geller, A. and J. Yan, *Could the Induction of Trained Immunity by β-Glucan Serve as a Defense Against COVID-19?* Frontiers in immunology, 2020. **11**: p. 1782-1782.

181. Rice, P.J., et al., *Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge.* Journal of Pharmacology and Experimental Therapeutics, 2005. **314**(3): p. 1079-1086.

182. Morales, A., D. Eidinger, and A. Bruce, *Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors.* The Journal of urology, 1976. **116**(2): p. 180-182.

183. van Puffelen, J.H., et al., *Trained immunity as a molecular mechanism for BCG immunotherapy in bladder cancer.* Nature Reviews Urology, 2020. **17**(9): p. 513-525.

184. Karaca, H., et al., *Positive effects of oral β-glucan on mucositis and leukopenia in colorectal cancer patients receiving adjuvant FOLFOX-4 combination chemotherapy.* Asian Pacific Journal of Cancer Prevention, 2014. **15**(8): p. 3641-3644.

185. Demir, G., et al., *Beta glucan induces proliferation and activation of monocytes in peripheral blood of patients with advanced breast cancer.* International Immunopharmacology, 2007. **7**(1): p. 113-116.

186. Liu, M., et al., *Transcription factor c-Maf is a checkpoint that programs macrophages in lung cancer.* The Journal of clinical investigation, 2020. **130**(4): p. 2081-2096.

187. Kalafati, L., et al., *Innate Immune Training of Granulopoiesis Promotes Anti-tumor Activity.* Cell, 2020. **183**(3): p. 771-785.e12.

188. Li, B., et al., *Orally administered particulate beta-glucan modulates tumor-capturing dendritic cells and improves antitumor T-cell responses in cancer.* Clin Cancer Res, 2010. **16**(21): p. 5153-64.

189. Polajzer, T., T. Jarm, and D. Miklavcic, *Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro.* Radiol Oncol, 2020. **54**(3): p. 317-328.

190. Braza, M.S., et al., *Inhibiting inflammation with myeloid cell-specific nanobiologics promotes organ transplant acceptance.* Immunity, 2018. **49**(5): p. 819-828. e6.

191. Shah, V.B., D.L. Williams, and L. Keshvara, *beta-Glucan attenuates TLR2- and TLR4-mediated cytokine production by microglia.* Neurosci Lett, 2009. **458**(3): p. 111-5.

192. Brown, G.D., et al., *Dectin-1 mediates the biological effects of β-glucans.* The Journal of experimental medicine, 2003. **197**(9): p. 1119-1124.

193. Tseng, D., et al., *Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response.* Proc Natl Acad Sci U S A, 2013. **110**(27): p. 11103-8.

194. Shankara Narayanan, J.S., et al., *Treatment of pancreatic cancer with irreversible electroporation and intratumoral CD40 antibody stimulates systemic immune responses that inhibit liver metastasis in an orthotopic model.* J Immunother Cancer, 2023. **11**(1).

195. Pushalkar, S., et al., *The Pancreatic Cancer Microbiome Promotes Oncogenesis by Induction of Innate and Adaptive Immune SuppressionMicrobiome Influences Pancreatic Oncogenesis.* Cancer discovery, 2018. **8**(4): p. 403-416.

196. Pushalkar, S., et al., *The Pancreatic Cancer Microbiome Promotes Oncogenesis by Induction of Innate and Adaptive Immune Suppression.* Cancer Discov, 2018. **8**(4): p. 403-416.

CURRICULUM VITAE

NAME: Matthew R. Woeste, M.D.

HOME ADDRESS: 1704 Ormsby Ln.
Louisville, KY 40222
C: (859) 496-1004
P: (502) 209-1366
matthew.woeste@louisville.edu

DATE OF BIRTH: April 13th, 1991

PLACE OF BIRTH: Fort Thomas, Kentucky, USA

MARITIAL STATUS**:** Married: Anna Christine Woeste, R.D.H.

Children:		
	Amelia Rosemary Woeste	06/06/2017
	Gwenyth Mae Woeste	03/22/2020
	Eleanor Louise Woeste	09/27/2022

DEGREE GRANTING EDUCATION

2009-2013 Northern Kentucky University
College of Arts and Sciences
Highland Heights, KY, USA,
Degree: B.S. Biology, Minor in Chemistry and Honors Studies

2013-2017 University of Louisville School of Medicine
Louisville, KY, USA
Degree: M.D.

2019-current University of Louisville School of Medicine
Department of Microbiology and Immunology
Louisville, KY, USA
PhD Candidate

POST GRADUATE TRAINING

2017-current	University of Louisville School of Medicine The Hiram C. Polk Jr., MD Department of Surgery Louisville, KY, USA Surgical Resident

CERTIFICATIONS AND LICENSURE

2017	State of Kentucky medical license number #R4549 DEA Certification #FW7984936 NPI#1144751702
2022	Basic Life Support Certification
2022	Advanced Cardiac Life Support Certification
2017	Advanced Cardiac Life Support Certification
2017	Advanced Trauma Life Support Certification
2015	United States Medical Licensing Exam Step 1 (243)
2017	United States Medical Licensing Exam Step 2 CK (249)
2017	United States Medical Licensing Exam Step 2 CS (PASS)

AWARDS AND RECOGNITIONS

2023	Graduate Dean's Citation
2023	Resident Surgical Skills Olympics, First place
2021	Hugh C. Williams Travel Scholarship, co-recipient
2020	American College of Surgeons, Kentucky Chapter, Resident Paper Competition, 1st place, "Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery"
2020	Hugh C. Williams Travel Scholarship, co-recipient
2020	Edelen-Hagan Resident Research Publication Award
2020	Resident Surgical Skills Olympics, Third place
2019	American College of Surgeons, Kentucky Chapter, Resident Paper Competition, Cancer, 1st place, "Identifying factors predicting prolonged opioid use after mastectomy"
2019	Resident Surgical Skills Olympics, Third place
2017	M.D., Cum Laude, University of Louisville School of Medicine
2017	Dr. Hiram C. Polk, Jr. Scholarship Award
2017	The Dean's Award for Leadership
2016	The Morgan Williams Award
2016	Greater Louisville Medical Society Foundation Scholarship
2014	The Doctors Murrel H., Martin Z., and Benjamin M. Kaplan Award
2013	B.S., Summa Cum Laude, Northern Kentucky University
2013	University Honors Scholar, Norther Kentucky University
2013	The Clara Richards Award

2012	Greaves Fellowship Recipient
2011	Michael Frances Zalla Presidential Memorial Travel Award
2010	Northern Kentucky University Homecoming Duke
2009	The Outstanding Freshman Award, Honors 101
2009-2013	President's Honors List
2009-2013	Commonwealth Excellence Scholarship

HONORARY AND PROFRESSIONAL SOCIETIES

2016 – current	Alpha Omega Alpha Honor Society, Membership #188146
2017 – current	American College of Surgeons, Resident member
2017 – current	Louisville Surgical Society

EMPLOYMENT

2017 – current	University of Louisville Department of Surgery, House staff
2011-2013	Northern Kentucky University: Science, Technology, Engineering, and Mathematics Ambassador
2010-2013	Northern Kentucky University, Greenhouse Manager

EXPERIENCE AND ADMINISTRATIVE SERVICE

University of Louisville School of Medicine, Hiram C. Polk Jr., M.D., Department of Surgery

2021-2022	House staff counsel, general surgery representative
2020	Website improvement team
2018	Resident Wellness Committee

University of Louisville School of Medicine

2013-2017	President, Class of 2017
2013-2015	Pre-Clinical Curriculum Committee
2015-2017	Clinical Curriculum Committee
2014-2015	Block Examination Challenge Committee
2014	LCME Standards Task Force Student Member

Northern Kentucky University

2011-2012	President, Health Professions Club
2012-2013	Secretary, Health Professions Club
2012-2013	Student Wellness Advisory Committee

TEACHING ACTIVITIES

2016-2017 General Surgery Medical Students as Teachers, instructor

SPEAKING OPPORTUNITIES

2017 University of Louisville School of Medicine, Commencement Student Speaker

2014 Class of 2018 White Coat Ceremony, Student Speaker

POSTER PRESENTATIONS

2021 **Woeste, M.R.,** Jacob, K., Duff, M.B., Donaldson, M., Sanders, M.G., McMasters, K.M., Ajkay, N., *Impact of routine expert breast pathology consultation and factors predicting discordant diagnosis.* American College of Surgeons Clinical Congress. October 23rd-27th, 2021. Virtual meeting.

2014 **Woeste, M**., Kimbrough, CW., Lakshmanan, J., Matheson, PJ., Gentile, A., Benns, MV., Zhang, B., Jason W Smith, JW., Harbrecht, BG. *Resveratrol regulates intracellular signaling in hepatoma and primary hepatocytes*. Summer Endocrine Research Training Program. Louisville, KY, USA.

ORAL PRESENTATIONS

2022 **Woeste, M.R.,** Jacob, K., Duff, M.B., Donaldson, M.A., McMasters, K.M., Ajkay, N., Identifying factors predicting margin status after mastectomy. American College of Surgeons Clinical Congress [QS# 5367] October 18th, 2022. San Diego, California, USA.

2021 **Woeste, M.R**., Strothman, P., Jacob, K., Egger, M.E., Philips, P. McMasters, K.M., Martin, R.C.G., Scoggins, C.R., Hepatopancreatobiliary readmission score out performs administrative LACE+ index as a predictive tool of readmission [QS15]. Southwestern Surgical Congress. September 3rd, 2021. Maui, Hawaii, USA.

2020 **Woeste, M.R,** Bhutiani, N., Donaldson, M., McMasters, K.M., Ajkay, N., Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery. American College of Surgeons, Kentucky Chapter Meeting. September 18th, 2020. Lexington, KY, USA.

2020 **Woeste, M.R,** Bhutiani, N., Donaldson, M., McMasters, K.M., Ajkay, N., Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery. Society of Surgical Oncology Annual Cancer Symposium, August 18th, 2020. Virtual meeting.

2019 **Woeste, M.R**., Bhutiani, N., Geller, A.E., Hindy HE., McMasters, K.M., Ajkay, N., Identifying Factors Predicting Prolonged Opioid Use After Mastectomy. American College of Surgeons, Kentucky Chapter Meeting. September 27th, 2019. Louisville, KY, USA.

2019 **Woeste, MR**., Bhutiani, N., Geller, AE., Hindy HE., McMasters, KM., Ajkay, N., Identifying Factors Predicting Prolonged Opioid Use After Mastectomy. Society of Surgical Oncology Annual Cancer Symposium, March 30th, 2019. San Diego, CA, USA.

2019 **Woeste, M.R.,** Philips, P., Egger ME., Scoggins, CR., McMasters, KM., Martin RCG., Optimal perfusion chemotherapy: A prospective comparison of mitomycin c and oxaliplatin for hyperthermic intraperitoneal chemotherapy in metastatic colon cancer. [QS#11]. Southwestern Surgical Congress. Huntington Beach, California, USA. 2019

2013 **Woeste, M.,** Bisphenol Structural Requirements for Inhibition and Proteolytic Digestion of Sarco/endoplasmic Reticulum Calcium ATPase. Senior Honors Capstone presentation. Northern Kentucky University's Annual Celebration of Research.

ANNOTATED BIBLIOGRAPHY

1. **Woeste, M**., Steller, J., Hofmann, E., Kidd, T., Patel, R., Connelly, K., Jayasinghe, M., Paula, S., 2013. Structural requirements for inhibitory effects of bisphenols on the activity of the sarco/endoplasmic reticulum calcium ATPase. *Bioorganic and Medicinal Chemistry*, 21(13): 3927-33. doi: 10.1016/j.bmc.2013.04.012

2. Paula, S., Elam, C., **Woeste, M**., Abell, J., Kempton, RJ., 2013. Hydroquinones with conformationally constrained substituents: synthesis, characterization, and evaluation as calcium-ATPase inhibitors. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 3(5): 535. doi:10.7763/IJBBB.2013.V3.271

3. Kimbrough, CW., Lakshmanan, J., Matheson, PJ., **Woeste, M**., Gentile, A., Benns, MV., Zhang, B., Jason W Smith, JW., Harbrecht, BG., Resveratrol

Decreases Nitric Oxide Production by Hepatocytes During Inflammation. *Surgery*, 2014. 158(4), 1095-101. doi:10.1016/j.surg.2015.07.012

4. **Woeste, M.R.** and W.G. Cheadle, Postoperative Infection—A Pervasive Mediator of Patient Mortality. *JAMA Surgery*, 2020. 155: p. 68-68. doi:10.1001/jamasurg.2019.4554

5. **Woeste, M.R**., Bhutiani, N., Geller, AE., Hindy HE., McMasters, KM., Ajkay, N., Factors predicting prolonged opioid use after mastectomy. *Annals of Surgical Oncology*, 2020. 27(4): p. 993-1001. doi:10.1245/s10434-019-08171-4

6. **Woeste, M.R.,** Philips, P., Egger ME., Scoggins, CR., McMasters, KM., Martin RCG., Optimal perfusion chemotherapy: A prospective comparison of mitomycin c and oxaliplatin for hyperthermic intraperitoneal chemotherapy in metastatic colon cancer. *Journal of Surgical Oncology*, 2020. 121(8): p. 1298-1305. doi:10.1002/jso.25920

7. **Woeste, M.R.,** Bhutiani, N., Hong, Y., Kim, W., Egger ME., Philips, P., McMasters, KM., Martin RCG., Scoggins, CR., Primitive neuroectodermal tumor incidence, treatment patterns, and outcome: An analysis of the National Cancer Database. *Journal of Surgical Oncology*, 2020. 122(6): p. 1145-1151. doi:10.1002/jso.26139

8. **Woeste, M.R.,** Bhutiani, N., Donaldson, M., McMasters, KM., Ajkay, N., Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery, *Journal of Surgical Oncology*. 2021. 123: p. 439-445. doi:10.1002/jso.26301

9. **Woeste, M.R.**, Geller, A.E., Martin, R.C.G., Optimizing the Combination of Immunotherapy and Trans-Arterial Locoregional Therapy for Stages B and C Hepatocellular Cancer. *Annals of Surgical Oncology*, 2021. 28(3): p. 1499-1510. doi:10.1245/s10434-020-09414-5

10. **Woeste, M.R.,** K.M. McMasters, and M.E. Egger, Stage IIIa Melanoma and Impact of Multiple Positive Lymph Nodes on Survival. *Journal of the American College of Surgeons*, 2021. 232(4): p. 517-524.e1. doi:10.1016/j.jamcollsurg.2020.11.015

11. Ryan, R., Tracy, B.M., He, K., Jacob, E., **Woeste, M.,** Teaching trainees how to write: Surgical resident opioid prescribing and perioperative pain management. 2021. Available at: https://bulletin.facs.org/2021/01/teaching-trainees-how-to-write-surgical-resident-opioid-prescribing-and-perioperative-pain-management/

12. Morrissey, SM., Geller, AE., Hu, X., Tieri, D., Cooke, EA., Ding, C., **Woeste, M.R.**, Zhang, H., Cavallazzi, R., Clifford, S.P., Chen, J., Cai, L., Kong, M., Watson, C.T., Huang, J., Yan, J., Emergence of Low-density Inflammatory

Neutrophils Correlates with Hypercoagulable State and Disease Severity in COVID-19 Patients. *JCI Insight*, 2021. 6. doi:10.1172/jci.insight.148435

13. **Woeste, M.R**., Strothman, P., Jacob, K., Egger, M.E., Philips, P. McMasters, K.M., Martin, R.C.G., Scoggins, C.R., Hepatopancreatobiliary readmission score out performs administrative LACE+ index as a predictive tool of readmission. *American Journal of Surgery*, 2021. doi:10.1016/j.amsurg.2021.09.037

14. **Woeste, M.R.**, Egger, M.E., Bhutiani, N. *Fecal microbial transplant and immunotherapy for melanoma*. What you should know: Selected readings in general surgery, 2021.

15. **Woeste, M.R.,** Wilson, KD., Kruse, EJ., Weiss, MJ., Christein, JD., White, RR., Huang, K., Martin RCG., Optimizing patient selection for irreversible electroporation of locally advanced pancreatic adenocarcinoma: Analyses of survival. *Frontiers in Oncology*, 2021. 11: p. 817220. doi.org/10.3389/fonc.2021.817220

16. Geller, A.E., Shrestha, R., **Woeste, M.,** Guo, H., Ding, C, Andreeva, K., Chariker, J., Hu, X., Zhou, M., Tieri, D., Watson, C., Mitchell, R., Zhang, H., Li, Y., Martin, R., Rouchka, E., Yan, J., The Induction of Peripheral Trained Immunity in the Pancreas Incites Anti-tumor Activity to Control Pancreatic Cancer Progression. *Nature Communications*, 2022. 13: p. 759. doi.org/10.1038/s41467-022-28407-4

17. Ding, C., Shrestha, R., Zhu, X., Geller, A.E., Wu, S., **Woeste, M.R**., Li, W., Wang, H., Yuan, F., Chariker, J.H., Hu, X., Li, H., Tieri, D., Puckett, A., Zhang, H., Rouchka, E.C., Mitchell. R., Siskind, L.J., Zhang, X., Xu, X., McMasters, K.M., Yu, Y., Yan, J., Induction of Trained Immunity in Lung Interstitial Macrophages Controls Cancer Metastasis through the Metabolic Sphingolipids-Mitochondrial Fission Pathway. *Nature Immunology*. 2023. 24:239-254. doi: 10.1038/s41590-022-01388-8

18. **Woeste, M.R.,** Jacob, K., Duff, M.B., Donaldson, M., Sanders, M.G., McMasters, K.M., Ajkay, N., Impact of routine expert breast pathology consultation and factors predicting discordant diagnosis. *Surgical Oncology*, 2022. 45:101860. doi: 10.1016/j.suronc.2022.101860

19. Hammaker, A., Slayer, C., Foote, D., l-Yafi, M., Smith, S., Callahan, Z., Marks, J., Elsaadi, A., Campbell, S., Stahl, C., Patel, P., **Woeste, M.,** Patel, J., Greenwall, K., Meister, K., Etheridge, J., Cho, J., Thursh, C., Kimbrough, K., Waqar Nasim, B., Willis, R., Adams, S., Dodwad, S., Stopenski, S., Nahmias, J., Kader, S., Abelson, J., Harvey, J., Farr, D., George, B., Postlewait, L., Sutton, J., Quillin, R.C., Cortez, A.R., A Multi-Institutional Study of Factors Associated with Pursuing General Surgery After Residency. *Surgery*. 2022. 172(3):906-912. doi: 10.1016/j.surg.2022.05.033

20. **Woeste, M.R.**, Salyer CE, Hammaker AC, Dodwad, S, Foote DC, Nahmias JT, Callahan ZM, Quillin RC, Cortez AR on behlalf of US ROPE Consortium. Do general surgery residents begin specializing prior to fellowship? A multi-institutional study from the US ROPE consortium. *Journal of the American College of Surgeons.* 2022. 235(5):p 799-808 doi: 10.1097/XCS.0000000000000311

21. **Woeste, M.R.,** Shrestha, R., Geller, A.E., Montoya-Durango, D., Li, Shu., Ding, C., Hu, X., Li, H., Puckett, A., Mitchell, R., Hayat, T., Tan, M., Li, Y., McMasters, K.M., Martin, R., Yan, J., Irreversible electroporation augments β-glucan induced trained innate immunity for the treatment of pancreatic ductal adenocarcinoma. 2023. *Journal of Immunotherapy for Cancer*. Accepted, In press.

22. Eruchalu, C.N., Etheridge, J.C., Hammaker, A.C., Kader, S., Abelson, J.S., Harvey, J., Farr, D., Stopenski, S.J., Nahmias, J.T., Elsaadi, A., Campbell, S.J., Foote, D.C., Ivascu, F.A., Montgomery, K.B., Zmijewski, P., Byrd, S.E., Kimbrough, M.K., Smith, S., Postlewait, L.M., Dodwad, S.M., Adams, S.D., Markesbery, K.C., Meister, K.M., **Woeste, M.R.,** Martin, R.C.G., Callahan, Z.M., Marks, J.A., Patel, P., Anstadt, M.J., Waqar Nasim, B., Willis, R.E.,Patel, J.A., Newcomb, M.R., Stahl, C.C., Al Yafi, M., Sutton, J.M., George, B.C., Quillin, R.C., Cho, N.L., Cortez, A.R., Race and Ethnic Disparities in Operative Experience Among General Surgery Residents: A Multi-Institutional Study from the US ROPE Consortium. 2023. *Annals of Surgery.* Accepted, online ahead of print.

23. Winer, L.K., Kader, S., Abelson, J.S., Hammaker, A.C., Eruchalu, C.N., Etheridge, J.C., Cho, N.L., Foote, D.C., Ivascu, F.A., Smith, S., Postlewait, L.M., Greenwell, K., Meister, K.M.,Montgomery, K.B., Zmijewski, P., Byrd, S.E., Kimbrough, M.K., Stopenski, S.J., Nahmias, J.T., Harvey, J., Farr, D., Callahan, Z.M., Marks, J.A., Stahl, C.C., Yafi, M.A., Sutton, J.M., Elsaadi, A., Campbell, S.J., Dodwad, S.M., Adams, S.D., **Woeste, M.R**., Martin, R.C.G., Patel, P., Anstadt, M.J., Nasim, B., Willis, R.E., Patel, J.A., Newcomb, M.R., George, B.C.,Quillin, R.C., Cortez, A.R., Gender Disparities in Operative Experience Among General Surgery Residents: A Multi-Institutional Study from the US ROPE Consortium. 2023. *Annals of Surgery*. Accepted, online ahead of print.

24. Sisak S, Price AD, Foote DC, Montgomery KB, Lindeman B, Cho NL, Sheu NO, Postlewait LM, Smith SR, Markesbery KC, Meister KM, Kader S, Abelson JS, Anstadt MJ, Patel PP, Marks JA, Callahan ZM, Kimbrough MK Byrd SE, Stopenski SJ, Nahmias JT, Patel JA, Wilt W, Dodward SM, Adams SD, Willis RE, Farr D, Harvey J, **Woeste MR**, Martin RCG, Al Yafi M, Sutton JM, Cortez AR, Holm TM. A Multi-Institutional Study from the US ROPE Consortium

Examining Factors Associated with Endocrine Surgery Exposure for General Surgery Residents. Surgery. *Under Review.*

25. Price AD, Whitrock JN, Foote DC, Eruchalu CN, Dodward SM, Woeste MR, Winer LK, Meister KM, Montgomery KB, Byrd SE, Kimbrough MK, Harvey J, Stahl CC, Al Yafi M, Farr D, Nahmias JT, Stopenski SJ, Campbell SJ, Postlewair LM, Smith SR, Adams SD, Cho NL, Marks JA, Anstadt MJ, Abelson JS, Patel JA, Sutton JM, Quillin RC, Cortez AR. Defining the Disparity: A Multi-Institutional Analysis of Factors Associated with Suboptimal Operative Volume During Residency. J Surg Res. *Under Review.*

26. **Woeste, M.R**., Jacob, K., Peters, M., McMasters, Wahlgreen, M., K.M., Ajkay, N., Identifying factors predicting margin status after mastectomy. Manuscript in preparation.

ABSTRACTS

1. Breunderman, E., **Woeste, M.R**., Miller, J.T., Block, S., Martin, R.C.G., Kehdy, F., *Demeester Score and Histopathologic Findings on Lower Esophageal Biopsy: Do They Correlate?* [Abstract #139]. Gastroenterology, 2019. 156(6):S-1380. doi:10.1016/S0016-5085[17]40476-9

2. **Woeste M.R.**, Bhutiani N., Geller A., Eldredge-Hindy H., McMasters K.M., Ajkay N., *Identifying Factors Predicting Long-Term Narcotic Use After Mastectomy* [Abstract #125]. 72nd Annual Cancer Symposium. Society of Surgical Oncology. March 27-30, 2019. San Diego, CA. Ann Surg Oncol 2019;26:S52-S53

3. **Woeste, M.R,** Bhutiani, N., Donaldson, M., McMasters, K.M., Ajkay, N., Evaluating the effect of neoadjuvant chemotherapy on surgical outcomes after breast conserving surgery [Abstract #118]. 73rd Annual Cancer Symposium. Society of Surgical Oncology. August 17-18, 2020. Virtual Meeting.

4. **Woeste, M.R.,** Philips, P., Egger ME., Scoggins, CR., McMasters, KM., Martin RCG., *Optimal perfusion chemotherapy: A prospective comparison of mitomycin c and oxaliplatin for hyperthermic intraperitoneal chemotherapy in metastatic colon cancer.* [QS Abstract #11]. Southwestern Surgical Congress. April 15th, 2019. Huntington Beach, California, USA 2019

5. **Woeste, M.R**., Strothman, P., Jacob, K., Egger, M.E., Philips, P. McMasters, K.M., Martin, R.C.G., Scoggins, C.R., *Hepatopancreatobiliary readmission score out performs administrative LACE+ index as a predictive tool of readmission*

[Abstract #984146]. Southwestern Surgical Congress. September 3rd, 2021. Maui, Hawaii, USA.

6. Hammaker, A., Slayer, C., Foote, D., Al-Yafi, M., Smith, S., Callahan, Z., Marks, J., Elsaadi, A., Campbell, S., Stahl, C., Patel, P., **Woeste**, **M.,** Patel, J., K. Greenwall , K. Meister, J. Etheridge , N. Cho , C. Thursh , K. Kimbrough , B. Waqar Nasim , R. Willis , S. Adams , S. Dodwad , S. Stopenski , J. Nahmias , S. Kader , J. Abelson , J. Harvey , D. Farr[18] , B. George[2] , L. Postlewait[4] , J. Sutton[3] , R.C. Quillin, A.R. Cortez. A Multi-Institutional Study from the US Resident Operative Experience (ROPE) Collaborative Studying Factors Associated with Entering Practice Upon Graduation. [ASC20221549]. Academic Surgical Congress.

7. Price, A.D., Al Yafi, M., Sutton, J., Markesbery, K., Meister, K.M., Smith, S., Postlewait, L., Harvey, J., Farr, D., Thrush, C., Kimbrough, M.K., Patel, P., Anstadt, M.J., Callahan, Z.M., Marks, J.A., Elsaadi, A., Campbell, S.J., Dodwad, S.M., Adams, S.D., , **M.R. Woeste[14]** , Foote, D.C., Kader, S., Abelson, J.S., Stopenski, S.J., Nahmias, J.T., Nasim, B.W., Willis, R.E., Stahl, C.C., Patel, J.A., Etheridge, J.C., Cho, N.L., Quillin, R.C., Cortez, A.R., **Finding the Outliers – Examining Variation in Operative Experience Among General Surgery Residents. [ASC20231236]. Academic Surgical Congress.**

8. Shindorf, M., **Woeste, M.,** Philips, P., Scoggins, C., Martin R., Chesney, J., McMasters, K., Egger M., Adoption of new therapies and overall survival for patients with cutaneous melanoma are improved in high-volume facilities. Western Surgical Association. November 7th, 2022.