1-1-2015

Induction of T Cell Immunity Overcomes Resistance to Pd-1 and Ctla-4 Blockade and Improves Survival in Pancreatic Cancer

Rafael Winograd
University of Pennsylvania, winograd@mail.med.upenn.edu

Induction of T Cell Immunity Overcomes Resistance to Pd-1 and Ctla-4 Blockade and Improves Survival in Pancreatic Cancer

Abstract
Disabling the function of immune checkpoint molecules can unlock T cell immunity against cancer, yet despite remarkable clinical success with monoclonal antibodies (mAb) that block PD-1 or CTLA-4 resistance remains common and essentially unexplained. Certain tumors, especially pancreatic carcinoma, are fully refractory to these antibodies. As reported in this thesis, I used a genetically engineered mouse model of pancreatic carcinoma in which spontaneous immunity is minimal, and found that PD-L1 is prominent in the tumor microenvironment, a phenotype confirmed in patients. Tumor infiltrating T cells express PD-1 even more prominently than T cells in a classical model of chronic infection, in which anti-PD-1 mAb mediates clinical benefit. Despite this striking expression of PD-1 and PD-L1 in the pancreatic tumor microenvironment, treatment with anti-PD-1 mAb, with or without anti-CTLA-4 mAb, fails in well-established tumors, recapitulating clinical results. Agonist anti-CD40 mAb with chemotherapy, deployed as a vaccine, induces T cell immunity and reverses the complete resistance of pancreatic tumors to anti-PD-1 and anti-CTLA-4. The combination of anti-CD40/chemotherapy plus anti-PD-1 and/or anti-CTLA-4 induces regression of subcutaneous tumors, improves overall survival, and confers curative protection from multiple rechallenges, consistent with immune memory not otherwise achievable. Combinatorial treatment nearly doubles survival of mice with spontaneous pancreatic cancers, revealing a clinical opportunity. These findings suggest that in non immunogenic tumors, epitomized by pancreatic carcinoma, baseline refractoriness to checkpoint inhibitors may be rescued by the priming of a T cell response with an antitumor vaccine. These studies indicate that understanding the immunobiology of differing tumor types may improve the ability to rationally design combinatorial immunotherapies in oncology.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Robert H. Vonderheide

Keywords
Cancer Immunotherapy, Pancreatic Cancer, Vaccines

Subject Categories
Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology | Oncology

INDUCTION OF T CELL IMMUNITY OVERCOMES RESISTANCE TO PD-1 AND CTLA-4 BLOCKADE AND IMPROVES SURVIVAL IN PANCREATIC CANCER

Rafael Winograd

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2015

Supervisor of Dissertation

*Signature*_____

Robert H. Vonderheide, Hanna Wise Professor in Cancer Research

Graduate Group Chairperson

*Signature*_____

Daniel S. Kessler, Associate Professor of Cell and Developmental Biology

Dissertation Committee

Anil K. Rustgi, T. Grier Miller Professor of Medicine

Ben Z. Stanger, Associate Professor of Medicine

Steven M. Albelda, William Maul Measy Professor of Medicine

James L. Riley, Research Associate Professor of Microbiology

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor, Dr. Robert Vonderheide for his support and guidance throughout the course of my thesis work. Without his encouragement and insight this work would not have been possible. I would also like to acknowledge Dr. Beatty for helping me as I began my study in this field; working with him familiarized me with important approaches and techniques which allowed me to develop the experimental methodology needed to ask the questions I was interested in. I would like to acknowledge the important contributions of our collaborators Dr. Wherry and Pamela Odorizzi whose expertise in interrogating T cells in chronic infection helped me to assess intratumoral T cells in comparison to a well characterized model. The analysis of primary human pancreatic cancer was carried out in collaboration with Dr. Furth and Anders Meyer in the department of Pathology. Finally, I would like to acknowledge my funding (T32 CA009140) which made this work possible.

ABSTRACT

INDUCTION OF T CELL IMMUNITY OVERCOMES RESISTANCE TO PD-1 AND CTLA-4 BLOCKADE AND IMPROVES SURVIVAL IN PANCREATIC CANCER

Rafael Winograd

Robert H. Vonderheide

Disabling the function of immune checkpoint molecules can unlock T cell immunity against cancer, yet despite remarkable clinical success with monoclonal antibodies (mAb) that block PD-1 or CTLA-4 resistance remains common and essentially unexplained. Certain tumors, especially pancreatic carcinoma, are fully refractory to these antibodies. As reported in this thesis, I used a genetically engineered mouse model of pancreatic carcinoma in which spontaneous immunity is minimal, and found that PD-L1 is prominent in the tumor microenvironment, a phenotype confirmed in patients. Tumor infiltrating T cells express PD-1 even more prominently than T cells in a classical model of chronic infection, in which αPD-1 mAb mediates clinical benefit. Despite this striking expression of PD-1 and PD-L1 in the pancreatic tumor microenvironment, treatment with αPD-1 mAb, with or without αCTLA-4 mAb, fails in well-established tumors, recapitulating clinical results. Agonist αCD40 mAb with chemotherapy, deployed as a vaccine, induces T cell immunity and reverses the complete resistance of pancreatic tumors to αPD-1 and αCTLA-4. The combination of αCD40/chemotherapy plus αPD-1

and/or αCTLA-4 induces regression of subcutaneous tumors, improves overall survival, and confers curative protection from multiple rechallenges, consistent with immune memory not otherwise achievable. Combinatorial treatment nearly doubles survival of mice with spontaneous pancreatic cancers, revealing a clinical opportunity. These findings suggest that in non immunogenic tumors, epitomized by pancreatic carcinoma, baseline refractoriness to checkpoint inhibitors may be rescued by the priming of a T cell response with an antitumor vaccine. These studies indicate that understanding the immunobiology of differing tumor types may improve the ability to rationally design combinatorial immunotherapies in oncology.

MANUSCRIPTS

The following manuscripts are discussed in this thesis:

Winograd, R., Byrne, K.T., Evans, R.A., Odorizzi, P.M., Meyer, A.R.L., Bajor, D.L., Clendenin, C., Stanger, B.Z., Furth, E.E., Wherry, E.J., and Vonderheide, R.H. Induction of T cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma

Beatty, G.L., Winograd, R., Evans, R.A., Long, K.B., Luque, S.L., Lee, J.W., Gladney, W.L., Guirnalda, P.D., and Vonderheide, R.H. Productive T cell immunity against pancreatic carcinoma in mice is regulated by $Ly6C^{low}$ $F4/80^{+}$ extratumoral macrophages

Condamine, T., Kumar, V., Ramachandran, I.R., Youn, J., Celis, E., Finnberg, N., El-Deiry, W.S., Winograd, R., Vonderheide, R.H., English, N.R., et al. (2014). ER stress regulates myeloid-derived suppressor cell fate through TRAIL-R-mediated apoptosis. J. Clin. Invest. *124*, 2626-39

Vonderheide, R.H., Bajor, D.L., Winograd, R., Evans, R.A., Bayne, L.J., and Beatty, G.L. (2013). CD40 immunotherapy for pancreatic cancer. Cancer Immunol. Immunother. *62*, 949-54

Manuscripts not discussed in this thesis:

King, C.E., Wang, L., Winograd, R., Madison, B.B., Mongroo, P.S., Johnstone, C.N., and Rustgi, A.K. (2011). LIN28B fosters colon cancer migration, invasion and transformation through let-7-dependent and –independent mechanisms. Oncogene. *30*, 4185-93

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1 – Introduction

Cancer immune surveillance

It has become evident that the microenvironment of solid malignancies are infiltrated by various populations of leukocytes which play roles in the promotion and regulation of tumor outgrowth (Hanahan and Weinberg, 2011). Postulation about the immune system's role in cancer dates back more than a century to Paul Ehrlich's hypothesis that the immune system holds malignancies at bay in long lived organisms (Ehrlich, 1909). This idea was revisited half a century later with the "immunological surveillance" hypothesis proposed by Sir Macfarlane Burnet and Lewis Thomas, arguing that control of early malignant lesions was an "evolutionary necessity" which could be orchestrated by the immune system (Burnet, 1957; Thomas, 1959; Burnet, 1970). Experimental verification of this hypothesis proved difficult until the development of inbred strains of mice that lacked key immune proteins (IFN-γ, perforin) or populations (Rag-2$^{-/-}$ mice without T, B, and natural killer T (NKT) cells) allowed for the unequivocal demonstration of immune control of tumor outgrowth (Shankaran et al., 2001; Street et al., 2001). The current principal hypothesis describing the immune system's influence on tumor development was first posited by Robert Schreiber who proposed that "immunoediting" of nascent tumors occurs in three phases: elimination (of small immunogenic malignancies), equilibrium (between the nascent tumor and the immune system), and escape (of the tumor from immune surveillance through various immunosuppressive mechanisms) (Dunn et al., 2002).

The immune system's role in controlling and editing cancer is not an artifact of murine model systems. Immunosuppressed populations, whether from AIDS or immunosuppressive regimens following organ transplantation, are known to exhibit higher rates of malignancies (Vesely et al., 2011). Clinical evidence of immune responses to cancer were first suggested by studies correlating the presence of tumor infiltrating lymphocytes (TILs; specifically $CD8^+$ T cells) with improved survival in patients with melanoma and ovarian and colorectal cancers (Clemente et al., 1996; Galon et al., 2006; Naito et al., 1998; Pagès et al., 2005; Zhang et al., 2003). More recently, the remarkable success of immune therapies in eliciting durable responses in cancer patients has laid the debate to rest, as combinatorial use of immune modulating antibodies and new targets are avidly being tested in preclinical and clinical studies (Brahmer et al., 2012; Hamid et al., 2013; Hodi et al., 2010; Odorizzi and Wherry, 2012; Page et al., 2013; Topalian et al., 2012).

The elimination phase of immunoediting hearkens back to the idea of tumor immune surveillance, in which somatic mutations that lead to malignant transformation and the accompanying disruption of normal tissue architecture allow for recognition of these cells by the immune system. Two of the "hallmarks of cancer" are the invasion of tissue and angiogenesis, processes which can lead to the production of proinflammatory molecules and the subsequent recruitment of innate immune cell populations such as NK cells and macrophages, among others (Dunn et al., 2004; Hanahan and Weinberg, 2011). Once these innate populations encounter tumor associated Natural killer group 2 member D (NKG2D) ligands, glycolipid-CD1d complexes, high-mobility-group box 1 (HMGB1), or other signals, their production of IFN-γ serves as a critical step in the mobilization of

the antitumor immune response, although various other cytokines and chemokines can augment this effect (Diefenbach et al., 2001; Dunn et al., 2002; Apetoh et al., 2007). Tumor cell death in the context of this inflammation allows for uptake of tumor antigens by the dendritic cells (DCs) and macrophages that have been recruited and activated by tumor associated cytokines; these antigen presenting cells (APCs) can then prime Th1 $CD4^+$ T cells, which in turn facilitate the cross-priming of $CD8^+$ cytotoxic T lymphocytes (CTLs) by the APCs (Yu et al., 2003; Huang et al., 1994). Once primed, effector T cells can traffic to the tumor microenvironment and target tumor cells in an antigen specific, major histocompatibility complex (MHC) class-I dependent manner, serving as an extrinsic tumor suppressor mechanism (Schreiber et al., 2011). The elimination of malignant cells expressing tumor antigens can lead to the eradication of a lesion, but can also act to select for those tumor cells lacking the tumor antigen or expression of MHC class-I.

Rare malignant cells which avoid immune mediated elimination may enter into equilibrium with the immune system, in which outgrowth of tumors is prevented by the immune system but complete eradication is also not achieved. Evidence for equilibrium comes from experiments in which carcinogen induced tumors fail to grow in immune competent mice for months, but upon ablation of T cells or IFN-γ (but not innate immune components such as NK cells or NKG2D) half of the mice rapidly develop tumors at the site of carcinogen injection (Koebel et al., 2007). During the equilibrium phase tumors may gain the ability to avoid or suppress the immune processes holding it at bay, allowing for occult malignancies to escape immune suppression and grow into clinically apparent lesions.

The escape of tumors from immune control can be due to a variety of molecular and cellular mechanisms which occur in the context of high cancer mutation rates and the Darwinian-like selective pressure exerted by immune surveillance of these lesions. Tumor cell intrinsic mechanisms such as loss of antigen, antigen processing function, or MHC class-I have been shown to render these selected-for malignant cells all but invisible to immune pressure (Khong and Restifo, 2002). Resistance to apoptosis, achieved through the upregulation of antiapoptotic genes, for example, is a common feature of malignant cells and can also confer functional resistance to immune pressure (Hanahan and Weinberg, 2011).

The establishment of an immunosuppressive microenvironment also allows for escape from immune surveillance. The secretion by tumor cells of immunosuppressive cytokines such as IL-10, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), among others, orchestrates the recruitment of regulatory immune and mesenchymal populations which in turn secrete factors which suppress effector immune functions and recruit further suppressive cells (Vesely et al., 2011). Various myeloid cell populations have been implicated in suppressing antitumor T cell immunity either directly or indirectly. Immature myeloid cells, also called myeloid derived suppressor cells (MDSCs), are increased in mice and humans with cancer, and can suppress T cell responses either directly by binding the inhibitory T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) for example, or indirectly by locally depleting key T cell nutrients such as L-arginine and L-cysteine or through the secretion of reactive oxygen species (ROS) in the tumor microenvironment (Gabrilovich et al., 2012). The polarization of tumor associated macrophages is also crucial to the

immunosuppressive microenvironment; tumor secreted factors promote the accumulation of macrophages with proangiogenic and tumor promoting properties. Unlike classical or 'M1' macrophages, these 'M2' polarized cells do not secrete IL-12 (which can promote NK and T cell activation) but rather produce high levels of IL-10, which promotes the development of T_H2 cells and the secretion of TGF-β and CCL22 which can drive regulatory T cell (Treg) development and recruitment, respectively (Gajewski et al., 2013; Vesely et al., 2011). Tregs, in turn, inhibit effector T cell function through the consumption of IL-2 and through the inhibition of normal APC function by secretion of IL-10 and trans-endocytosis of the co-stimulatory molecules CD80/CD86 (Josefowicz et al., 2012; Qureshi et al., 2011). While every cancer type and especially every individual tumor are different, patterns have emerged as to the immunosuppressive mechanisms employed. Given the immense complexity and heterogeneity of malignancies, it is likely that the exact pattern and interplay of immune, mesenchymal and malignant components will never be completely understood, but tractable and druggable targets for therapeutic intervention have emerged and allow for the translation of basic biological findings into novel treatment for cancer patients.

Negative immune checkpoints and cancer

One of the most successful translations of cancer immunology into treatments is the recent demonstration that monoclonal antibodies blocking the programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) can improve survival in patients with melanoma and the subsequent FDA approval of these agents (Hamid et al., 2013; Hodi et al., 2010). Both PD-1 and CTLA-4 are cell surface

receptors expressed on T cells which are crucial to the normal regulation of the immune system and the prevention of rampant autoimmunity. The major role of PD-1 is to limit T cell activity in peripheral tissues during immune responses to pathogens by preventing the targeting of normal cells (Pardoll, 2012). Genetic loss of PD-1 in mice leads to the development of autoimmune cardiomyopathy and mortality, indicating the important role for this pathway in the prevention of autoimmunity (Nishimura et al., 2001). The main ligands for PD-1 are programmed death-ligand 1 (PD-L1) and programmed death-ligand 2. PD-L1 is broadly expressed on many epithelial tissues, while both ligands can be expressed by various leukocyte subsets (Keir et al., 2008). Ligation of PD-1 on T cells leads to the recruitment of the Src homology 2 domain-containing tyrosine phosphatase (SHP-2), counteracting key kinases downstream of the T cell receptor (TCR) which drive T cell activation (Chemnitz et al., 2004; Freeman et al., 2000). PD-1 is transiently increased upon T cell activation and notably expressed at very high levels on exhausted $CD4^+$ and $CD8^+$ T cell populations (Blackburn et al., 2009; Crawford et al., 2014; Nishimura et al., 2001). CTLA-4 is a crucial immune checkpoint regulator expressed on T cells which out-competes CD28 for the ligation of CD80 and CD86. Unlike PD-1, which is thought to mediate its effect primarily at the effector stage, ligation of CTLA-4 on effector cells inhibits the gene regulation effects immediately downstream of the CD28 costimulatory pathway, blunting the activation of the T cell (Parry et al., 2005). CTLA-4 can also downregulate T cell responses indirectly by the sequestration of CD80 and CD86 from APCs through trans-endocytosis (Qureshi et al., 2011). CTLA-4 plays a crucial role in controlling autoimmune responses during normal biology as genetic loss of CTLA-4 leads to rampant lymphoproliferation, autoimmunity, and death in mice (Tivol

et al., 1995). Expression of CTLA-4 is found on effector T cells, but is most prominent on Tregs (its expression is partially driven by forkhead box P3 (FOXP3) the master transcriptional regulator of Tregs) where it enhances their immunosuppressive activity (Peggs et al., 2009; Wing et al., 2008).

The antibody mediated blockade of negative immune checkpoints to improve antitumor immunity was pioneered by the Allison group with the demonstration that an αCTLA-4 mAb led to antitumor immune responses in an immunogenic murine colon carcinoma model (Leach et al., 1996). Crucially, their experiments assuaged the fear that blockade of CTLA-4 would unleash rampant autoimmune toxicity as had been observed in mice genetically lacking CTLA-4 (Tivol et al., 1995). Further murine studies indicating the potential efficacy of targeting this pathway led to the development of clinical reagents and their testing in patients with advanced malignancies. The first report of tumor regressions in patients following treatment with an αCTLA-4 mAb (ipilimumab) was a study of patients with metastatic melanoma treated with both ipilimumab and a peptide vaccine against the gp-100 melanoma-associated antigen; two complete responses were reported, although 43% of patients experienced grade III/IV autoimmune toxicities, notably colitis and hypophysitis (αCTLA-4 mediated hypophysitis was later shown to be due to direct targeting of CTLA-4 expressed on cells in the pituitary gland (Iwama et al., 2014)) (Phan et al., 2003). In a phase III trial, treatment with ipilimumab led to a survival benefit in patients with metastatic melanoma, and importantly, 18% of patients were reported to survive long term (>2 years; compared to 5% in the other arm) despite being treated for only 3 months up front, indicating the potential for immune therapies to induce long lived antitumor memory responses (Hodi et al., 2010). When

added to the chemotherapeutic dacarbazine, ipilimumab significantly improved survival compared to dacarbazine alone; the results of these two studies led to FDA approval for ipilimumab (Robert et al., 2011). Recent work in murine models has led to an improved understanding of the mechanism of action of αCTLA-4 treatment; studies show that antitumor efficacy is due to both inhibition of negative signaling in effector cells as well as the depletion of Tregs, findings that are likely relevant to ipilimumab which is a human IgG1 mAb capable of mediating antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) of bound target cells (Peggs et al., 2009; Simpson et al., 2013). Recent analysis of melanoma patients treated with αCTLA-4 has demonstrated that the treatment primarily acts to induce new antitumor $CD8^+$ T cell responses rather than merely expanding those antitumor $CD8^+$ T cells present prior to therapy, providing the first human *in vivo* evidence of this mechanism of action (Kvistborg et al., 2014).

CTLA-4 is expressed systemically on T cells and this lack of tumor specificity led to early doubts about whether a therapeutic window existed for this target. While PD-L1 is also systemically expressed, it has been found to be significantly overexpressed in the microenvironment of numerous solid tumors. Increased expression of PD-L1 has been demonstrated on myeloid populations in patients as well as on tumor cells themselves (Curiel et al., 2003; Dong et al., 2002; Duraiswamy et al., 2013). PD-L1 expression in tumors can be intrinsic, driven by oncogene activation such as EGFR in lung cancers or loss of the tumor suppressor PTEN in gliomas (Akbay et al., 2013; Parsa et al., 2006). Analyses of histologic sections of human melanoma and carcinomas of the lung, kidney, and head and neck has revealed a strong spatial correlation between the presence of TILs

and the expression of PD-L1 in these tumors (Lyford-Pike et al., 2013; Taube et al., 2012; 2014; Velcheti et al., 2013). These data suggest that tumor cells upregulate PD-L1 in response to immune pressure (specifically IFN-γ which is known to drive PD-L1 expression in normal tissue) from infiltrating $CD8^+$ T cells, a hypothesis termed 'adaptive resistance' (Keir et al., 2008; Sznol and Chen, 2013; Taube et al., 2012). Experimental evidence supporting this hypothesis was recently published in the B16 model of melanoma; using a genetic knockout and antibody depletion, tumor PD-L1 expression was shown to be dependent on both IFN-γ and $CD8^+$ T cells (Spranger et al., 2013).

Multiple mAbs blocking PD-1 and PD-L1 are in clinical development, and most have demonstrated comparable single agent clinical activity. Nivolumab is a fully human IgG4 αPD-1 mAb; in a large phase I study of multiple solid malignancies, objective responses were seen in 31%, 16%, and 29% of patients with metastatic melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma (RCC), respectively (Topalian et al., 2012). A competing αPD-1, pembrolizumab, recently received accelerated FDA approval for the treatment of melanoma after demonstrating an overall response rate (ORR) of 26% in a randomized expansion of a phase I trial (Hamid et al., 2013; Robert et al., 2014). The αPD-L1 MPDL3280A was recently shown to have a 26% ORR as a single agent in metastatic bladder cancer, and ORRs of 30%, 23%, and 14% in melanoma, NSCLC, and RCC, respectively (Herbst et al., 2014; Powles et al., 2014). Concurrent treatment with nivolumab and ipilimumab was recently found to lead to an ORR of 40% in patients with melanoma, which is better than the historical response rates of either agent alone, but was not compared to single agent treatment in this phase I study (Wolchok et al., 2013).

Despite remarkable response rates in patients with some malignancies to single agent treatment with checkpoint inhibitors, other tumor types have demonstrated much less sensitivity to such intervention. Furthermore, even in 'sensitive' neoplasms most patients fail to respond to checkpoint inhibitor therapy; it is unclear whether the mechanisms of resistance for these two populations are similar or different. A few analyses of treated patient cohorts offer insights into *in vivo* mechanisms of action and predictors of response to antibodies blocking negative checkpoint molecules. Melanoma, for which the best response rates with single-agent checkpoint inhibition have been observed, segregates into distinct subpopulations based on the tumor immune microenvironment, and a pre-treatment immune gene signature was shown to predict response to ipilimumab in melanoma patients in a retrospective analysis (Gajewski et al., 2010; Ji et al., 2011). These data suggest that αCTLA-4 acts to improve upon baseline immune responses to the tumor. However, a recent analysis of melanoma-specific $CD8^+$ T cells in the peripheral blood of patients treated with ipilimumab found that although treatment significantly increased the number of detectable melanoma-specific $CD8^+$ T cells responses, this was not due to an increase in the magnitude of pre-existing melanoma-specific $CD8^+$ T cells (Kvistborg et al., 2014). Treatment with αCTLA-4 led to a broader repertoire of anti-melanoma $CD8^+$ T cells but did not expand those melanoma-specific $CD8^+$ T cells which were present prior to treatment (just as it did not expand virus specific $CD8^+$ T cell responses). Taken together, these data suggest that αCTLA-4 may be facilitating the induction of novel antitumor T cell clones, but that this effect only has meaningful antitumor activity in patients in which a baseline antitumor immune response is already present. Whether this antitumor immune response allows for

better priming of antitumor T cells (i.e. through the availability of tumor antigen, presence of IFNs to facilitate proper T cell activation), or whether it facilitates trafficking and effector function of new antitumor T cells (localized secretion of chemokines or IL-2) is unclear. One study of patients treated with tremelimumab, another αCTLA-4 mAb, indicates that most patients (14/18) had increased TILs after treatment, but that this infiltrate does not correlate with response to treatment, further complicating the understanding of which metrics best predict response and resistance to αCTLA-4 treatment (Huang et al., 2011).

Even less is understood about the mechanisms underlying sensitivity and resistance to αPD-1/αPD-L1 mAbs. Early hypotheses suggested that simply the presence of PD-L1 in the tumor microenvironment may predict responses, a finding that was true in a small cohort examined in a phase I study (Topalian et al., 2012). This same correlation did not hold true in a study of αPD-1 plus αCTLA-4 in melanoma patients, where objective responses were noted in 4/10 PD-L1 positive and 8/17 PD-L1 negative patients, however the concurrent treatment with αCTLA-4 confounds this data set (Wolchok et al., 2013). Two recent studies of patients treated with αPD-L1 indicated that pretreatment PD-L1 expression on tumor-infiltrating immune cells, but *not* on tumor cells, correlated with response in bladder cancer and NSCLC (Herbst et al., 2014; Powles et al., 2014). A complicating factor in these analyses is that PD-L1 expression correlates strongly with TILs in multiple malignancies; it is therefore difficult to determine whether responses to blockade of the PD-1/PD-L1 axis correlate with the presence of TILs or the expression of PD-L1 (Lyford-Pike et al., 2013; Taube et al., 2012; 2014; Tumeh et al., 2014). The most recent analysis of melanoma patients treated with αPD-1 indicated that

the presence of $CD8^+$ T cells, PD-1, and PD-L1 in pretreatment biopsies all strongly correlated with response (Tumeh et al., 2014). Histologic analyses have demonstrated that the tumor PD-L1 and TIL correlation is true beyond melanoma (specifically in head and neck squamous cell carcinoma (HNSCC), RCC, and NSCLC) and responses to αPD-1/αPD-L1 have been observed in patients with lung and renal cancers (and one HNSCC patient (Herbst et al., 2014)), lending credence to the idea that a baseline antitumor immune response may predict the success of αPD-1 therapy (Brahmer et al., 2012; Lyford-Pike et al., 2013; Taube et al., 2014; Topalian et al., 2012; Tumeh et al., 2014). Specifically, the presence of a more clonal intratumoral $CD8^+$ T cell population correlates with responses in melanoma patients treated with αPD-1, suggesting that unlike αCTLA-4, targeting of the PD-1/PD-L1 axis works best on an already present antigen specific antitumor $CD8^+$ T cell population, rather than simply an inflammatory tumor microenvironment (TME) (Tumeh et al., 2014; Kvistborg et al., 2014).

Recent work in murine models supports the notion that checkpoint blockade acts on an existing immune response. The augmentation of antitumor T cell responses with vaccines, peritumoral poly(I:C), or intratumoral oncolytic virus has been shown to improve baseline responses to checkpoint inhibitors in murine models of melanoma, ovarian cancer, and colon cancer (Bald et al., 2014; Duraiswamy et al., 2013; Zamarin et al., 2014; Zhou et al., 2010). Additionally, a recent study by Dr. Schreiber and colleagues indicates that in a murine sarcoma model αPD-1 or αCTLA-4 act to induce expansion and activation of tumor antigen-specific $CD8^+$ T cells (Gubin et al., 2014). Taken together, the preclinical and clinical data suggest that responses to αPD-1/αPD-L1 and αCTLA-4 depend on a preexisting antitumor immune responses, that αCTLA-4, specifically,

induces novel tumor-specific CD8 T cells, and that therapeutic induction of antitumor T cells can augment the percentage of responders in tumor systems with baseline sensitivity to checkpoint inhibitors. However, some tumor types, such as pancreatic ductal adenocarcinoma (PDA), are fully refractory to checkpoint inhibitors in clinical studies (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). It is unclear to date whether any therapeutic approaches can extend the range of checkpoint inhibitor therapy to those tumor types that are resistant.

Pancreatic ductal adenocarcinoma and the KPC model

PDA is a lethal, aggressive, and common disease with the lowest 5-year patient survival rate of any tumor type routinely tracked (6%) (Howlander et al., 2013). Currently PDA ranks fourth in cancer deaths per year, but the incidence of PDA is rising, fueled by an aging population and the increasing prevalence of obesity and diabetes, and it is calculated to become the second leading cause of cancer death in the United States by 2020 (Hidalgo, 2010; Rahib et al., 2014). Although the causes of pancreatic cancer are unknown, several factors have been shown to increase risk; tobacco use increases risk the most (2.5-3.6 fold) while obesity, diabetes, and alcohol use also confer increased risk (Hidalgo, 2010). Several factors contribute to the high death rate in patients with PDA; the retroperitoneal location of the pancreas allows for the tumors to develop relatively unnoticed, causing nonspecific symptoms such as back pain and fatigue so that the vast majority (~80%) of patients have advanced disease upon diagnosis, precluding surgical resection (Hidalgo, 2010). Pancreatic cancer is also remarkably resistant to most therapeutic interventions; despite recent approval of two new chemotherapeutic regimens

(FOLFIRINOX and gemcitabine + nab-paclitaxel), patient response rates remain low (31.6% and 23%, respectively) and durability of responses is tragically short (Conroy et al., 2011; Von Hoff et al., 2013). Furthermore, both regimens, but especially FOLFIRINOX, can be difficult for patients to tolerate.

PDA develops progressively from precursor lesions termed pancreatic intraepithelial neoplasia (PanIN) which have been divided into stages based on histologic analysis of their epithelial polarity and nuclear aberrations into PanIN-1a, PanIN-1b, PanIN-2, and PanIN-3 (carcinoma *in situ*) (Rustgi, 2006). Each progressive stage is associated with greater frequency of mutations in prototypical oncogenes and tumor suppressors. Other pancreatic lesions such as mucinous cystic neoplasia and intraductal papillary mucinous neoplasia have also been identified as precursor lesions for PDA (Kopp et al., 2012). Strikingly, over 90% of pancreatic ductal tumors bear an activating mutation in the *KRAS2* oncogene; this mutation locks this key GTP-ase in its active form, leading to constitutive signaling down the PI3K and MEK/ERK pathways, among others (Hidalgo, 2010; Rustgi, 2006). The high frequency of this mutation, and the importance of these signaling pathways in driving cell proliferation and survival, have led to a general consensus that *Kras* is the driving mutation in PDA, a theory confirmed when induction of $Kras^{G12D}$ (constitutively active) in the pancreata of mice led to progressive development of PanIN lesions and PDA (Hingorani et al., 2003). Interestingly, despite the ductal appearance of PDA lesions, recent work in mice has demonstrated that the induction of oncogenic *Kras* leads to more PanINs when induced in acinar cells (through the promoter *Ptf1a*) compared to ductal/centroacinar cells (*Sox9* promoter), suggesting that acinar cells may be the cell of origin for most pancreatic tumors (Kopp et al., 2012).

The transcription factor *Prxx1*, transiently induced with pancreatitis or pancreatic injury, upregulates *Sox9* and contributes to acinar-to-ductal metaplasia, a key feature of the transformation of acinar cells to PDA upon $Kras^{G12D}$ expression, pancreatitis, or both (Kopp et al., 2012; Reichert et al., 2013). Other than *Kras*, inactivation of the tumor suppressors *TP53* and *CDKN2A* ($p16^{Ink4A}$) is very common in PDA (50%-75% and 90%-95%, respectively) and each of these has been shown to cooperate with $Kras^{G12D}$ to induce PDA in genetic mouse models (Aguirre et al., 2003; Hingorani et al., 2005).

The genetically engineered PDA model that we used in our studies is the KPC model in which $Kras^{G12D}$ and $TP53^{R172H}$ (a dominant-negative isoform analogous to the cancer predisposing mutation found in the Li-Fraumeni syndrome) are targeted to the endogenous loci and rendered quiescent by floxed transcriptional and translational silencing cassettes expressed in the promoter. Pancreas specific Cre recombinase, driven by the *Pdx-1* promoter, excises the silencing cassettes leading to expression of $Kras^{G12D}$ and $TP53^{R172H}$ in all cells of the pancreatic lineage (Hingorani et al., 2005). These KPC mice stochastically develop pancreatic adenocarcinoma with 100% penetrance within the first 6 months of life. The KPC model recapitulates the molecular, histopathologic, and clinical features of human pancreatic adenocarcinoma, including the progression through PanIN lesions and metastasis to peritoneum, liver and lung (Beatty et al., 2011; Clark et al., 2008; Hingorani et al., 2005).

Histologically, PDA is distinguished by a dense desmoplastic stroma, rich in fibroblasts, extracellular matrix (ECM), and inflammatory leukocytes. The cellular populations in the stroma play many roles in pancreatic cancer development, progression, invasion, and immune evasion, and the non-cellular components, including collagen,

fibronectin and hyaluronic acid, contribute to the elevated intratumoral hydrostatic pressure thought to cause poor drug delivery (Provenzano et al., 2012; Shepard et al., 2012). Reprogramming of pancreatic fibroblasts during tumorigenesis is a key pathway in the induction of pathologic fibrosis and the fibroblasts in human PDA upregulate genetic programs involved in ECM deposition, inflammation, and growth factor secretion (Sherman et al., 2014).

Since the stromal compartment has not undergone malignant transformation it may be more susceptible to intervention. Therapeutic ablation of hyaluronic acid decreases intratumoral hydrostatic pressure, increases intratumoral drug levels, and can synergize with gemcitabine to enhance survival in preclinical studies of a genetic mouse model of PDA, prompting a currently ongoing clinical study (Provenzano et al., 2012) (NCT01839487). Another approach to targeting PDA stroma is through inhibition of the paracrine signaling of the hedgehog pathway. Secretion of hedgehog ligands by PDA cells leads to increased activation of stromal cells, and pharmacologic inhibition of the Smoothened receptor in KPC mice decreases the desmoplastic stroma and improves survival when combined with gemcitabine (Olive et al., 2009). However, unlike the promising interim data of the hyaluronidase trial, a clinical study of Smoothened inhibitor with gemcitabine in PDA patients was halted when interim analysis showed that patients receiving the Smoothened inhibitor fared worse than those treated with gemcitabine alone, raising questions as to the role of the tumor stroma in PDA (Amakye et al., 2013). Recent work demonstrates that stromal ablation in KPC mice, either through genetic depletion of αSmooth muscle actin^{+} (αSMA) myofibroblasts or through deletion of *Sonic hedgehog* (*Shh*) in tumor cells, leads to the development of poorly differentiated

pancreatic tumors and accelerated death compared to control KPCs (Rhim et al., 2014; Özdemir et al., 2014). These studies indicate that tumor stroma in PDA may in fact be protective; this is potentially part of a wound-healing response gone awry, a mechanism thought to be involved in solid tumorigenesis (Hanahan and Weinberg, 2011). PDA desmoplasia may slow the outgrowth of undifferentiated tumors, but several groups demonstrate that depletion of the stroma, or the fibroblasts responsible for generating the stromal response, in fact potentiates other therapeutic modalities. The Stanger group demonstrated that in KPC mice lacking *Shh*, an αVEGFR2 mAb prolonged survival, while it did not in *Shh* sufficient KPCs; also, the depletion of fibroblast activating protein^{+} (FAP) or αSMA^{+} fibroblasts potentiates checkpoint inhibitor mediated antitumor effects in two studies of KPC mice (Feig et al., 2013; Rhim et al., 2014; Özdemir et al., 2014). Lastly, the vitamin D analogue calcipotriol was recently shown to bind the vitamin D receptor on pancreatic fibroblast and induce a reversion of their pro-tumorigenic gene signature, leading to fibroblasts resembling quiescent pancreatic stellate cells (Sherman et al., 2014). The use of calcipotriol (in combination with gemcitabine) in KPC mice led to decreased collagen and increased vasculature in pancreatic tumors, and was able to prolong survival compared to gemcitabine alone (Sherman et al., 2014).

While some of these data may seem contradictory, some general conclusions can be made about fibroblasts and ECM in PDA. During pancreatic tumorigenesis signals from tumor cells (such as *Shh* ligands) and local inflammation can induce quiescent pancreatic fibroblasts to become activated; these fibroblasts upregulate αSMA and undergo genetic reprogramming, likely programs that are part of normal wound healing responses. However, in these cancer associated fibroblasts the activated genetic program

is maintained, as the 'wound' does not heal, and their secretion of ECM, proinflammatory signals, and growth factors becomes pathologic and contributes to the formation of the dense fibrotic stroma associated with PDA. This response may actually slow the outgrowth of pancreatic tumor cells, but also has immunosuppressive effects and prevents successful drug delivery to tumor cells. For therapeutic purposes, ablation of the stromal compartment may potentiate other therapeutic approaches, but must be approached with caution given the potential to accelerate tumor growth.

Immunologically, the pancreatic tumor microenvironment is notable for a robust leukocytic infiltrate, one recapitulated in the KPC model (Beatty et al., 2011; Clark et al., 2008; 2007). Both human and murine PDA conspicuously lack a notable $CD8^+$ T cell infiltrate; rather, many of the TILs in PDA are Tregs (Bernstorff et al., 2001; De Monte et al., 2011; Fukunaga et al., 2004; Hiraoka et al., 2006; Clark et al., 2007; Zhang et al., 2014). In the KPC model, Tregs are present as early as the PanIN stage, and other immunosuppressive populations such as tumor associated macrophages (TAMs) and MDSCs are also increased in the pancreata of KPC mice harboring PanIN lesions (Clark et al., 2007). In pancreatic tumors, TAMs and MDSCs each make up, on average, 10% of the cellular composition of these tumors, highlighting the prominent role these suppressive myeloid populations occupy in PDA (Clark et al., 2007).

The immunosuppressive role of TAMs and MDSCs in this model of PDA has been well characterized. MDSC proliferation in KPC mice is driven by tumor derived granulocyte-macrophage colony-stimulating factor (GM-CSF), secretion of which is driven by oncogenic $Kras^{G12D}$ (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). GM-CSF drives extramedullary hematopoiesis in the spleens of KPC mice, and MDSCs

accumulate systemically and are recruited to the tumor and metastases (Bayne et al., 2012). These immature myeloid cells suppress $CD8^+$ T cell responses; they exhibit high levels of arginase and inducible nitric oxide synthase (iNOS) activity, both known mechanisms of MDSC suppression (Bayne et al., 2012; Gabrilovich et al., 2012).

Recently, Dr. Vonderheide and I collaborated with the Gabrilovich group to show that KPC MDSCs express increased *Trailr2*, a death receptor which is upregulated in MDSCs upon ER stress, and which is responsible for increased MDSC turnover in tumor bearing hosts, stimulating the proliferation of MDSC precursors in a feed-forward mechanism (Condamine et al., 2014). Importantly, in an implantable tumor model the overexpression of *Trailr2* allowed for selective depletion of MDSCs (leaving normal monocytes and neutrophils unaffected) in tumor bearing hosts, potentiating an antitumor T cell response (Condamine et al., 2014). TAMs in the KPC model also have immunosuppressive propreties, secreting IL-10 and IL-6 and expressing little MHC class II; treatment with an agonist αCD40 can transiently reprogram these macrophages and induce upregulation of MHC class II and the costimulatory molecule CD86, and imbue them with tumoricidal properties *in vitro* (Beatty et al., 2011). Treatment of KPC mice with αCD40 leads to an increase in systemic IL-12, TNF-α, and IFN-γ and causes macrophage-dependent tumor regression with a notable involution in the tumor stroma; a clinical study of αCD40 (with gemcitabine) treatment of PDA patients also led to partial responses in 4/21 patients (Beatty et al., 2011; 2013). Depletion of TAMs in an orthotopic model, using KPC or $Kras^{G12D}$ $p16^{Ink4A}$ derived cell lines, by inhibition of colony-stimulating factor 1 receptor (CSF1R; a chemokine receptor important for macrophage trafficking into tissues) also reversed their immunosuppressive effects and

allowed for improved $CD8^+$ T cell responses against the orthotopic tumors (Zhu et al., 2014).

The prevalence of populations of Tregs, TAMs, MDSCs, and fibroblasts in PDA tumors lends credence to the notion that the PDA TME is particularly immunosuppressive, but increasingly, there is an appreciation from studies in KPC and other PDA models of an underlying sensitivity of PDA tumor cells to T cell cytotoxicity (Vonderheide and Bayne, 2013). The dearth of effector T cells at all stages of tumorigenesis, combined with the appearance of immunosuppressive populations as early as the PanIN stages suggest that PDA tumors may be functionally immune privileged sites. Nevertheless, the lack of PDA immunogenicity does not necessarily indicate an inherent lack of antigenicity of PDA tumor cells; indeed, to the extent that effector T cells minimally encounter PDA tumor cells during the entire natural history of this cancer, PDA cells might be unexpectedly sensitive to T cell killing because they have not been exposed to Darwinian-like T cell selective pressure *in vivo*. Without T cell pressure, T cell escape and classical immunoediting may not be necessary for pancreatic tumor growth as it is for highly immunogenic tumors (Schreiber et al., 2011; Vonderheide and Bayne, 2013). This may explain the ability to induce T cell responses against pancreatic cancers when suppressive populations are deleted or inhibited (Bayne et al., 2012; Feig et al., 2013; Zhu et al., 2014; Özdemir et al., 2014).

Goals and key findings of this thesis project

While checkpoint blockade has produced remarkable single agent results in patients with melanoma, lung cancer, and kidney cancer, most patients fail to respond. Furthermore,

certain tumors, notably pancreatic cancer, are fully refractory to these agents to date. In the studies reported here, I tested the hypothesis that failed immune recognition or poor T cell priming underlies weak clinical responses to checkpoint therapy, i.e. induction of T cell immunity is required to potentiate tumor regressions not otherwise achievable with checkpoint blockade alone in minimally immunogenic tumors such as PDA. I used the KPC mouse model of spontaneous PDA which recapitulates the molecular, histologic, and immune parameters of the human disease and has predicted clinical responses (Bayne et al., 2012; Beatty et al., 2011; Clark et al., 2007; Hingorani et al., 2005; Provenzano et al., 2012; Sherman et al., 2014). Both the KPC and KPC derived subcutaneous tumor models were used to assess the relevance of the PD-1/PD-L1 and CTLA-4 pathways in pancreatic cancer, and to assess tumor responses to mAbs that block these key negative immune checkpoints. Analysis of human PDA tumors was performed to confirm the clinical relevance of our findings in the murine models. I induced T cell immunity using an agonistic αCD40 in combination with chemotherapy (Elgueta et al., 2009; Nowak et al., 2003), and compared the impact and mechanism of αPD-1/αCTLA4 mAb with or without this vaccine, examining only well-established tumors. Thus the main goal of this project was to model the clinical resistance of pancreatic cancer to checkpoint inhibitor therapy and to determine whether the induction of a T cell response could overcome this resistance and lead to immune mediated regression and rejection of PDA.

CHAPTER 2 – Materials and Methods

*Most of the methods in this chapter have been described in manuscripts:

Winograd et al., Beatty et al. (see page v for full citations)

Mice

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{LSL\text{-}R172H/+}$, *Pdx1-Cre* (KPC) mice (Hingorani et al., 2005), and their littermates *Trp53*$^{LSL\text{-}R172H/+}$, *Pdx1-Cre* (PC) mice were used for studies in Chapter 4. KPC mice and *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{LSL\text{-}R172H/+}$, *Pdx1-Cre, LSL-Rosa-YFP* (KPC-Y) (Rhim et al., 2012) were backcrossed 10 generations on the C57BL/6 background for studies in Chapter 3. Six- to eight-week-old female C57BL/6 and B6.129S7-Ifngtm1Ts/J (IFN-γ ko) mice used for implantable tumor studies were from Jackson Laboratories.

Patient Samples and Analysis

Formalin-fixed, paraffin-embedded tissue samples were prepared after surgical resection of patients with resectable pancreatic carcinoma according to an IRB-approved protocol. Immunohistochemistry was performed using antibodies against CD8 (Clone C8/144B; Dako M7103; 1:40) and PD-L1 (Clone E1L3N; Cell Signaling 13684; 1:75). Staining was performed on a Leica BondTM instrument using the Bond Polymer Refine Detection System (Leica AR9800). Heat-induced epitope retrieval was done for 20 minutes in either ER1 solution (Leica AR9961) for CD8 or ER2 solution (Leica AR9640) for PD-L1. PD-L1 staining required extended incubation for primary antibody (1 hour) and

polymer (20 minutes). Sections were analyzed by a senior pancreatic cancer clinical pathologist. All slides were scanned using the Aperio System. Utilizing the virtual slides, three fields within and outside the tumor bed were randomly selected for each case; care was taken to exclude fields encompassing lymphoid aggregates (follicles). For each field, the area was calculated by the imaging system and the number of $CD8^+$ cells was counted manually. For each field, the number of positive cells per area was calculated and the average of the three fields was calculated for each case to normalize for differing size fields, thereby eliminating any size field counting bias. PD-L1 staining (both membranous and cytoplasmic) was scored as 1+ (minimal expression), 2+ (moderate expression), 3+ (high expression), or 4+ (intense expression).

Collection of Tissue Samples from Mice

The entire pancreas (KPC) or subcutaneous tumor was washed in PBS, minced into small fragments, and incubated in collagenase solution (1 mg/ml collagenase V in DMEM) at 37°C for 45 min. Dissociated cells were passed through a 70 μM cell strainer twice and washed three times in DMEM. Spleens and lymph nodes were homogenized and passed through a 70 μM cell strainer to achieve single cell suspensions. For spleens, red blood cells were lysed using ACK Lysis Buffer (BioWhittaker).

Cell Lines

PDA cell lines from KPC (backcrossed or not) or KPC-Y mice were derived from single cell suspensions of PDA tissue, as previously described (Beatty et al., 2011). Dissociated

cells were plated in a 6-well dish with serum free DMEM. After 2 weeks, media was changed to DMEM + 10% FCS. After 4-10 passages, cells were used in experiments.

In vivo Mouse Studies

For implantable tumor experiments, PDA tumor cells ($5x10^5$ for studies in Chapter 3; $1x10^6$ for studies in Chapter 4) were injected subcutaneously in PBS onto the flanks of mice and allowed to grow 9-12 days until tumor volumes averaged 30-100mm^3. Mice were then enrolled into treatment groups such that cohorts were balanced for baseline tumor size. Mice were treated intraperitoneally (i.p.) with αPD-1 (RMP1-14, BioXcell; 200μg per dose) on days 0, 3, 6, 9, 12, 15, 18, and 21 (after enrollment) and/or αCTLA-4 (9H10, BioXcell; 200μg per dose) on days 0, 3, and 6. All antibodies were endotoxin free. Clinical grade gemcitabine (Eli Lilly) was purchased through the Hospital of the University of Pennsylvania Pharmacy; clinical grade nab-paclitaxel was either purchased or a kind gift from Celgene. Chemotherapy vials were resuspended and diluted in sterile PBS, and injected i.p. at 120 mg/kg (for each chemotherapeutic) on day 1. As a control for the human albumin component of nab-paclitaxel, control cohorts were treated with human albumin at the same dose as the albumin component of nab-paclitaxel (108 mg/kg) on day 1 (Sigma Life Science). Agonistic αCD40 (FGK45, BioXcell; 100μg) was given i.p. on day 3. For T cell depletion studies, αCD8 (2.43, BioXcell; 200μg per dose) and αCD4 mAbs (GK1.5, BioXcell; 200μg per dose) were injected i.p. twice weekly for the duration of the experiment, starting on day 0 (day of enrollment). For isotype controls, rat IgG2a (2A3, BioXcell; 100μg) and rat IgG2b (LTF-2, BioXcell; 200μg per dose) were used i.p.. This approach achieved >98% depletion of $CD8^+$ and $CD4^+$ T cells

in peripheral blood and tumor tissue compared to control mice, as monitored by flow cytometry. For tumor rechallenge studies, αCD8 or isotype control antibodies were injected i.p. the day before the second rechallenge and continued twice weekly until day 60 or until the mouse was sacrificed for tumor burden**.** To monitor growth of subcutaneous tumors, tumor diameters were measured by calipers and volume calculated by 0.5 x L x W^2 in which L is the longest diameter and W is the perpendicular diameter. Endpoint criteria for the survival studies included tumor volume exceeding 1,000 mm^3 or tumor ulceration. Mice that died suddenly or developed vestibular signs, as described in Figure 30, with minimal tumor burden were censored on the day of death or euthanasia.

For studies using the KPC model, young KPC mice were monitored by abdominal palpation and/or ultrasonography as previously described (Beatty et al., 2011) (Vevo 2100 Imaging System with 55MHz MicroScan transducer, Visual Sonics) for the development of pancreatic tumors. Mice with ultrasound diagnosed tumors of volume 30-150 mm^3 were enrolled and block randomized into treatment groups. Tumors were visualized and reconstructed for quantifying tumor volume using the integrated Vevo Workstation software package. Baseline tumor volume was not significantly different across cohorts. KPC mice were treated with the same dose and schedule of antibodies and chemotherapeutics as noted above in the subcutaneous model. Mice were censored from study if they developed a secondary malignancy (n=1). Endpoint criteria included tumor volume exceeding 1,000 mm^3 (by ultrasonography), severe cachexia, or extreme weakness and inactivity.

For explanted tumor studies, tumors from non-backcrossed KPC mice were dissected and 3x3 mm tumor chunks were implanted subcutaneously in PC mice. In the

"two tumor" models, explanted pancreatic tumor chunks or a PDA cell line (as described above) were injected subcutaneously in non-backcrossed KPC mice bearing ultrasound diagnosed tumors. Mice were treated starting on day 12-13 as further described in Figures 33 and 36.

Antibodies

The following monoclonal antibodies were used for flow cytometry: from BD Biosciences, αCD45 (30-F11; V500), αCD3 (145-2C11; PerCP, APC-Cy7), αCD19 (1D3; APC, V450), αCD8 (53-6.7; APC-Cy7, PerCP-Cy5.5), αCD11c (HL3; V450, APC), αCD4 (RM4-5; V450, PerCP-Cy5.5), αFoxP3 (FJK-16S; APC), αCD31 (MEC13.3; PE, FITC, APC); αH-2K^{b} (MHC class I) (AF6-88.5; PE); from eBiosciences, αF4/80 (BM8; FITC, PerCP, PE-Cy7), αCD45(30-F11; eFluor605), αFoxP3 (FJK-16S; PE), αLag-3 (C9B7W; APC), αB220 (RA3-6B2; APC-eFluor780), αNK1.1 (PK136; APC-eF780); and from Biolegend, αCD274 (PD-L1) (10F.9G2; PE), αCD279 (PD-1) (29F.1A12; FITC; RMP1-30; PE-Cy7), αCD90.2 (53-2.1; PerCP), αLag-3 (C9B7W; PerCP-Cy5.5), αCD8 (53-6.7;PE-e610), αKi-67 (16A8; FITC). Viability was assessed using either 7-aminoactinomycin D (7-AAD; BD Biosciences) or Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies).

Flow Cytometry

Single cell suspensions were stained with fluorochrome-labeled antibodies at 4°C for 15 min in PBS/1% FCS/2mM EDTA. Intracellular staining was done using a fixation/permeabilization kit (eBioscience). Cells were acquired on a FACSCanto or LSR

II flow cytometer (BD Biosciences) and were analyzed using BD FACSDiva software (BD Biosciences) or FlowJo (TreeStar).

In vitro IFN-γ stimulation of tumor cells

KPC-derived PDA cell lines were plated, allowed to rest overnight, and had media replaced the next day with complete media containing IFN-γ (R&D Systems) at 50 ng/mL. Cells were cultured with or without IFN- γ for 24 or 48 hours and then collected for flow cytometric analysis.

Immunohistochemistry

For analysis of CD3 (AbD Serotec, KT3, 1:100), CD4 (BioXCell, GK1.5, 15 μg/mL), CD8 (BioXcell, 2.43, 15 μg/mL), and Foxp3 (EBioscience, FJK-16s, 1:40), frozen sections fixed in 100% methanol were analyzed as previously described (Bayne et al., 2012). For quantification, the number of cells was counted per 40x field with a minimum of 4 fields per tumor quantified.

LCMV Clone 13 infection

Lymphocytic choriomeningitis virus (LCMV) clone 13 was propagated, titered and used as previously described (Blackburn et al., 2009). C57BL/6 mice were infected intravenously with $4x10^6$ PFU of LCMV clone 13. Mice were sacrificed on day 30 post infection and tissues harvested for analyses.

Statistical Analysis

Differences between two groups were analyzed by two-tailed Student's T test. Differences between three or more groups were analyzed by one-way ANOVA with Bonferonni's multiple comparison test used as a post hoc test to assess differences between any two groups. Tumor growth curves were analyzed by two-way ANOVA, with Tukey multiple comparisons of means used as a post hoc test to assess differences between any two groups. Survival curves were assessed by Log-rank (Mantel-Cox). Correlation between two groups was assessed by Spearman's Rank Correlation Coefficient. Significance of tumor regressions on 'waterfall plots' was determined using Fisher's Exact test. All statistical analyses were performed on GraphPad Prism 6 (GraphPad), except 2-way ANOVA and related post hoc testing, which were performed on R Statistical Software (R Core Team). $p \leq 0.05$ was taken as significant.

CHAPTER 3 – Induction of T cell immunity with chemotherapy and agonist αCD40 overcomes resistance to checkpoint inhibitors in pancreatic cancer

*The majority of the results described in this chapter are in a manuscript currently undergoing revisions at Cancer Immunology Research: Winograd, R., Byrne, K.T., Evans, R.A., Odorizzi, P.M., Meyer, A.R.L., Bajor, D.L., Clendenin, C., Stanger, B.Z., Furth, E.E., Wherry, E.J., and Vonderheide, R.H. Induction of T cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma

INTRODUCTION

It is now well-appreciated that T cells are key mediators of antitumor immunity and regulate the outcome of tumor immune surveillance (Schreiber et al., 2011). Critical to this regulation are lymphocyte inhibitory receptors such as PD-1 and CTLA-4 which restrain T cell antitumor immunity (Odorizzi and Wherry, 2012; Page et al., 2013; Pardoll, 2012; Sznol and Chen, 2013). Monoclonal antibodies that block the interaction of PD-1 or CTLA-4 with their ligands induce T-cell dependent tumor regression in many experimental systems (Page et al., 2013; Pardoll, 2012). In the clinic, unprecedented rates of tumor regressions have been observed in patients with melanoma and carcinomas of the lung and kidney following treatment with mAb against CTLA-4, PD-1, or PD-L1 (Brahmer et al., 2012; Hamid et al., 2013; Hodi et al., 2010; Topalian et al., 2012).

Although mAbs that block these immune checkpoint molecules represent a new therapeutic paradigm for cancer, mechanisms of PD-1 or CTLA-4 resistance are poorly

understood. Pre-existing T cell antitumor immunity has been hypothesized as a prerequisite (Gajewski et al., 2010; Ji et al., 2011; Zamarin et al., 2014). The majority of cancer patients treated with these agents alone do not clinically respond, and some tumor types, such as pancreatic carcinoma, are fully refractory (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). Although the combination of αPD-1 and αCTLA-4, either together or in sequence, may improve tumor response rates in melanoma, as suggested by results of a recent clinical trial, a large fraction of patients on this trial still failed to respond (Wolchok et al., 2013). Melanoma, for which the best response rates with single-agent checkpoint inhibition have been observed, segregates into distinct subpopulations based on the tumor immune microenvironment, and an immune gene signature predicts response to ipilimumab in melanoma patients (Gajewski et al., 2010; Ji et al., 2011). Tumor PD-L1 expression in melanoma correlates spatially with the presence of infiltrating $CD8^+$ T cells, suggesting that tumor cells upregulate PD-L1 in response to immune pressure, a hypothesis termed adaptive immune resistance (Taube et al., 2012). Evidence of similar pathophysiology has been observed in carcinomas of the lung, kidney, and head and neck (Lyford-Pike et al., 2013; Taube et al., 2014). Recent work in a mouse model of melanoma demonstrates that $CD8^+$ T cell-derived IFN-γ drives PD-L1 expression in malignant cells; this work is corroborated by a recent report indicating that $CD8^+$ T cells, PD-1, and PD-L1 all correlate with survival in patients treated with αPD-1, and that this immune signature can predict responses (Spranger et al., 2013; Tumeh et al., 2014) . Furthermore, IFN-γ and IFN-γ-inducible genes are upregulated in pretreatment biopsies of responding melanoma patients compared to nonresponders treated with αPD-L1 (Herbst et al., 2014). These data suggest that the efficacy of checkpoint inhibitors may

require the presence of an endogenous antitumor T cell response. In fact, the augmentation of antitumor T cell responses with vaccines, peritumoral poly(I:C), or intratumoral oncolytic virus has been shown to improve baseline responses to checkpoint inhibitors in murine models of melanoma, ovarian cancer, and colon cancer (Bald et al., 2014; Duraiswamy et al., 2013; Zamarin et al., 2014; Zhou et al., 2010). In models which are fully refractory to αPD-1 or αCTLA-4 alone, however, it remains unclear whether a vaccine approach can potentiate tumor rejection and long-term survival.

In the studies reported here, I tested the hypothesis that failed immune recognition or poor T cell priming is responsible for the lack of responses to checkpoint inhibitors in PDA. I focused on the KPC mouse model of spontaneous PDA in which expression of oncogenic $Kras^{G12D}$ and mutant *p53* is targeted to the pancreas by Cre recombinase under the control of the pancreatic specific promoter *Pdx-1* (Hingorani et al., 2005). This model recapitulates the molecular, histologic and immune parameters of the human disease (Bayne et al., 2012; Beatty et al., 2011; Clark et al., 2007; 2008; Hingorani et al., 2005). The clinical relevance of our findings in the murine models was confirmed by analysis of human PDA tumors. I induced T cell immunity using an agonistic αCD40 in combination with chemotherapy (Elgueta et al., 2009; Nowak et al., 2003), and compared the impact and mechanism of αPD-1/αCTLA4 mAb with or without this "vaccine", examining only well-established tumors.

RESULTS

PD-1/PD-L1 axis is highly expressed in murine and human PDA

To understand the biology of the PD-1/PD-L1 pathway in pancreatic carcinoma, I first interrogated the expression of PD-1 and PD-L1 using the KPC spontaneous genetic model of PDA. Within the microenvironment of KPC tumors few infiltrating T cells were observed, as has previously been reported (Figure 1) (Beatty et al., 2011; Clark et al., 2008). However, these T cells prominently expressed PD-1 in all subsets including $CD8^+$, $CD4^+$, and regulatory ($Foxp3^+$) T cells, as determined by flow cytometry (Figure 2). For each subset, PD-1 expression was significantly higher in the tumor than in the corresponding populations in the spleens of the same tumor-bearing mice (Figure 2). In the absence of a distinct marker for pancreatic epithelial cells in the KPC model, I identified tumor cells with negative gating, excluding leukocytes (CD45), endothelial cells (CD31), and mesenchymal populations (CD90) by flow cytometry of single cell suspensions of KPC tumors. KPC pancreatic tumor cells exhibited moderate expression of PD-L1 on more than 40% of the identified tumor cells (Figure 3). PD-L1 was also expressed by 10%-50% of normal pancreatic epithelial cells identified in C57BL/6 wild-type mice in the absence of cancer. In contrast to moderate expression of PD-L1 on tumor cells in the KPC model, DCs and macrophages in the KPC tumor microenvironment expressed very high levels of PD-L1, statistically significantly higher compared to PD-L1 expression of these same APC populations in the spleens of KPC mice (Figure 4).

To assess whether these findings in the KPC model were consistent with human PDA, I collaborated with Dr. Furth and developed a validated immunohistochemical assay for PD-L1 expression in human tissues and examined human PDA samples for PD-L1 expression. In primary tumors from patients with resected PDA, we observed moderate to intense expression of PD-L1 on tumor cells in 4 of 8 (50%) resection

specimens (Figure 5). We also observed that T cells in human PDA were relatively rare within malignant foci (mean ratio of $CD8^+$ T cells per μm^2 of tumor vs. non-tumor areas was 0.065 ± 0.052, range of 0.000-0.170) – again consistent with the KPC model (Beatty et al., 2011). PD-L1 expression on tumor cells in human samples did not correspond spatially with the presence of $CD8^+$ T cells (Figure 5). There was no statistical correlation between intensity or extent of tumor PD-L1 expression and intratumoral $CD8^+$ T cell infiltration ($p=0.69$) (Figure 6). For example, of the two tumors with the most intratumoral $CD8^+$ T cells, one had intense and the other had minimal PD-L1 expression (Figure 6). These data in human PDA are in contrast to the correspondence of tumor PD-L1 expression and T cell infiltration previously reported for tumors from patients with melanoma or kidney or head and neck carcinoma (Lyford-Pike et al., 2013; Taube et al., 2012; 2014).

PD-1 is as highly expressed in murine PDA as it is in chronic LCMV infection

To evaluate the potential role of the PD-1/PD-L1 axis in mediating immune suppression in PDA, I first generated a PDA cell line from a backcrossed KPC mouse and established subcutaneous PDA tumors in immune competent C57BL/6 mice. Histopathological examination of established tumors from this model showed recapitulation of both the cellular and extracellular components of spontaneous KPC tumors, with prominent deposition of a dense desmoplastic stroma and comparable populations of infiltrating immunosuppressive leukocytes, including a robust infiltrate of $F4/80^+$ macrophages (Figure 7). I then examined expression of PD-1 on T cells from subcutaneous tumor-bearing mice, but did so by simultaneously examining PD-1 expression on T cells from a

parallel cohort of mice in which chronic LCMV infection had been established with LCMV clone 13 (Figure 8). In many ways, this model of chronic LCMV infection has served as a gold standard for understanding the transcriptional basis and phenotype of exhausted $CD4^+$ and $CD8^+$ T cells (Barber et al., 2006; Blackburn et al., 2009; Crawford et al., 2014; Doering et al., 2012; Paley et al., 2012; Wherry et al., 2007). Two of the most highly upregulated genes mechanistically linked to T cell exhaustion in response to chronic infection in this model are PD-1 and Lag-3 (Blackburn et al., 2009). I therefore compared coexpression of these markers on intratumoral and splenic T cells in mice bearing established subcutaneous PDA tumors with splenic T cells from mice with chronic LCMV (Figure 8). Intratumoral $CD8^+$, $CD4^+$, and regulatory T cells co-expressed PD-1 and Lag-3 at levels comparable to the corresponding T cell populations in LCMV-infected mice (Figure 9). Furthermore, PD-1 expression was statistically higher on tumor infiltrating T cells than on T cells from chronically infected mice, demonstrating the prominence of this inhibitory receptor in the PDA microenvironment (Figure 10). This phenotype was restricted to the tumor, as splenic T cells from tumor-bearing mice did not coexpress PD-1 or Lag-3. Thus, T cell expression of PD-1 is higher in the PDA tumor microenvironment than it is in chronic LCMV infection.

I also examined PD-L1 expression in the subcutaneous PDA model. About 60% to 70% of tumor cells isolated from established tumors expressed PD-L1 (Figure 11), similar to the expression of PD-L1 on this cell line grown *in vitro* (Figure 14). These findings were confirmed using a YFP^+ tumor cell line established from a pancreatic tumor isolated from a KPCY genetically engineered mouse; in this model, YFP serves as a validated lineage tracer for pancreatic epithelium (Rhim et al., 2012). After

subcutaneous tumor implantation and growth in syngeneic hosts for 14 days, I found that on average 66.7% of YFP^+ tumors cells expressed PD-L1, as measured by flow cytometry (Figure 12). Moreover, high levels of PD-L1 on both DCs and macrophages were observed in the tumor microenvironment of the KPC subcutaneous tumors (Figure 13), mirroring PD-L1 expression on these APC subsets in spontaneous tumors of KPC mice. Both a higher percentage of $PD\text{-}L1^+$ APCs and a higher (~3-4-fold) mean fluorescence intensity (MFI) of PD-L1 was observed compared to the corresponding APC populations in the spleens of the same mice (Figure 13). These data indicate that PD-L1 expression is prevalent in the PDA microenvironment, as these APC populations make up as much as 8%-25% of the cellular composition of these tumors (Figure 7).

Tumor PD-L1 expression in PDA is not IFN-γ dependent

PD-L1 expression in human melanoma and HNSCC correlates spatially with T cell infiltration (Lyford-Pike et al., 2013; Taube et al., 2012), and ,in melanoma, tumor expression of PD-L1 is dynamically upregulated in response to IFN-γ secreted by these infiltrating $CD8^+$ T cells (Spranger et al., 2013). To determine whether this same mechanism is responsible for PD-L1 expression in PDA, I first assessed the ability of our PDA cell lines to upregulate PD-L1 in response to IFN-γ; *in vitro*, IFN-γ stimulation resulted in increased expression of PD-L1 by PDA cells in each of 8 separate KPC derived cell lines (Figure 14). To then assess the role of this pathway *in vivo*, I evaluated tumor and APC PD-L1 expression in the presence or absence of T cells and IFN-γ. Subcutaneous PDA tumors were established in mice that were genetically lacking IFN-γ, systemically depleted of $CD4^+$ and $CD8^+$ T cells, or both. Tumor growth *in vivo* was the

same for each condition compared to control (Figure 15). Analysis of these tumors showed no significant change in tumor PD-L1 expression (either percentage or MFI) with regard to IFN-γ or T cell status (Figure 16). Analysis of the APC populations in these tumors indicated that IFN-γ plays a minor role in the regulation of PD-L1 expression on intratumoral DCs and macrophages. Small but statistically significant differences were observed in the percentage and MFI of PD-L1 expression on intratumoral APCs between IFN-γ sufficient and deficient hosts (Figure 17). In contrast, the presence or absence of T cells did not affect PD-L1 expression of APCs regardless of host IFN-γ status (Figure 17), recapitulating the lack of correspondence between $CD8^+$ T cells and PD-L1 expression in human PDA (Figure 6).

T cell stimulation with CD40/gemcitabine/nab-paclitaxel converts PDA from being fully refractory to highly sensitive to checkpoint blockade

Given the prominent expression of PD-1 and PD-L1 in the PDA tumor microenvironment, I tested the antitumor *in vivo* efficacy of PD-1 blocking mAb either with or without CTLA-4 blocking mAb (Figure 18). Even with αCTLA-4, αPD-1 did not impact tumor growth or survival (Figure 19), even though a comparable αPD-1 dosing schedule reproducibly improves clinical outcomes in mice chronically infected with LCMV clone 13 (Barber et al., 2006; Blackburn et al., 2008). This finding was surprising given the fact that tumor associated T cells express even more PD-1 than the T cells in LCMV infected mice (Figure 10). However, this lack of antitumor efficacy is similar to the lack of responses observed to date in patients with advanced PDA treated with αPD-

L1 or αCTLA-4, suggesting that this model recapitulates this immunologic aspect of the human disease (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010).

These same reagents have shown efficacy in patients in other malignancies; one possible distinction may be the presence of an antitumor immune response at baseline in subsets of these patients (Gajewski et al., 2010; Galon et al., 2013). I therefore hypothesized that the induction of a T cell response would be required to overcome refractoriness to αPD-1 and αCTLA-4 in PDA and achieve clinical benefit. It has been well-established that an agonist αCD40 antibody facilitates cancer vaccines (Cho and Celis, 2009) and can synergize with chemotherapy-induced immunogenic cell death to initiate a T cell-dependent antitumor immune response and tumor regression, providing a vaccine effect in model systems for which a tumor-rejection antigen is not characterized (Buhtoiarov et al., 2010; Nowak et al., 2003). Here, we chose to examine the chemotherapeutic combination of gemcitabine and nab-paclitaxel because these agents are recently FDA-approved for the treatment of metastatic PDA (Von Hoff et al., 2013), and gemcitabine has been previously been shown to cooperate immunologically with αCD40 for the induction of T cell immunity (Nowak et al., 2003). Treatment of mice with established subcutaneous PDA tumors with αCD40/chemotherpay altered the phenotype of tumor infiltrating T cells, although the percent of T cells infiltrating the tumors did not change. There were statistically significantly fewer $CD8^+$ T cells that co-expressed the inhibitory PD-1 and Lag-3 markers in treated tumors, and more proliferating $CD4^+$ and $CD8^+$ T cells were found in the tumors of treated mice compared to controls (Figure 20).

As is true for the vast majority of patients with metastatic PDA, the combination of gemcitabine and nab-paclitaxel at the maximum tolerated dose did not induce regression of established subcutaneous PDA tumors (data not shown); however, the addition of αCD40 to this chemotherapy regimen inhibited tumor growth and improved survival compared to control-treated tumor-bearing mice (Figure 21). These effects were T cell-dependent, as αCD40 plus chemotherapy failed to impact tumor growth in mice depleted of $CD8^+$ and $CD4^+$ cells (Figure 21). The addition of αPD-1, αCTLA-4, or both to treatment with αCD40/chemotherapy significantly improved the ability of αCD40/chemotherapy to inhibit tumor growth, and led to an increase in survival in mice bearing subcutaneous PDA tumors (Figure 21). Moreover, the addition of checkpoint inhibitors to αCD40/chemotherapy led to complete rejection of established tumors and long-term tumor-free survival in significant proportions of mice treated (Figure 22). The highest rates of tumor regression were observed in mice treated with both αPD-1 and αCTLA-4, with 39% (17 of 44) of mice achieving long-term complete remission and survival after treatment with all three antibodies plus chemotherapy (Figure 22). Tumor growth was delayed in nearly all mice treated with αCD40/chemotherapy and αPD-1/αCTLA-4, even in those mice not completely rejecting their tumors, suggesting that the tumor response rate is even higher than the tumor rejection rate in this model (Figure 23).

Rejection of PDA tumors by αCD40/chemotherapy and checkpoint blockade is T cell-mediated

To determine whether the antitumor effect I observed was T cell-mediated, I repeated the study with a cohort of mice depleted of $CD4^+$ and $CD8^+$ T cells, starting on the day prior

to the initiation of therapy. In the absence of T cells, there was a loss of the tumor growth inhibition and survival advantage of treatment, and no T-cell depleted mice rejected the tumor or survived long-term (Figure 24).

To understand the effect of our treatment on intratumoral T cell populations, I treated cohorts of tumor-bearing mice with αPD-1/αCTLA-4, αCD40/chemotherapy, both, or neither (control), and sacrificed mice one week after treatment with αCD40 (or control) to analyze tumors for T cell infiltration. Tumors from mice treated with αPD-1/αCTLA-4 plus αCD40/chemotherapy had a significantly increased (7-fold) CD8:Treg ratio compared to control-treated mice (Figure 25). This phenotype was also seen in some of the mice treated with αCD40/chemotherapy or αPD-1/αCTLA-4, although neither of these groups exhibited as consistent of an increase in the CD8:Treg ratio as the mice treated with αPD-1/αCTLA-4 plus αCD40/chemotherapy (Figure 25). The CTLA-4 mAb clone 9H10 partly mediates its antitumor effect by depletion of Tregs, which express CTLA-4 (Simpson et al., 2013); however, I observed that mice treated with αPD-1/αCTLA-4 alone did not have a significantly decreased percentage of Tregs among $CD4^+$ T cells (Figure 25) or among total $CD45^+$ cells (data not shown). Rather, the administration of αCD40/chemotherapy (either with or without αPD-1/αCTLA-4) was associated with a significant decrease in Treg percentages compared to control treated mice (Figure 25). These data suggest that αCD40/chemotherapy changes the immune microenvironment in this PDA model and leads to a decreased percentage of Tregs and increased CD8:Treg ratio, an effect that is augmented further with the addition of checkpoint blockade. The greatest changes in Treg percentage and CD8:Treg ratio were

associated with the highest rates of complete remission and long-term survival across cohorts reported in Figure 22.

One theoretical benefit of cancer immunotherapy is the induction of an antitumor memory T cell response with the capability to reject recurrent or metastatic disease. To test whether mice that had completely rejected established PDA tumors had developed immune memory, I rechallenged cohorts of mice that were in long-term complete remission with the same number of cells of the same PDA tumor line but on the opposite flank (Figure 26). I observed that 67% to 86% of such mice rejected the PDA tumor cells implanted on the opposite flank without any therapy (Figure 26), consistent with immunological memory. Because the most likely effector memory T cell population mediating this effect is a $CD8^+$ T cell, I further studied mice that had rejected both the initial tumor and the first rechallenge on the opposite flank, and either depleted these mice of $CD8^+$ T cells or administered an isotype control. All mice were then rechallenged with the same number of cells of the same cell line on the original flank. All mice depleted of $CD8^+$ T cells rapidly developed progressively growing tumors at the site of second rechallenge, whereas 4 of 6 isotype-treated mice rejected this second tumor rechallenge (Figure 27). This effect translated into a significant difference in overall survival after second rechallenge (Figure 27). These data indicate that combination immunotherapy can establish CD8-dependent immunological memory against PDA with curative potential.

Given the mechanistic understanding of the agonist αCD40 mAb and the chemotherapeutics gemcitabine and nab-paclitaxel, it seemed likely that the αCD40 arm of the treatment was key to potentiating the efficacy of checkpoint inhibitors in this

model (Diehl et al., 1999; French et al., 1999). In order to test this hypothesis, I treated tumor bearing mice with αCD40, gemcitabine/nab-paclitaxel, or both, and treated all mice with both αPD-1 and αCTLA-4, monitoring the mice for tumor growth and survival. Both cohorts treated with chemotherapy showed an early stabilization of tumor size in the aftermath of therapy, as compared to the cohort not receiving chemotherapy (Figure 28A). However, in the mice not receiving αCD40 treatment the tumor growth inhibition of the chemotherapy was short lived and the tumors continued to grow after a few days. In both groups treated with αCD40 and checkpoint inhibitors, tumors began to regress within a few days of αCD40 therapy, regardless of whether they had been treated with chemotherapy (Figure 28A). Ultimately, tumors were completely rejected in all 3 groups, but a clear pattern emerged; mice treated with αCD40 and checkpoint inhibitors rejected their tumors at much higher rates than the cohort which was treated with just chemotherapy and αPD-1/αCTLA-4 (Figure 28B). These data indicate that αCD40 is more important than chemotherapy in potentiating the efficacy of checkpoint inhibitors in this tumor model. Nevertheless, given that gemcitabine and nab-paclitaxel are a standard of care for patients with PDA, I chose to move forward with my studies in the KPC model using the combination of chemotherapy and αCD40 rather than αCD40 alone to maintain the translational relevance of this work.

αCD40/chemotherapy cooperates with PD-1 blockade to improve survival of mice with established tumors in the KPC genetic model of PDA

Having observed that the induction of T cell immunity via αCD40/chemotherapy potentiates the efficacy of checkpoint inhibitors in the subcutaneous model of PDA, I

tested this approach in the autochthonous KPC model of PDA. Observations in the KPC model have previously been shown to predict clinical responses in PDA patients treated with the same or homologous agent (Beatty et al., 2011; Provenzano et al., 2012; Rhim et al., 2014; Shepard et al., 2012). I therefore performed a randomized, controlled study of checkpoint inhibition in combination with αCD40/chemotherapy in cohorts of tumor-bearing KPC mice. Given the striking expression of PD-1 and PD-L1 in the KPC tumor microenvironment, I chose to test our hypothesis using the αPD-1 mAb. Mice diagnosed with pancreatic tumors of 30 mm^3-150 mm^3 were randomized to treatment with αCD40 plus gemcitabine/nab-paclitaxel, αPD-1, αCD40/chemotheray plus αPD-1, or control (as described in Materials and Methods and summarized in Figure 29A), using the same dose and schedule as used for mice in the subcutaneous PDA studies. I observed a statistically significant increase in overall survival for mice receiving αCD40/chemotherapy plus αPD-1 compared to control (p=0.015, log-rank Mantel-Cox) (Figure 29B). The effect was large: combination treatment nearly doubled median overall survival from 23 days in the control arm to 41.5 days in the experimental arm with a hazard ratio of 0.334 (0.0584-0.657, 95% confidence interval). Neither PD-1 alone nor αCD40/chemotherapy significantly improved overall survival. These data suggest that as predicted by my findings in the subcutaneous PDA model, the induction of a T cell response is needed to observe antitumor effects using αPD-1 in PDA.

Although treatment was well tolerated in the vast majority of mice, in 6.3% of mice treated with αCD40/chemotherapy and at least one checkpoint blocking mAb I noted clinical deterioration consistent with an infectious syndrome (Figure 30).

CONCLUSIONS AND DISCUSSION

The clinical success of checkpoint inhibitors, including FDA approval of ipilimumab and pembrolizumab in melanoma, has prompted investigations to replicate this result even more broadly in oncology. Early findings, however, suggest that many tumors are resistant, with some tumors such as pancreatic carcinoma appearing completely refractory to checkpoint blockade alone (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). Elucidation of the biological mechanisms underlying this resistance, and strategies to overcome it therapeutically, are only beginning to emerge. Here, using a genetically engineered mouse model (GEMM) of PDA, which like human PDA exhibits minimal spontaneous immunity, I demonstrate that despite robust expression of PD-1 and PD-L1 in the tumor microenvironment, treatment with αPD-1 with or without αCTLA-4 fails to improve the survival of mice or slow the growth of PDA tumors. These results replicate the lack of effect observed to date in PDA patients treated with analogous agents (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). However, in the context of αCD40/chemotherapy deployed as a vaccine, I demonstrate that the induction of T cell immunity converts PDA from a tumor that is completely refractory to αPD-1 and/or αCTLA-4 into one in which checkpoint blockade controls tumor growth and significantly improves survival in a $CD8^+$ T cell dependent manner. In particular, αCD40/chemotherapy plus αPD-1 nearly doubles the median overall survival in genetically engineered KPC mice with pre-established spontaneous pancreatic tumors. Moreover, the capability of treated mice to reject second and third subcutaneous tumor challenges in a $CD8^+$ T cell-dependent fashion, thereby rendering long-term survival, suggests the establishment of antitumor immune memory with curative potential. These

findings indicate that poorly immunogenic tumors, epitomized by the KPC pancreatic tumor model, can nevertheless be controlled by the adaptive immune system provided a dual approach of therapeutic T cell induction and checkpoint blockade is utilized.

Immunologically, the PDA tumor microenvironment is considered especially suppressive, but increasingly, there is an appreciation from studies in KPC and other PDA models of an underlying sensitivity of PDA tumor cells to T cell cytotoxicity (Vonderheide and Bayne, 2013). Unlike melanoma, PDA does not commonly present with a robust tumor infiltration of $CD8^+$ T cells (Bernstorff et al., 2001; De Monte et al., 2011; Fukunaga et al., 2004; Hiraoka et al., 2006). Instead of effector T cell infiltration in the tumor, Dr. Vonderheide and others have observed in genetically engineered mouse models of PDA, a prominent network of immunosuppressive macrophages, MDSCs, and Tregs that becomes dominant even at the earliest stages of disease (Bayne et al., 2012; Beatty et al., 2011; Clark et al., 2007; 2008; Zhang et al., 2014). I demonstrated that PD-1 is expressed on more T cells in the KPC tumor microenvironment than it is systemically in mice chronically infected with LCMV in which treatment with αPD-1 successfully reverses T cell exhaustion (Figure 10) (Barber et al., 2006). I propose therefore, that the lack of responses to treatment with checkpoint inhibitors in KPC mice likely reflects a tumor microenvironment without an underlying antitumor T cell response, making a response to αPD-1 or αCTLA-4 alone mechanistically unlikely.

Thus, in this study I interpret the antitumor effects of αCD40/chemotherapy plus αPD-1/αCTLA-4 as a strategy that overcomes acquired immune privilege in PDA. To be sure, other pathways in the PDA tumor microenvironment may also be "targetable" as part of novel immunotherapeutic approaches. For example, derailing non-tumor cell

intrinsic immunosuppressive elements in the PDA microenvironment (such as macrophages, fibroblasts, and MDSC) permits trafficking of $CD8^+$ T cells into the tumor and can induce tumor regression (Bayne et al., 2012; Feig et al., 2013; Zhu et al., 2014). These strategies can now be rationally combined with αCD40/chemotherapy plus αPD-1/αCTLA-4 and tested for synergy and survival benefit in the KPC model.

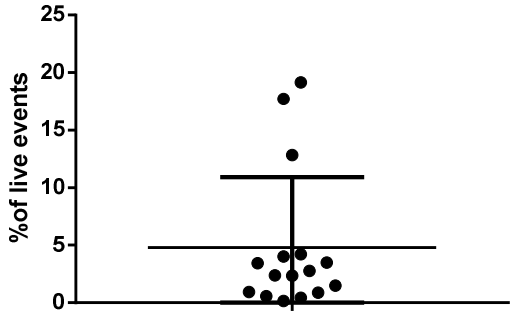
Despite the 80% increased overall survival observed in KPC mice treated with αCD40/chemotherapy and αPD-1 compared to controls, all mice succumbed to their disease. It is worth noting that there are no published reports of cures of KPC mice bearing established invasive tumors. A few groups have reported improved overall survival (without cures) with treatment in this model, including the recent demonstration that the vitamin D analogue calcipotriol improves survival by 57% (Olive et al., 2009; Provenzano et al., 2012; Sherman et al., 2014). Given the difference I observed in the response to treatment between the subcutaneous and KPC PDA models, I hypothesize that there is additional complexity in the tumor microenvironment of spontaneous KPC tumors which limits therapeutic responses. Potential other immunosuppressive pathways contributing to treatment resistance include MDSCs, macrophages, and FAP^+ mesenchymal cells, among others (Bayne et al., 2012; Feig et al., 2013; Pylayeva-Gupta et al., 2012; Zhu et al., 2014). My observations have relevance to patients with PDA not only because of our observations of PD-L1 expression in human PDA but also because of the high fidelity of the KPC model to human pancreatic cancer and its capability to predict the clinical potential of reagents (Beatty et al., 2011; Provenzano et al., 2012; Rhim et al., 2014; Sherman et al., 2014).

In the absence of a defined tumor antigen in our KPC model, I therapeutically induced T cells with chemotherapy followed by an agonist αCD40, a sequence previously described to injure tumor cells, release tumor antigen, and license APCs (Nowak et al., 2003). Although non-chemotherapeutic agents, such as targeted therapies, may also synergize with αCD40 (Ho et al., 2014), gemcitabine in particular cooperates with αCD40 (Nowak et al., 2003). Here, I added nab-paclitaxel given the recent regulatory clinical approval of gemcitabine with nab-paclitaxel for the treatment of metastatic PDA. Although, as previously shown, αCD40 alone can generate an antitumor macrophage (but not T cell) response *in vivo* (Beatty et al., 2011), the addition of gemcitabine, nab-paclitaxel, and αPD-1 enabled a T cell response. I demonstrated that while αCD40 is necessary for the potentiation of checkpoint inhibitor efficacy, chemotherapy alone does not allow checkpoint inhibitor treatment to induce rejection of tumors and long term survival (Figure 28). In this study, a greater percentage of mice treated with αCD40/αPD-1/αCTLA-4 rejected their tumors than those who also were treated with chemotherapy, although this difference was not significant; this could potentially be due to chemotherapeutic ablation of rapidly proliferating immune cells. Nevertheless, for the sake of translational relevance I conducted most of the studies in the setting of chemotherapy as it is standard of care for PDA patients; further investigation is needed to determine the role of chemotherapy in the induction of a T cell response. Moreover, I made these observations in the setting of pre-established tumors and an autochthonous tumor microenvironment, two additional elements of this experimental system relevant to translation to the clinic.

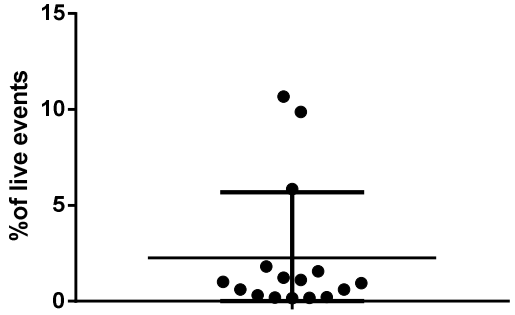
With increasingly potent immune therapies, toxicity can become an important and limiting issue. For example, the combination of nivolumab (αPD-1) with ipilimumab (αCTLA-4) is associated with a higher rate of grade 3 and 4 toxicities than either agent alone (Wolchok et al., 2013). During the studies reported here, I noted a few cases of treatment-related opportunistic infection of the CNS. This pathology was observed only in mice treated with both CD40/chemotherapy plus one or more checkpoint inhibitors, but even then only in certain cohorts of mice imported from one vendor and not in other imported mice of the same genetic background that received the same treatment (Figure 30). Mice bred in our facility and treated with this same combination of reagents never presented with this clinical syndrome (n=24). Pathologic analyses of mice exhibiting clinical deterioration indicated inflammation of the CNS which was neutrophilic and associated with evidence of bacterial infection, and not lymphocytic or otherwise notable for T cell autoimmunity. The overall impression was that of vendor-related commensal bacteria which became pathologic in the context of immune-altering treatment, providing a note of caution as new immunotherapy combinations are tested in patients in early phase trials.

In summary, induction of T cell immunity overcomes resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma. My work suggests than an understanding of the underlying immunobiology of solid tumors may help in determining which malignancies may benefit from checkpoint inhibition, and which may necessitate combinatorial immunotherapy to first induce an antitumor T cell response.

A



B



C

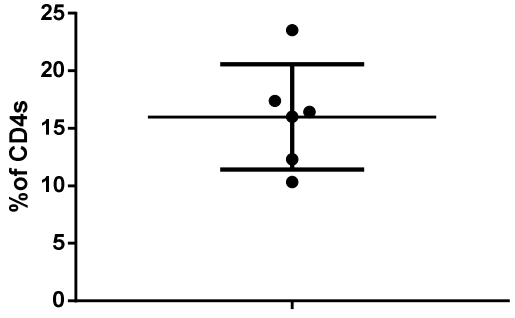


Figure 1. Tumor infiltrating T cells are rare in pancreatic tumors of KPC mice. Pancreatic tumors of KPC mice were evaluated by flow cytometry for the presence of **(A)** $CD4^+$ (gated on live, $CD3^+CD4^+$) and **(B)** $CD8^+$ (gated on live, $CD3^+CD8^+$) T cells. $CD4^+$and $CD8^+$ T cells are quantified as percentage of all live events (A and B). **(C)** Regulatory T cells (Tregs; gated on live, $CD3^+$ $CD4^+$ $FoxP3^+$) are shown as percentage of $CD4^+$ T cells.

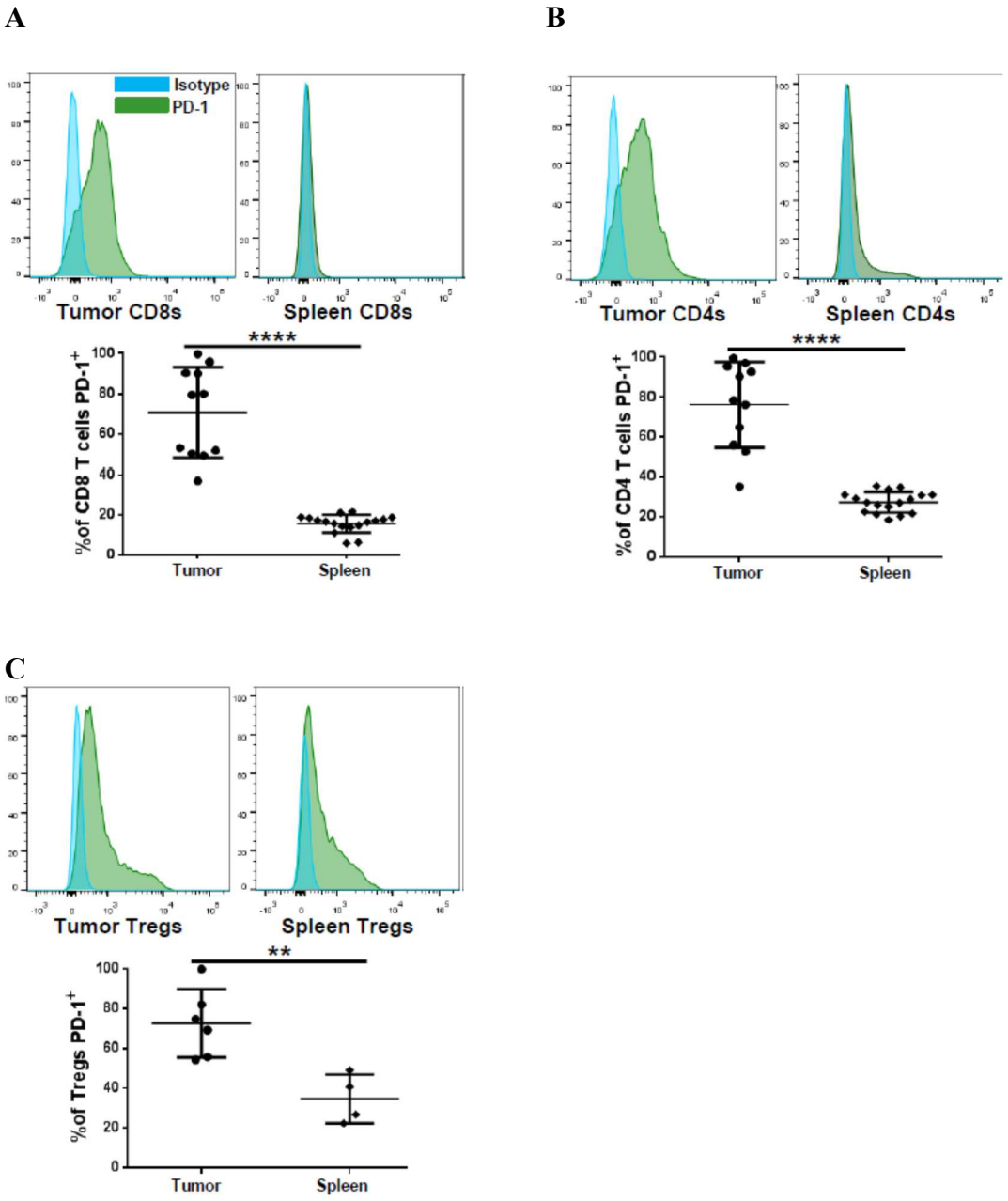


Figure 2. PD-1 is highly expressed on all tumor infiltrating T cell subsets in KPC tumors. Representative histograms and quantification of PD-1 expression on tumor infiltrating **(A)** $CD8^+$ (gated on live, $CD45^+$, $CD3^+$, $CD8^+$), **(B)** $CD4^+$ (gated on live, $CD45^+$, $CD3^+$, $CD4^+$), or **(C)** regulatory (Tregs; gated on live, $CD45^+$, $CD3^+$, $CD4^+$, $FoxP3^+$) T cells in tumors (n=6-11) or spleens (n=4-17) from tumor bearing KPC mice. **p≤ 0.01, ****p≤ 0.0001.

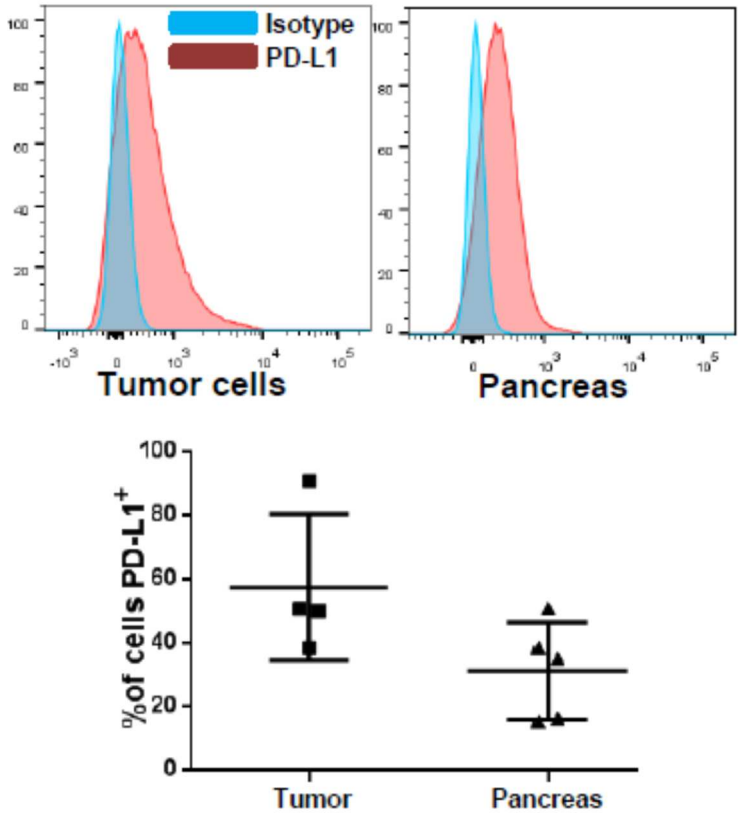


Figure 3. PD-L1 is moderately expressed on pancreatic tumor cells *in vivo* in KPC tumors. Representative histograms and quantification of PD-L1 expression on tumor cells and normal pancreatic epithelial cells (gated on live, $CD45^{neg}$, $CD31^{neg}$, $CD90^{neg}$) in tumors (n=4) from tumor bearing KPC mice and normal pancreata (n=5) from healthy C57BL/6 mice.

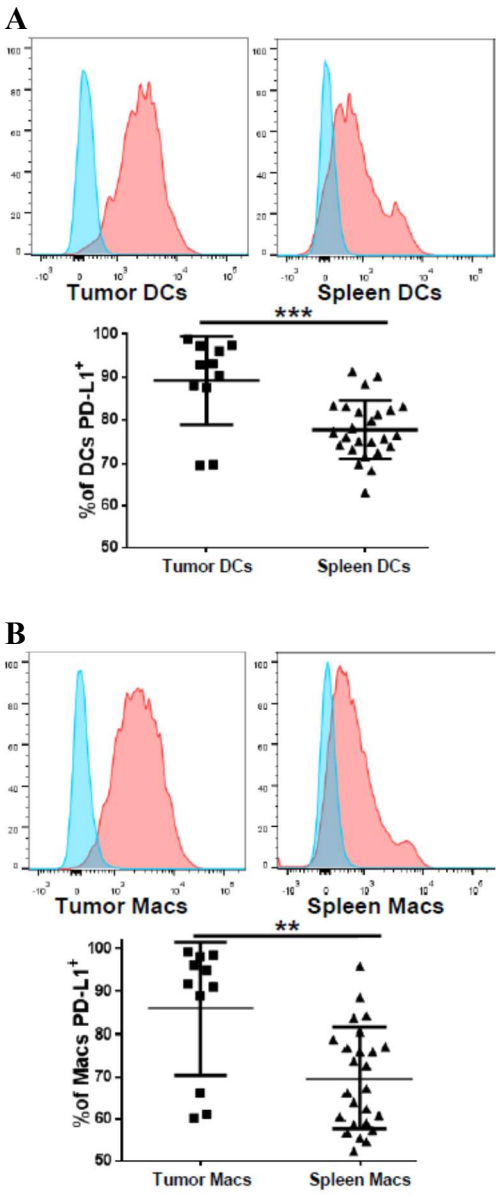


Figure 4. PD-L1 is highly expressed on dendritic cells and macrophages in pancreatic tumors of KPC mice. Representative histograms and quantification of PD-L1 expression on **(A)** dendritic cells (DCs; gated on live, $CD45^{+}$, $F4/80^{neg}$, $CD19^{neg}$, $CD11c^{+}$) and **(B)** macrophages (Macs; gated on live, $CD45^{+}$, $F4/80^{+}$) in tumors (n=11) or spleens (n=25) from tumor bearing KPC mice. **p≤ 0.01, ***p≤ 0.001.

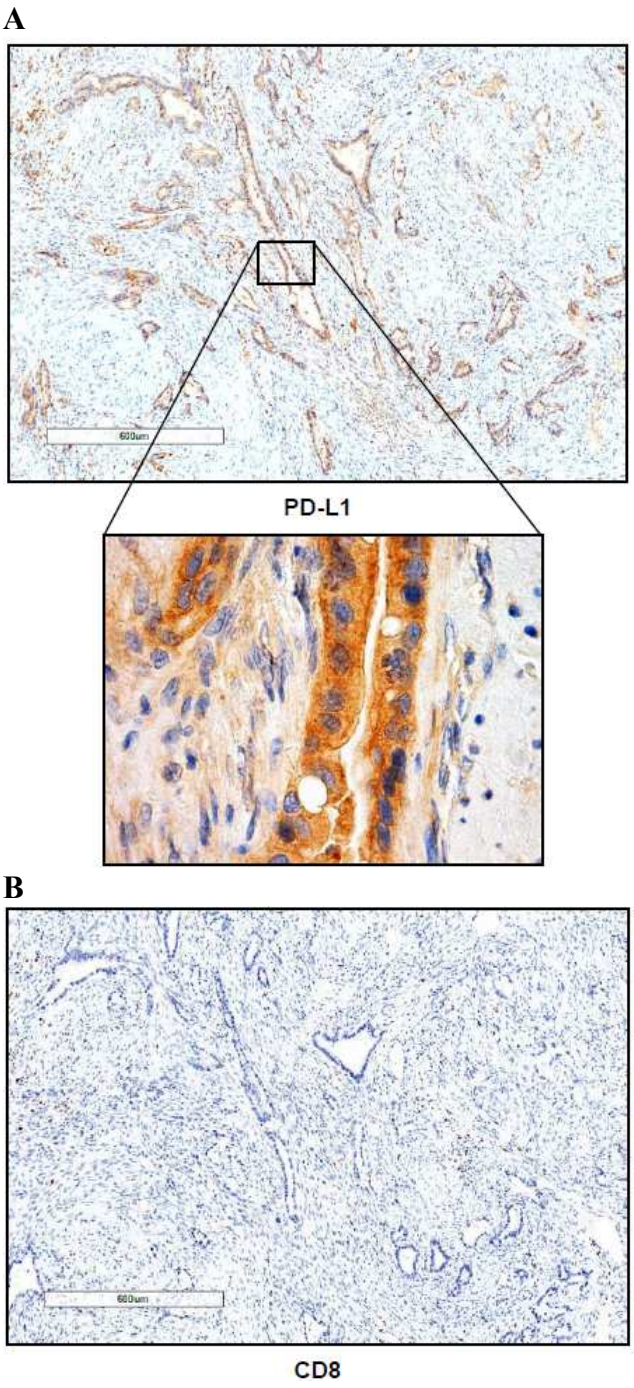


Figure 5. PD-L1 is expressed in human PDA; few CD8$^+$ T cells infiltrate human PDA. Histology of PD-L1 expression and CD8$^+$ T cell infiltration in human pancreatic cancer sections. **(A)** PD-L1 expression on malignant cells of a PDA tumor (PD-L1 expression score of 4+ (intense), see Materials and Methods; 40x and 400x magnification for top and bottom panels, respectively). **(B)** CD8 expression in serial section of the tumor in (A), demonstrating few tumor-infiltrating CD8$^+$ T cells (40x magnification).

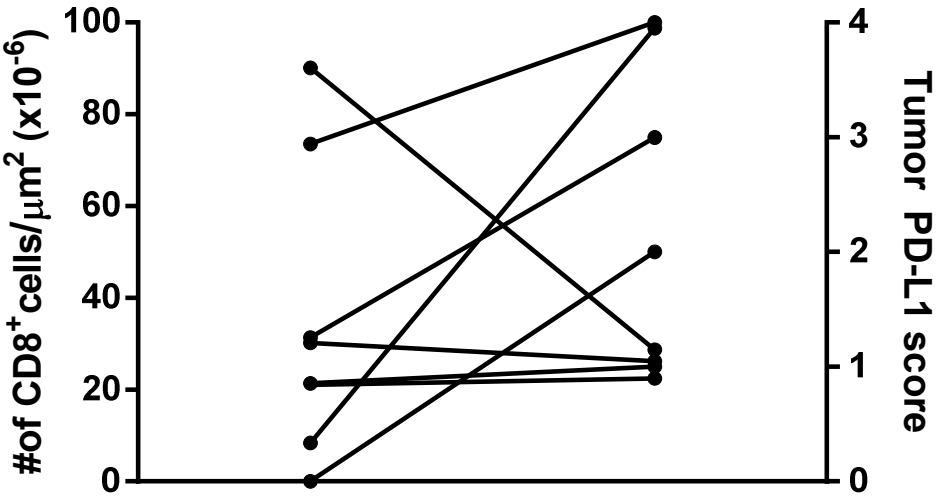


Figure 6. In human PDA there is no correlation between PD-L1 expression and CD8$^+$ T cell infiltration. Plot describing correlation between intratumoral CD8 count and tumor PD-L1 score (n=8). p=0.69.

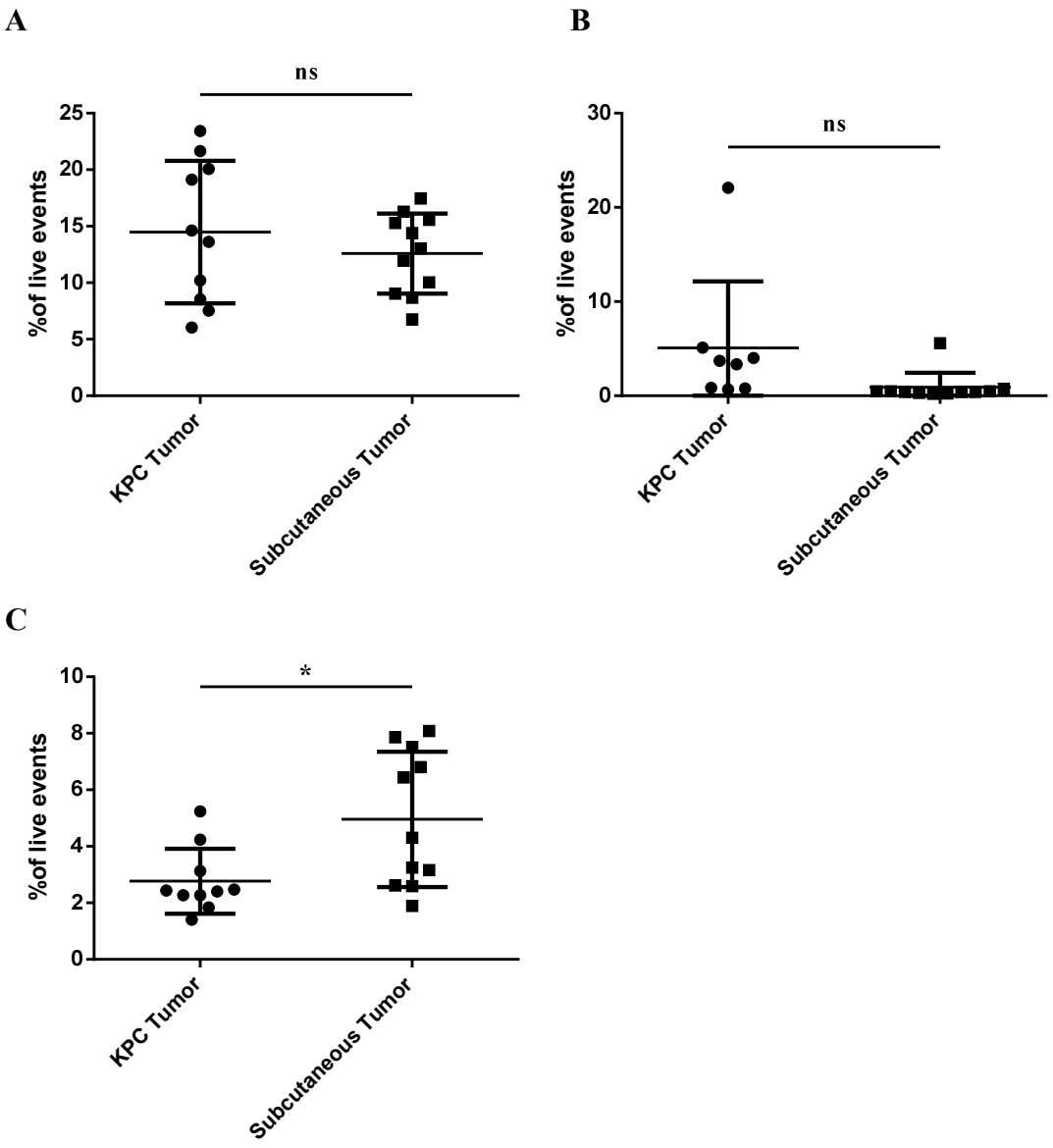


Figure 7. Subcutaneous PDA tumors recapitulate KPC tumor immune microenvironment. Pancreatic tumors of KPC mice and subcutaneously grown PDA tumors were evaluated by flow cytometry for the presence of **(A)** macrophages (gated as above), **(B)** B Cells (gated on live, $CD45^{+}$ $F4/80^{neg}$ $CD19^{+}$), and **(C)** DCs (gated as above). All populations are quantified as percentage of all live events. *$p \leq 0.05$.

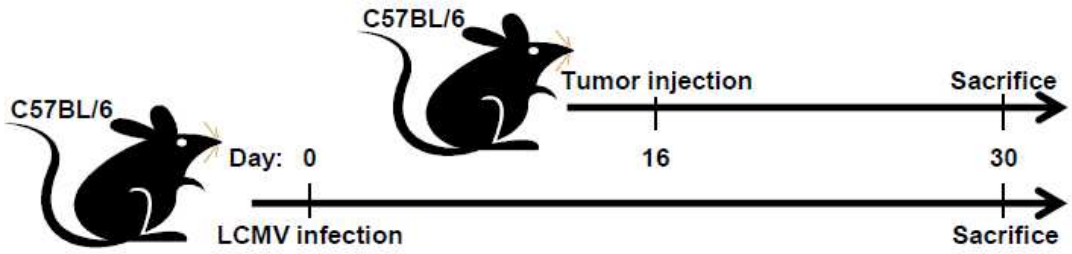


Figure 8. Experimental design for establishment of subcutaneous PDA tumors or chronic LCMV clone 13 infection simultaneously in 2 cohorts of C57BL/6 mice.

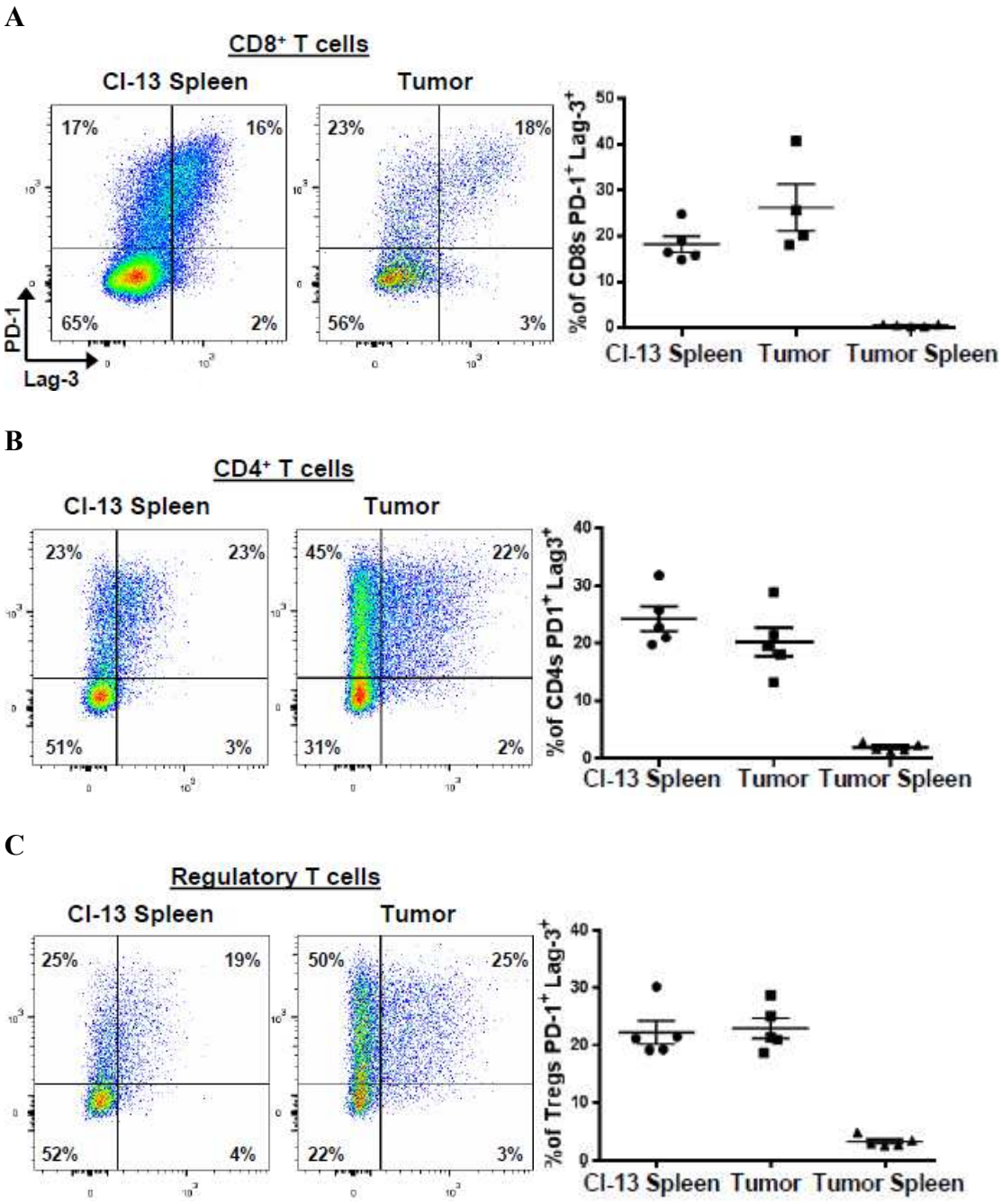


Figure 9. Co-expression of PD-1 and Lag-3 on T cell populations from mice with LCMV Clone 13 infection or subcutaneous PDA tumors. Representative flow plots and quantification of co-expression of PD-1 and Lag-3 on **(A)** $CD8^+$ (gated on live, lymphocytes, $B220^{neg}$, $NK1.1^{neg}$, $CD8^+$), **(B)** $CD4^+$ (gated on live, lymphocytes, $B220^{neg}$, $NK1.1^{neg}$, $CD4^+$) and **(C)** regulatory (Tregs; gated on live, lymphocytes, $B220^{neg}$, $NK1.1^{neg}$, $CD4^+$, $FoxP3^+$) T cells from spleens of mice infected with LCMV Clone 13 (Cl-13; day 30) or the tumors and spleens of mice bearing PDA tumors (day 14).

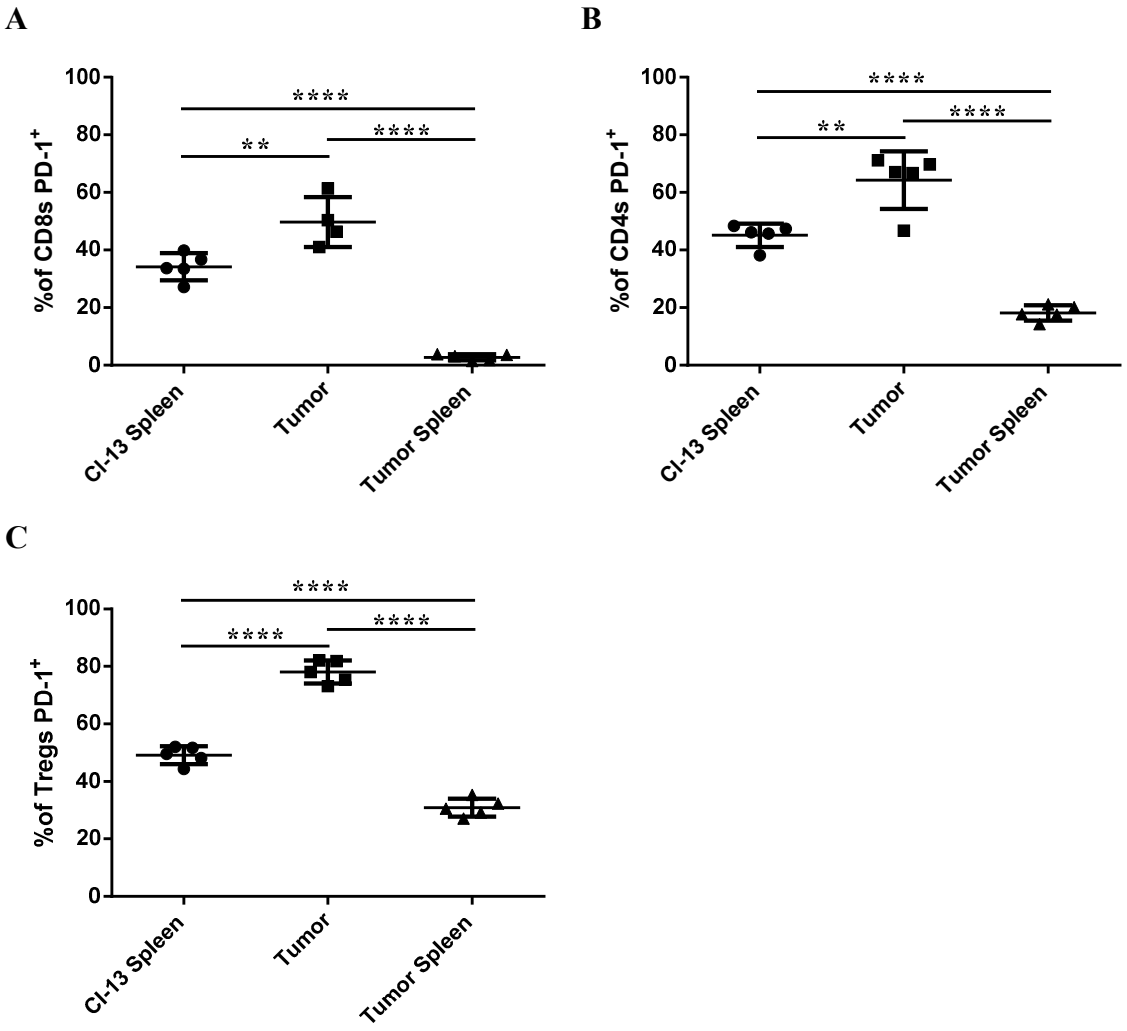


Figure 10. PD-1 expression is greater on tumor infiltrating T cells than corresponding populations in LCMV infected mice. Quantification of PD-1 expression on **(A)** $CD8^+$, **(B)** $CD4^+$, and **(C)** regulatory T cells (all gated as above) from spleens of mice infected with LCMV Cl-13 (day 30) or the tumors and spleens of mice bearing PDA tumors (day 14). One-way ANOVA: %of CD8s $PD\text{-}1^+$ **(A)**, $p \leq 0.0001$; %of CD4s $PD\text{-}1^+$ **(B)**, $p \leq 0.0001$; %of Tregs $PD\text{-}1^+$ **(C)**, $p \leq 0.0001$. Post hoc test p values are indicated where significant as $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$.

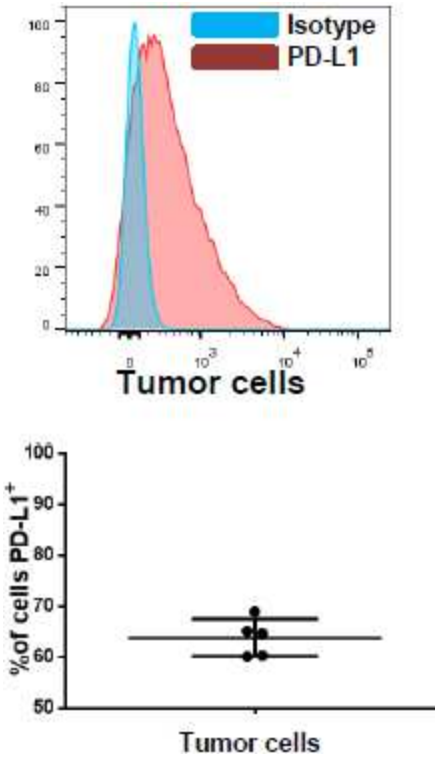


Figure 11. PD-L1 is moderately expressed *in vivo* on tumor cells in subcutaneous PDA tumors. Representative histograms and quantification of PD-L1 expression on tumor cells in subcutaneous PDA tumors (day 14), gated as in Figure 3.

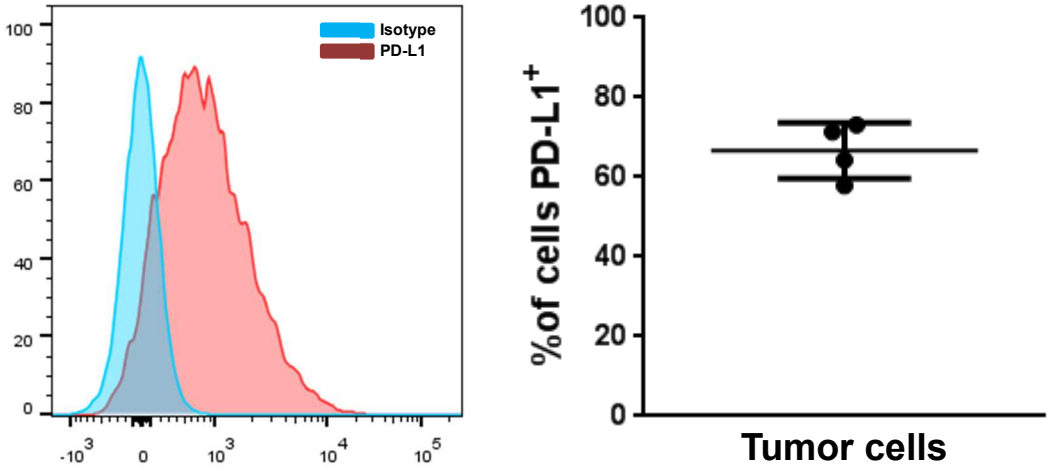


Figure 12. Moderate PD-L1 expression on lineage labeled PDA tumor cells *in vivo*. A PDA cell line generated from a backcrossed KPCY mouse (as described in Materials and Methods) was implanted in immune competent C57BL/6 mice (n=4) and mice were sacrificed on day 15 after implantation. Representative flow plot and quantification of PD-L1 expression on lineage labeled tumor cells is shown (gated on Live, $CD45^{neg}$ YFP^{+}).

A

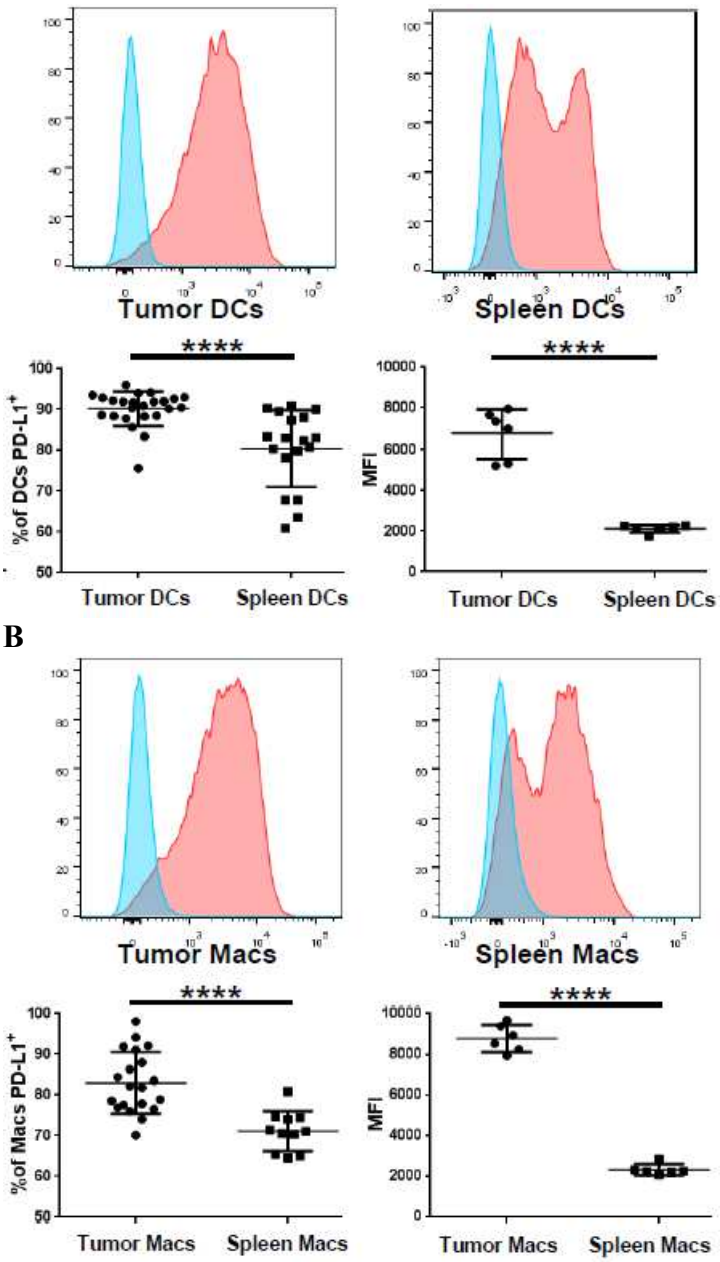
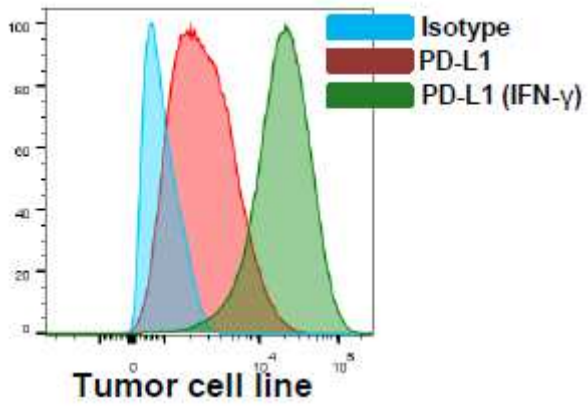


Figure 13. PD-L1 is highly expressed on dendritic cells and macrophages in subcutaneous PDA tumors. Representative histograms and quantification of PD-L1 expression on **(A)** dendritic cells (DCs) and **(B)** macrophages (Macs) in subcutaneous PDA tumors or spleens from the same mice (day 14), gated as above. (MFI=mean fluorescence intensity). ****p≤ 0.0001.

A



B

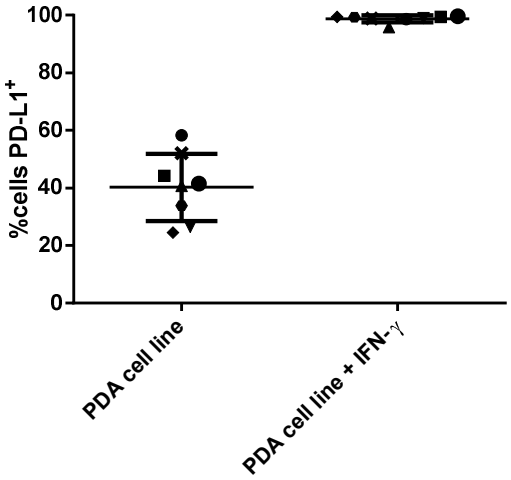


Figure 14. PD-L1 expression on PDA cell lines can be upregulated by IFN-γ *in vitro*. **(A)** Representative histogram of a KPC-derived PDA cell line interrogated for PD-L1 expression *in vitro* with or without IFN-γ in the culture, and **(B)** quantification of 8 distinct KPC-derived PDA cell lines interrogated for PD-L1 expression *in vitro* with or without IFN-γ.

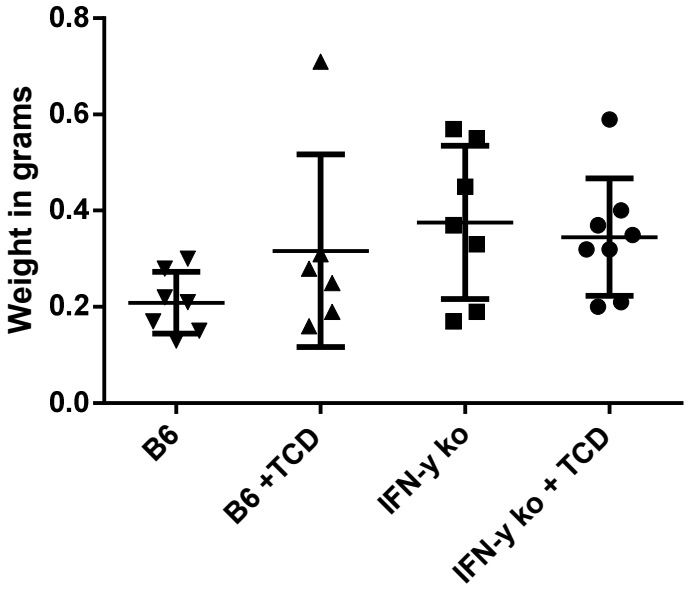


Figure 15. Tumor growth of subcutaneous PDA tumors is not significantly affected by host IFN-γ or T cell status. Tumor weights in grams of tumors grown in C57BL/6 (B6) or IFN-$\gamma^{-/-}$ (IFN-γ ko) mice with or without $CD4^+$ and $CD8^+$ T cell depletion (TCD) (day 16; n=6-8 mice per cohort).One-way ANOVA: p=0.486.

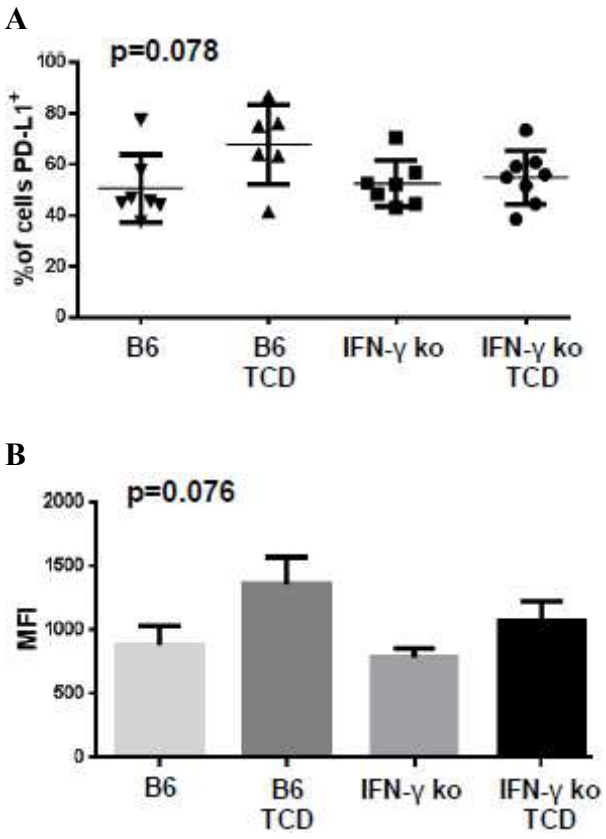


Figure 16. Tumor cell PD-L1 expression is not affected by IFN-γ or T cell status of host *in vivo*. (A) Quantification and **(B)** MFI of PD-L1 expression on tumor cells from subcutaneous PDA tumors established in either C57BL/6 (B6) or IFN-$\gamma^{-/-}$ (IFN-γ ko) mice with or without $CD4^+$ and $CD8^+$ T cell depletion (TCD) (day 16; n=6-8 mice per cohort). One-way ANOVA p values indicated.

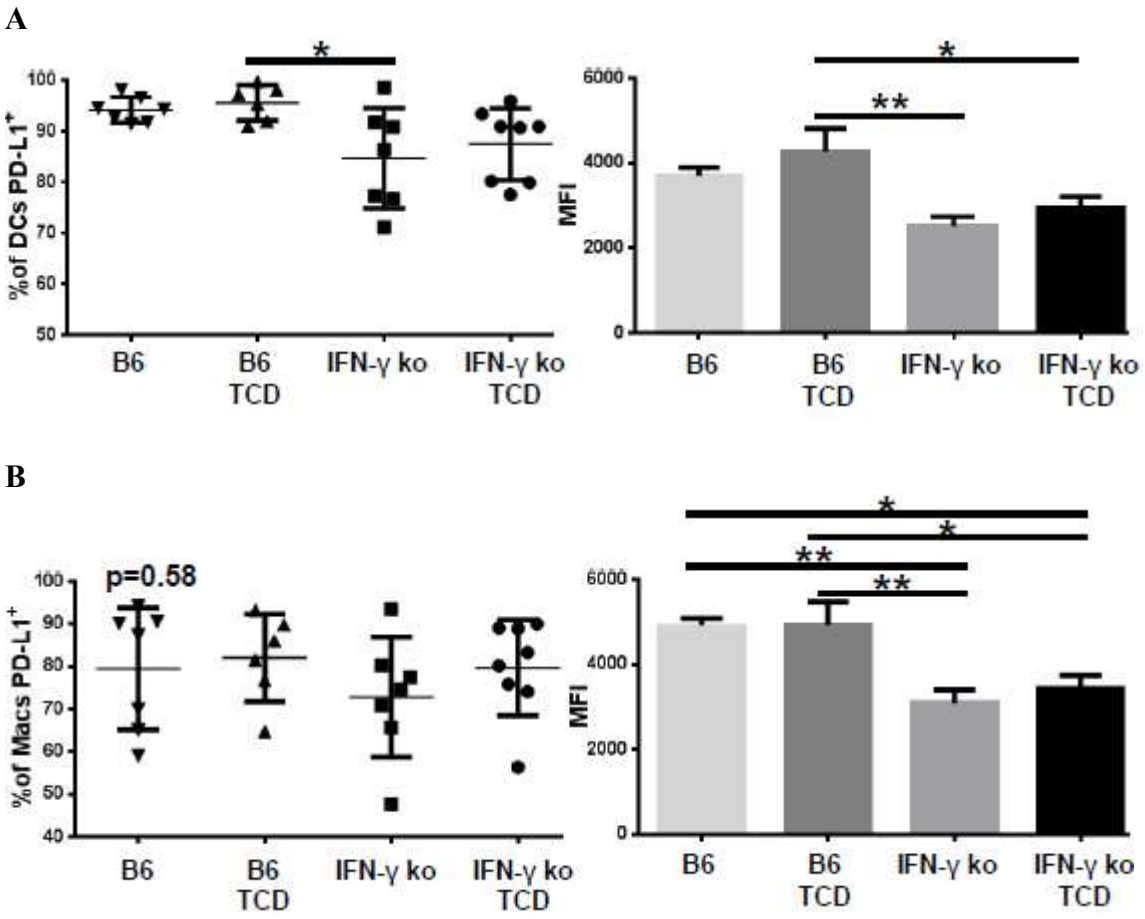


Figure 17. Host IFN-γ status has small effect on dendritic cell and macrophage PD-L1 expression in subcutaneous PDA tumors. Quantification and MFI of PD-L1 expression on **(A)** dendritic cells (DCs) and **(B)** macrophages (Macs) in subcutaneous PDA tumors grown in either B6 or IFN-γ ko mice with or without TCD (day 16; n=6-8 mice per cohort). One-way ANOVA: %DCs PD-L1$^+$ **(A)**, p=0.015; DC PD-L1 MFI **(B)**, p=0.0039; %Macs PD-L1$^+$ **(C)**, p=0.58; Macs PD-L1 MFI **(D)**, p=0.0007. Post hoc test p values are indicated where significant as *p≤ 0.05, **p≤ 0.01.

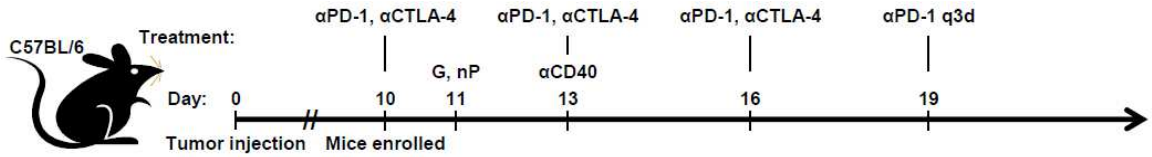


Figure 18. Experimental design for experiments of subcutaneous PDA tumors treated with checkpoint inhibitors and αCD40/chemotherapy. Further described in Materials and Methods. (G=gemcitabine; nP=nab-paclitaxel; q3d= antibody administered every 3 days).

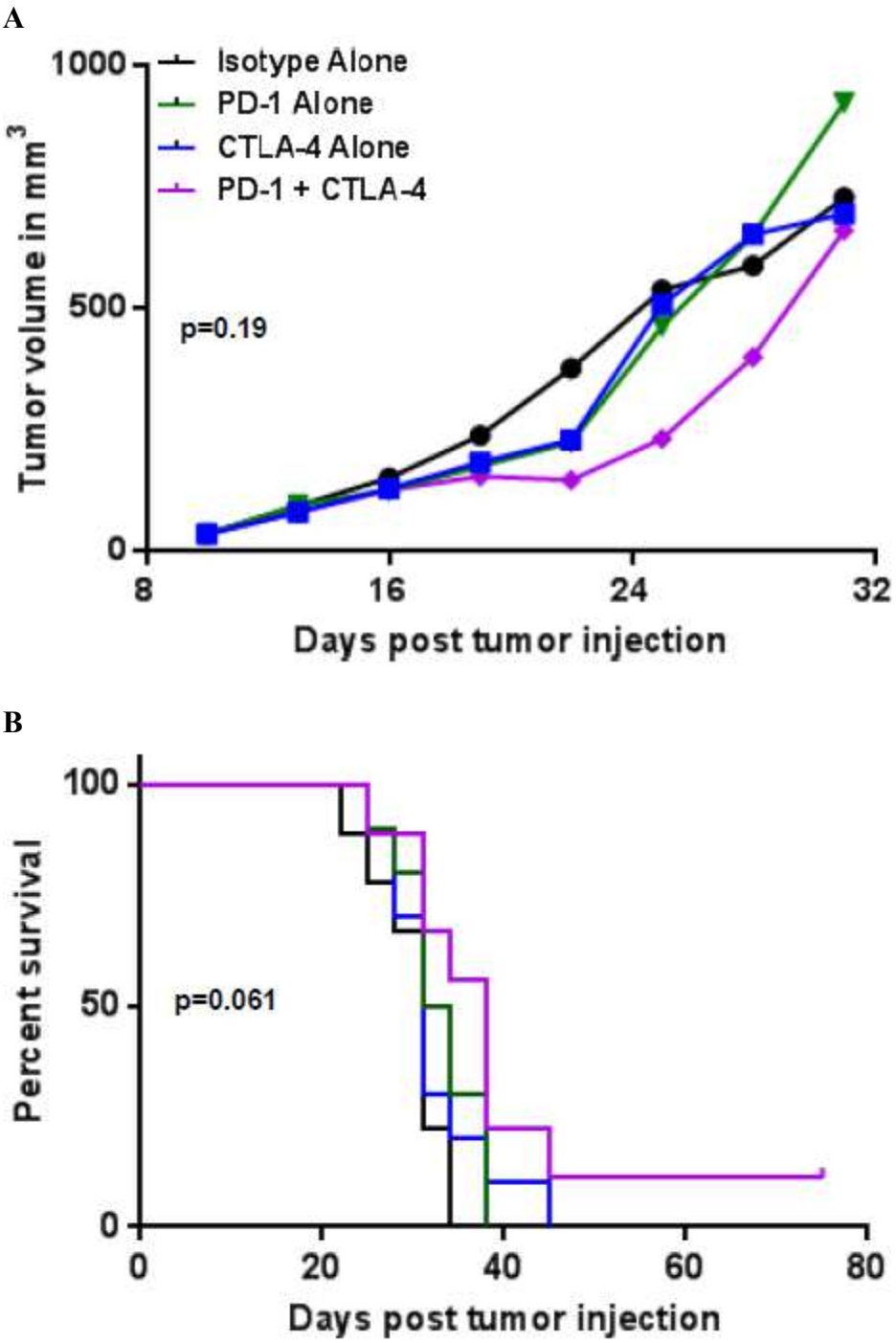


Figure 19. Checkpoint inhibitors alone do not inhibit tumor growth or improve survival in a subcutaneous PDA model. Tumor growth **(A)** and survival **(B)** analyses of mice bearing subcutaneous PDA tumors treated as indicated (n=9-10 per cohort; results for control and αPD-1+αCTLA-4 cohorts representative of 3 independent experiments). Two-way ANOVA **(A)** and log-rank **(B)** p values indicated. See also figure 23.

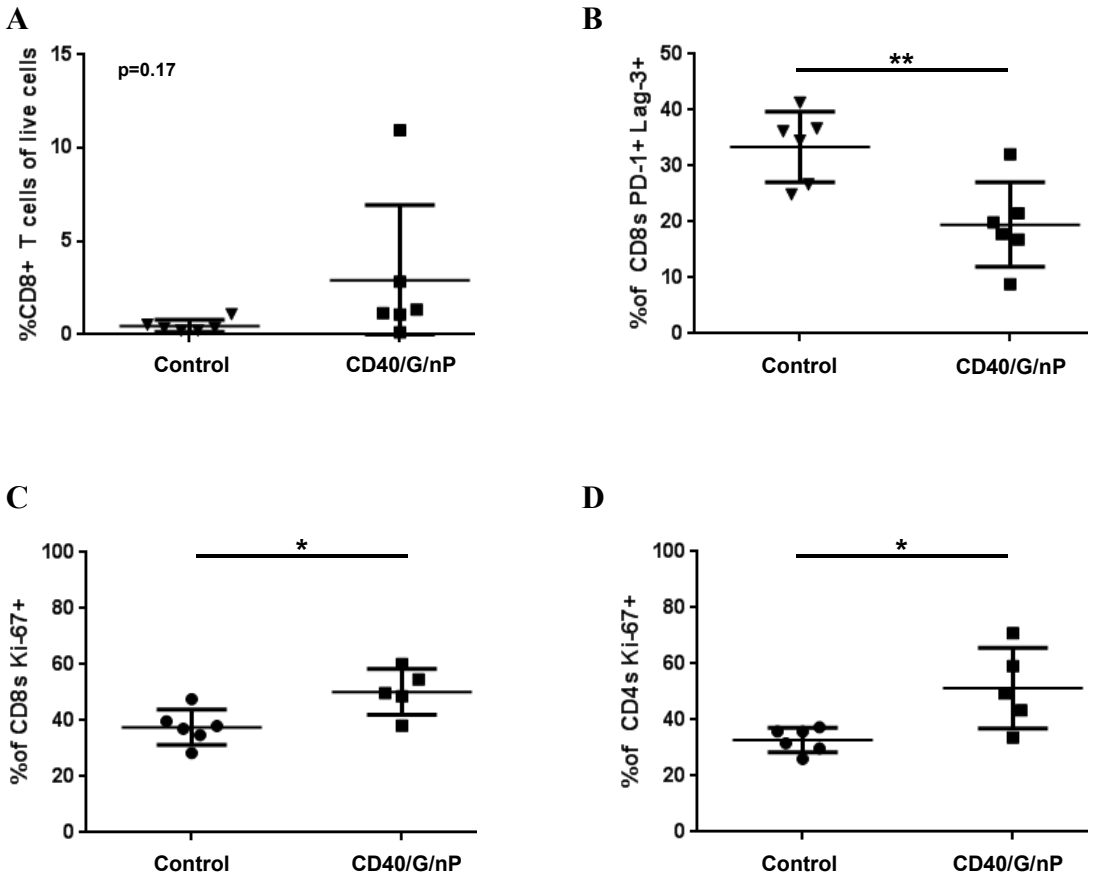


Figure 20. Agonist αCD40 and chemotherapy induces changes in tumor infiltrating T cells in subcutaneous PDA tumors. Flow cytometric analysis of mice bearing subcutaneous PDA tumors treated as indicated (day 7-8 after αCD40 treatment; G=gemcitabine; nP=nab-paclitaxel). **(A)** $CD8^+$ T cells make up an increased percent of cells in the tumor after treatment. **(B)** Fewer $CD8^+$ T cells co-express the exhaustion markers PD-1 and Lag-3 after αCD40/chemotherapy. More intratumoral $CD8^+$ **(C)** and $CD4^+$ **(D)** T cells are proliferating after αCD40/chemotherapy. *$p \leq 0.05$, **$p \leq 0.01$.

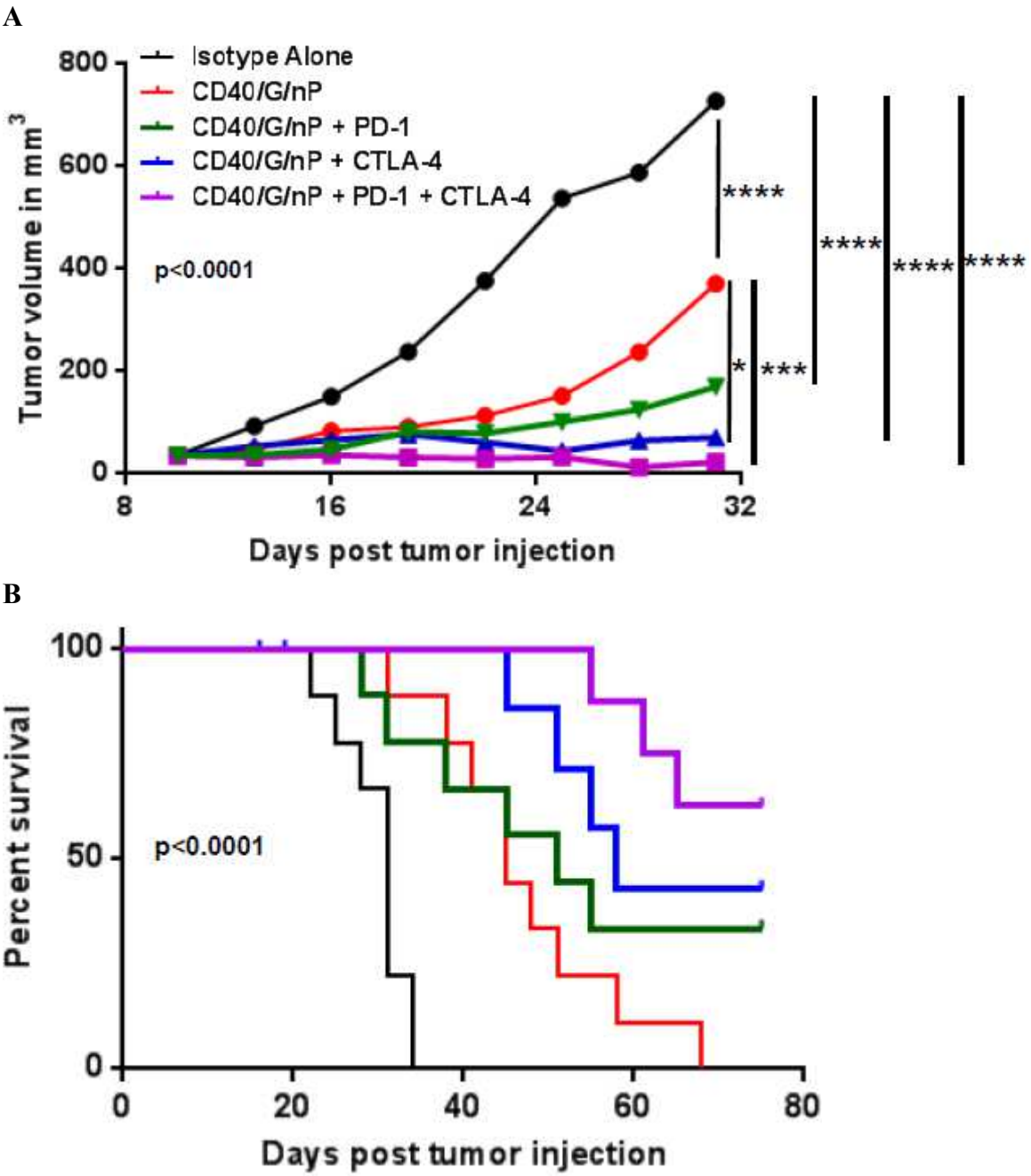


Figure 21. Vaccination with αCD40 and chemotherapy potentiates the efficacy of αPD-1 and αCTLA-4 mAbs. Tumor growth **(A)** and survival **(B)** analyses of mice bearing subcutaneous PDA tumors treated as indicated (n=9-10 per cohort; findings representative of 3 independent experiments). Two-way ANOVA **(A)**: p≤0.0001. Post hoc test p values indicated where significant as *p≤ 0.05, ***p≤ 0.001, ****p≤ 0.0001. Log-rank **(B)** p value indicated. See also figure 23.

Treatment Cohort	Long term survivors (%)
Isotype Alone	0/25 (0%)
PD-1 Alone	0/16 (0%)
CTLA-4 Alone	0/10 (0%)
PD-1+CTLA-4	1/19 (5%)
CD40/G/nP	3/25 (12%)
CD40/G/nP+PD-1	6/23 (26%)
CD40/G/nP+CTLA-4	7/22 (32%)
CD40/G/nP+PD-1+CTLA-4	17/44 (39%)

G:gemcitabine; nP:nab-paclitaxel

Figure 22. Therapeutic combination of αCD40/chemotherapy with one or more checkpoint inhibitors leads to rejection of significant proportion of tumors. Percentage of mice bearing subcutaneous PDA tumors treated with indicated regimens that rejected their tumors and survived tumor-free long-term (median follow-up of 42 days, range 23 to 146 days). Data compiled from 5 independent experiments.

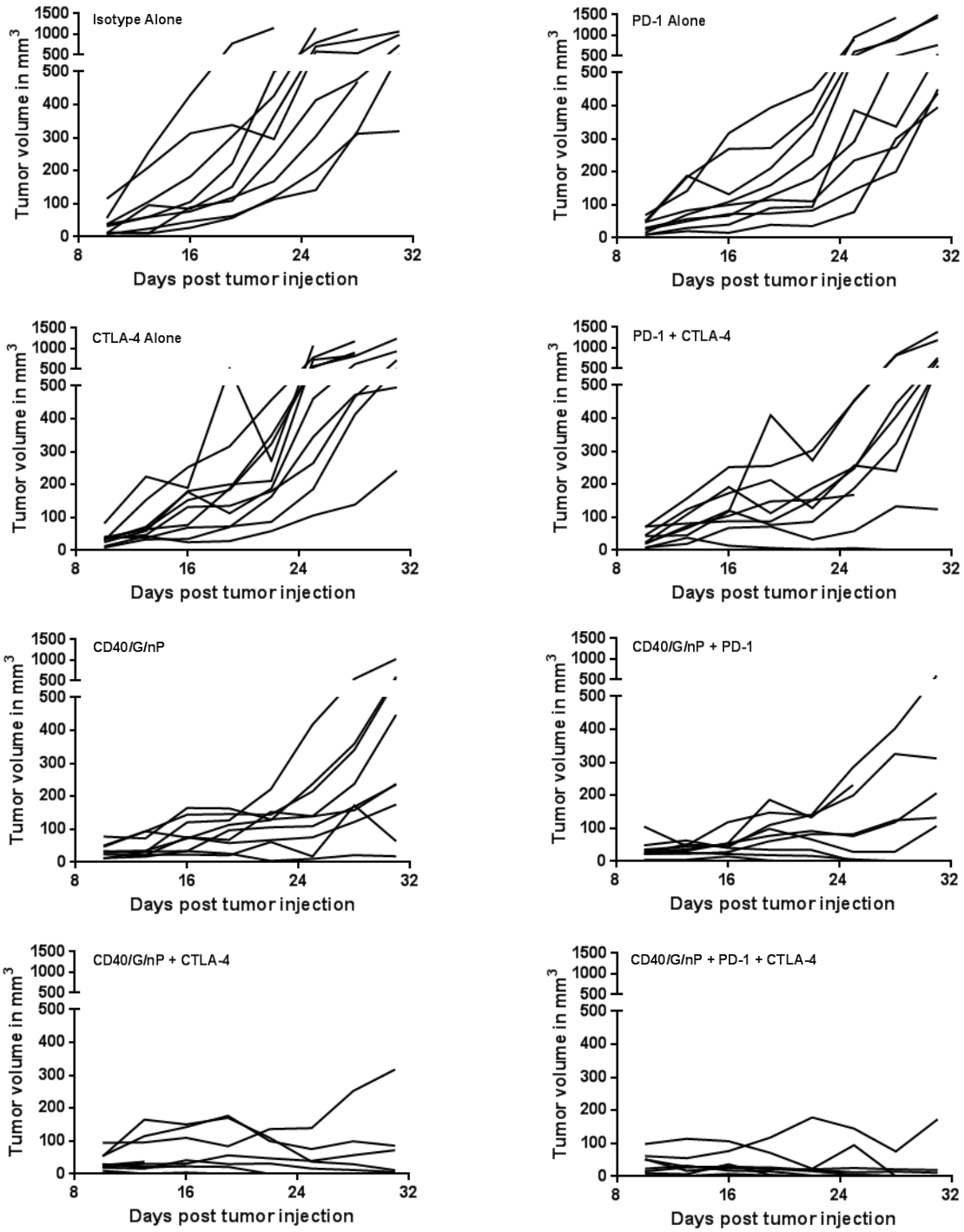


Figure 23. Tumor growth curves of individual mice with subcutaneous PDA treated as indicated. Mice bearing subcutaneous PDA tumors, treated as described in Figures 19 and 21 were assessed for tumor growth. Each graph represents all mice treated with indicated regimen; each line represents an individual mouse.

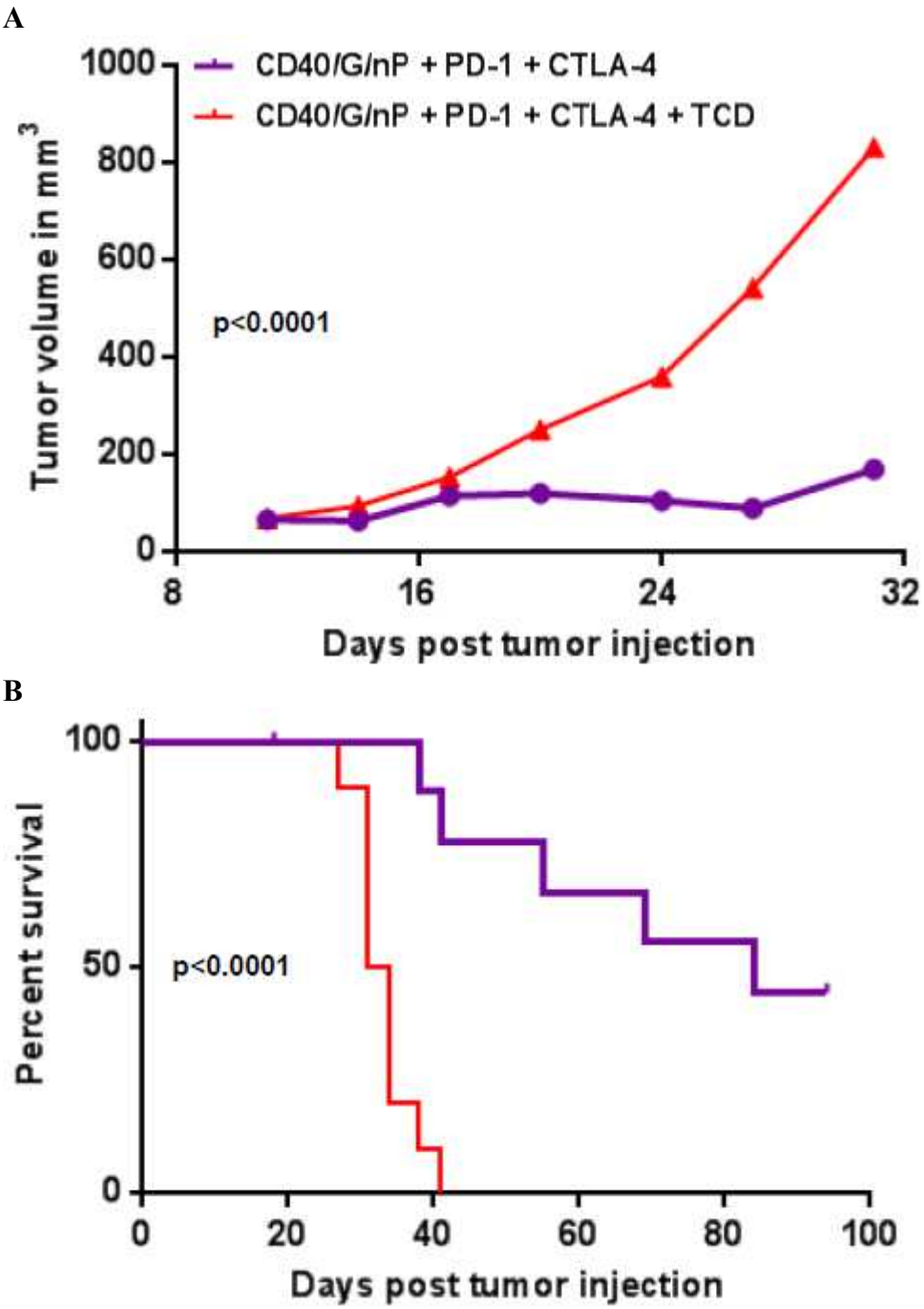


Figure 24. Tumor rejection and improved survival with vaccine and checkpoint inhibitor treatment is T cell dependent. Tumor growth **(A)** and survival **(B)** analyses of mice bearing subcutaneous PDA tumors treated as indicated (n=9-10 per cohort; G=gemcitabine; nP=nab-paclitaxel; TCD=CD4/CD8 depletion). Two-way ANOVA **(A)** and log-rank **(B)** p values indicated.

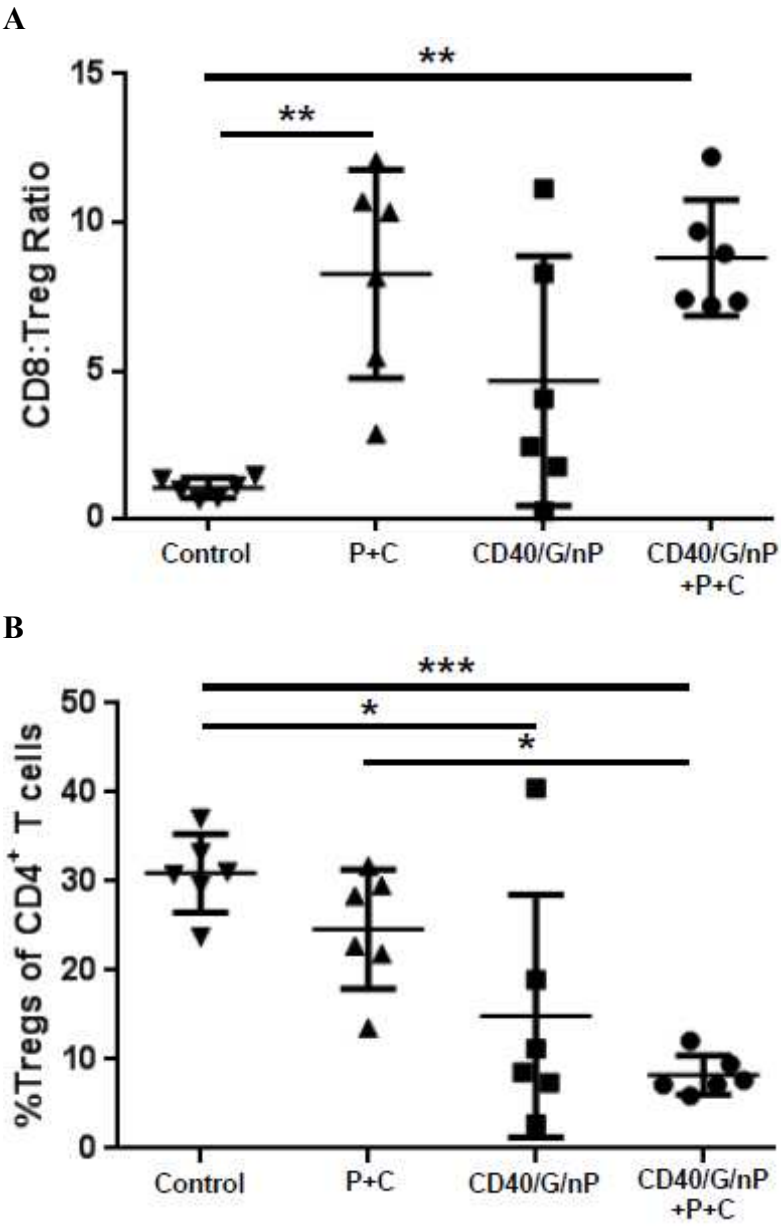
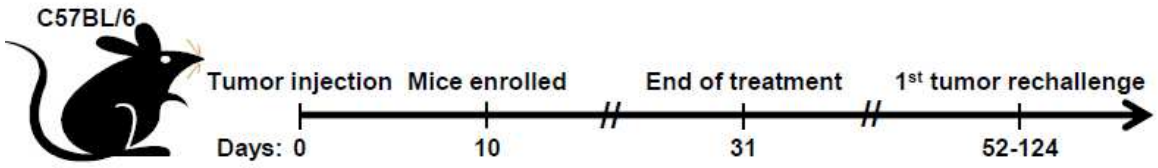


Figure 25. Immunotherapy improves CD8:Treg ratio and decreases intratumoral Treg percentage. Flow cytometric analysis of subcutaneous PDA tumors treated as indicated (day 18 after tumor injection, day 7 after αCD40 treatment; P=αPD-1; C=αCTLA-4). One way ANOVA: CD8:Treg Ratio **(A)**, p=0.0005; %Tregs of $CD4^+$ T cells **(B)**, p=0.0004. Post hoc test p values indicated where significant as *p≤ 0.05, **p≤ 0.01, ***p≤ 0.001.

A



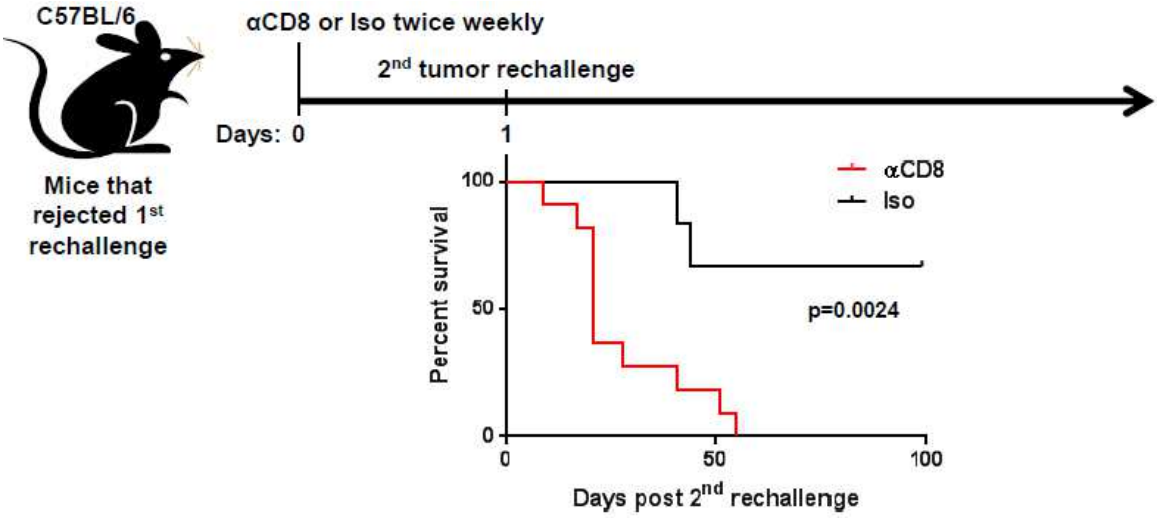
B

Treatment Cohort	Reject 1st rechallenge
PD-1+CTLA-4	0/1 (0%)
CD40/G/nP	2/3 (67%)
CD40/G/nP+P	5/6 (83%)
CD40/G/nP+C	6/7 (86%)
CD40/G/nP+P+C	6/9 (67%)

G:gemcitabine; nP:nab-paclitaxel;
P:αPD-1; C:αCTLA-4

Figure 26. Majority of mice that rejected first tumor after immunotherapy reject tumor rechallenge with no further treatment. (A) Experimental design for 1st tumor rechallenge experiments. **(B)** Table quantifies fraction and percentage of mice that rejected tumor rechallenge in mice that had rejected the initial tumor implantation and were tumor-free for at least 43 days. Data compiled from 3 independent experiments.

A

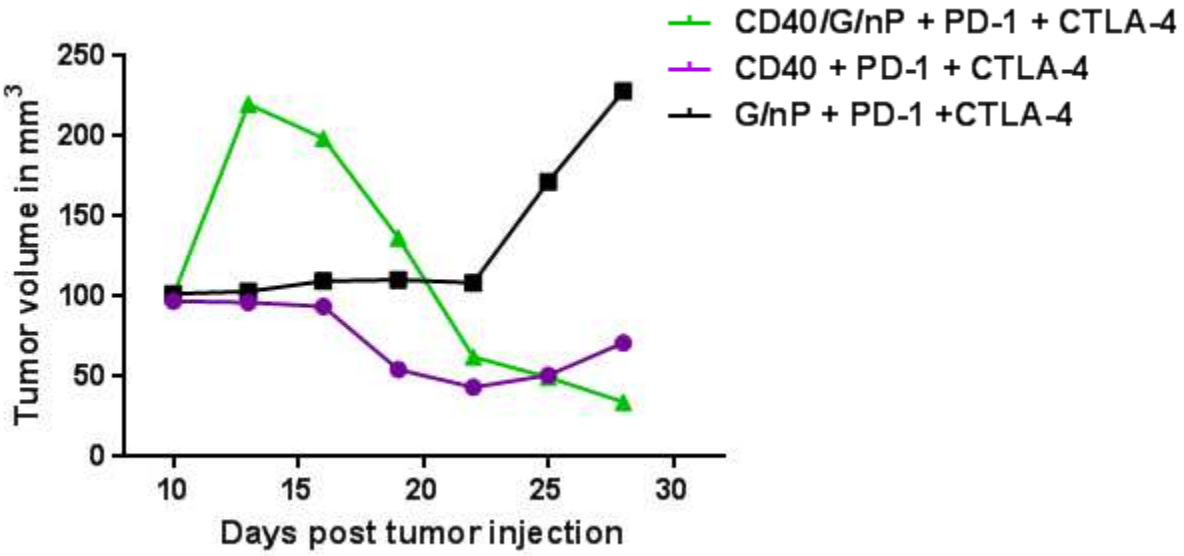


B

Treatment Cohort	Reject 2nd rechallenge	
	αCD8	Iso
PD-1+CTLA-4	ND	ND
CD40/G/nP	ND	1/2
CD40/G/nP+P	0/3	1/2
CD40/G/nP+C	0/3	2/2
CD40/G/nP+P+C	0/5	ND

Figure 27. The rejection of tumor rechallenges is CD8 T cell dependent. (A) Experimental design for 2nd tumor rechallenge experiment. The 2nd rechallenge occurred on day 31-49 after first rechallenge. **(B)** Table quantifies fraction and percentage of mice that rejected 2nd tumor rechallenge in mice that had rejected a 1st tumor rechallenge. Host mice in this experiment were either treated with αCD8 (n=11) or isotype (Iso; n=6) antibodies. Survival analysis of mice after 2nd rechallenge with or without CD8 depletion is shown **(A)**. Log-rank p value is indicated **(A)**. Data compiled from 2 independent experiments.

A



B

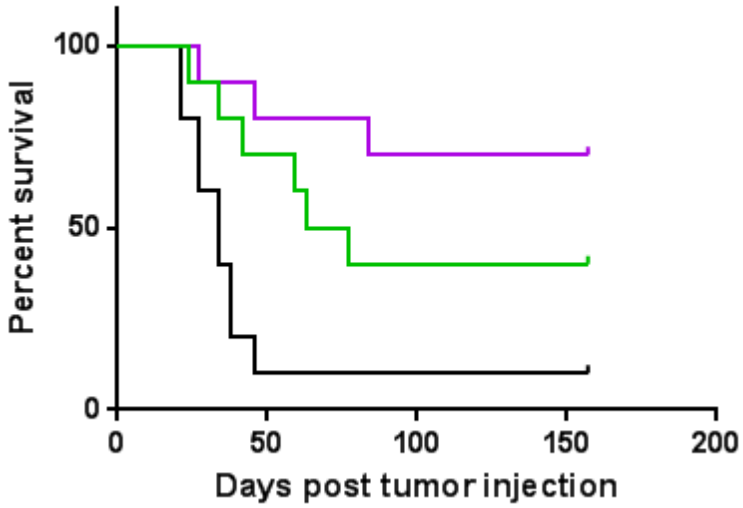
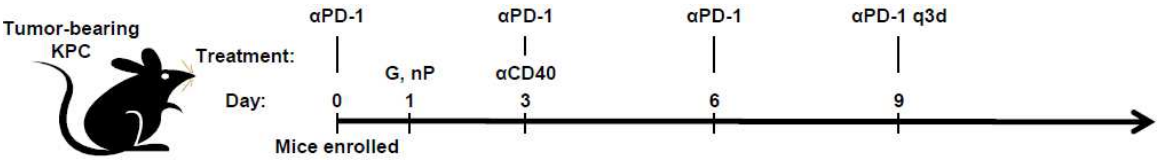
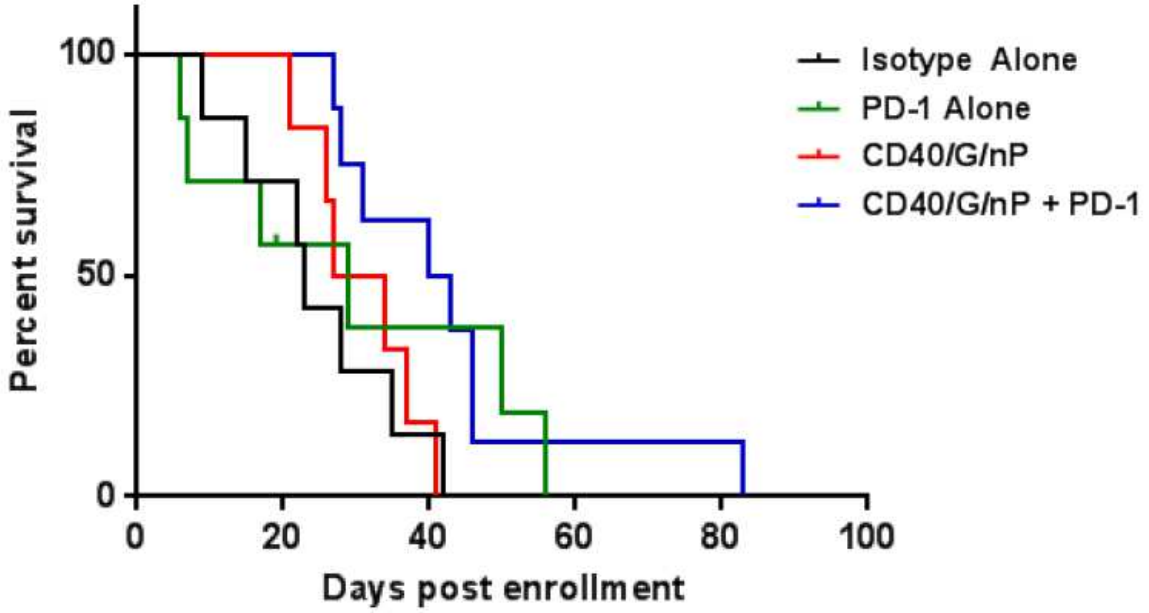


Figure 28. CD40 mAb but not chemotherapy alone potentiates tumor rejection by checkpoint inhibitors. Tumor growth **(A)** and survival **(B)** analyses of mice bearing subcutaneous PDA tumors treated as indicated (n=10 per cohort; G=gemcitabine; nP=nab-paclitaxel; same dose and schedule as Figure 18). Two-way ANOVA **(A)**: p=0.272; log-rank **(B)**: p<0.01.

A



B



C

Median Overall Survival			
Isotype Alone	PD-1 Alone	CD40/G/nP	CD40/G/nP + PD-1
23	29	30.5	41.5

Figure 29. The combination of αCD40/chemotherapy and checkpoint blockade improves survival in the KPC model of PDA. (A) Experimental design for randomized, controlled study in tumor-bearing KPC mice, treated with αCD40/chemotherapy and αPD-1, as described in Materials and Methods. (G=gemcitabine; nP=nab-paclitaxel; q3d= antibody administered every 3 days) (**B**) Overall survival analysis of tumor-bearing KPC mice treated as indicated (n=6-8 per cohort). αPD-1 alone vs. isotype alone p=0.39; CD40/G/nP vs. isotype alone p=0.76; CD40/G/nP + αPD-1 vs. isotype alone p=0.015. (**C**) Median overall survival of tumor-bearing KPC mice treated as indicated.

Treatment Cohort	Days post CD40	Clinical Signs	Pathology
CD40+G+nP + CTLA-4	**3**	**Found dead**	
CD40+G+nP + CTLA-4	**3**	**Found dead**	
CD40+G+nP + CTLA-4	**3**	**Generally depressed and hunched; 1 day later developed vestibular signs (rolling, unable to stand); died 2 days later**	**Intralesional bacterial cocci seen. Bilateral suppurative otitis externa and media, mild focally extensive suppurative encephalitis and meningitis, and severe multifocal to focally extensive necrosuppurative sialoadenitis, cellulitis and myositis.**
CD40+G+nP + CTLA-4	**4**	**Found dead**	
CD40+G+nP + PD-1	**4**	**Generally depressed, vestibular signs; died 1 day later**	
CD40+G+nP + PD-1	**4**	**Generally depressed, vestibular signs; euthanized 3 days later**	**Pseudomonas aeruginosa grown from inner ear. Marked, bilateral inflammatory exudate filling the external and middle canals and the nasolacrimal duct unilaterally. Unilaterally, at the base of the ear, there is focal necrotizing vasculitis with abundant neutrophils and fibrosis. The meninges are extensively and moderately expanded by neutrophils. Unilaterally the bulbar conjunctiva is infiltrated by abundant neutrophils.**
CD40+G+nP + PD-1	**7**	**Mild vestibular signs; recovered and survived long term**	
CD40+G+nP + PD-1 + CTLA-4	**3**	**Generally depressed; died 3 days later**	

Figure 30. Non-tumor related events and deaths in tumor bearing C57BL/6 mice after immunotherapy.C57BL/6 mice bearing subcutaneous tumors were treated as indicated. Mice bearing small tumors were occasionally found dead or ill; the presentation included clinical depression, hunching, poor appetite, and vestibular signs characterized by abnormalities of gait or posture and rolling behavior. For two moribund mice in which an extensive pathological characterization was performed, we diagnosed neutrophilic inflammation in the central nervous system (CNS). Evidence for bacterial infection was noted in both mice (*Pseudomonas aeruginosa* in one mouse; bacterial cocci, not otherwise specified, in the other).

CHAPTER 4 – T cell infiltration into spontaneous pancreatic tumors after induction of antitumor T cell response outside pancreatic microenvironment

*The majority of the results described in this chapter are in a manuscript currently undergoing revisions at Gastroenterology: Beatty, G.L., Winograd, R., Evans, R.A., Long, K.B., Luque, S.L., Lee, J.W., Gladney, W.L., Guirnalda, P.D., and Vonderheide, R.H. Productive T cell immunity against pancreatic carcinoma in mice is regulated by $Ly6C^{low}$ $F4/80^{+}$ extratumoral macrophages

INTRODUCTION

In order to understand the mechanism of resistance of PDA to checkpoint inhibitor therapy I studied two mouse models, the genetically engineered KPC spontaneous tumor model, and subcutaneously implanted KPC derived PDA cell lines. Phenotypically, both models exhibited comparable high expression of the inhibitory PD-1 and PD-L1 axis on tumor associated T cells and APCs, respectively. Likewise, both models recapitulated the clinical resistance to single agent checkpoint inhibitor therapy seen to date in treated PDA patients (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). And while in both models the addition of a vaccine to induce an antitumor T cell response potentiated the efficacy of checkpoint inhibitors, the types of responses observed in the two models were markedly different. In the subcutaneous PDA model large proportions of tumor bearing mice treated with vaccine and checkpoint inhibitors completely rejected their tumors and developed immune memory strong enough to reject subsequent tumor rechallenges. However, in the KPC model, while the combination of

αCD40/chemotherapy and αPD-1 was able to significantly improve overall survival, I observed no long term survivors or rejection of tumors.

The KPC model (as well as related Kras $p16^{Ink4A}$ model), has been widely adopted as the new murine standard for studying pancreatic cancer as it recapitulates the salient molecular, histopathologic, and clinical features of the human disease. As a preclinical model, the KPC GEMM has proved effective, as several agents shown to have antitumor efficacy in KPC mice have been tested in patients to similar results (Beatty et al., 2011; 2013; Provenzano et al., 2012). It is worth noting, however, that despite numerous studies of therapeutic interventions there is no published report of complete tumor rejection in a KPC tumor bearing mouse. The difference in the responses I observed between the KPC and subcutaneous models could be due to differences between the host mice, or to immunologic differences between the tumors that arise stochastically in the pancreas and the bolus of malignant cells implanted under the skin.

In order to determine whether the difference in responses is due to the host, I worked with Dr. Beatty, at the time a postdoctoral fellow in the lab of Dr. Vonderheide, who established a two tumor model by implanting a PDA cell line under the skin of KPC mice bearing pancreatic tumors. By treating these two tumor mice we could determine whether KPC resistance to immune therapy is due to systemic host immune deficiencies or instead due to local factors that abrogate responses that are successful against PDA tumors when located subcutaneously.

RESULTS

αCD40 and chemotherapy can reject subcutaneous PDA tumors in a T cell dependent manner

In a previously published study using the KPC model Dr. Vonderheide demonstrated that the combination of αCD40 and gemcitabine regressed tumors, but did so in a T cell independent manner (Beatty et al., 2011). No T cell response was seen in histologic analyses of these KPC tumors, and the regressions were shown to depend on the reprogramming of TAMs. The combination of αCD40 and gemcitabine as a vaccine has previously been reported, and in Chapter 3 I used the combination of gemcitabine, nab-paclitaxel, and αCD40 above to induce T cell responses to subcutaneous tumors (Nowak et al., 2003). To determine whether this lack of a T cell response was specific to the KPC mice Dr. Beatty implanted a KPC derived cell line onto the flanks of normal littermates. In these studies we used littermate PC mice as hosts (as described in Materials and Methods). The previously published αCD40/gemcitabine KPC studies were done in KPC mice *not* backcrossed onto the C57BL/6 background (Beatty et al., 2011). In order to compare the results in the subcutaneous tumor studies described here to those previously published, we chose to adhere to mice with this mixed genetic background for these studies. We used a PDA cell line derived from a non-backcrossed KPC mouse and implanted the tumors onto KPC littermates of the same mixed background. Mice with subcutaneous PDA tumors were treated and monitored for tumor growth. Tumor regression was observed in a large proportion of mice treated with the combination of αCD40 and gemcitabine, however, depletion of $CD4^+$ or $CD8^+$ T cells abolished the this treatment effect (Figure 31). Histologic analysis of tumors from treated mice indicated

that a robust T cell infiltrate followed treatment, a finding distinct from what was found in the tumors of KPC mice treated the same way (Figure 32) (Beatty et al., 2011). These data indicate that αCD40 /gemcitabine induces a T cell dependent regression of subcutaneous PDA tumors, while this same treatment regimen does not induce a T cell response in KPC tumor bearing mice (Beatty et al., 2011).

Pancreatic tumor bearing KPC mice are capable of mounting a T cell response against subcutaneous PDA tumors

The differences between the immune effects of vaccination using chemotherapy and αCD40 in the two PDA models could be due to systemic immunological differences between KPC mice and control mice with implanted tumors. In order to determine whether KPC mice are systemically incapable of mounting a T cell response against a PDA tumor, we implanted the same PDA cell line subcutaneously in KPC mice bearing ultrasound diagnosed pancreatic tumors (Figure 33). After 12 days of growth, the KPC mice bearing two tumors were treated with the same dose and schedule of gemcitabine and αCD40 and monitored for tumor growth (Figure 33). In the absence of treatment implanted PDA tumor cell lines grew progressively in the KPC mice (Figure 34). Upon treatment with αCD40/gemcitabine, however, the subcutaneous tumors in KPC mice regressed (Figure 34). Histological analysis of these subcutaneous tumors revealed a strong T cell infiltrate in treated tumors (Figure 35A). The regressing tumors were marked by a statistically significant increase in both $CD4^+$ and $CD8^+$ T cells, while Tregs were unchanged (Figure 35B). This finding demonstrates that pancreatic tumor bearing

KPC mice are not systemically immunosuppressed, as they are capable of mounting an antitumor T cell response when the tumor is outside the pancreatic microenvironment.

Explanted pancreatic KPC tumors remain susceptible to antitumor T cells induced by αCD40/gemcitabine

Various cellular populations in the KPC tumor microenvironment have been reported to inhibit antitumor T cell responses, including fibroblasts, macrophages, and MDSCs (Bayne et al., 2012; Feig et al., 2013; Zhu et al., 2014). I have previously shown that the microenvironment of subcutaneous KPC derived PDA tumors resembles autochthonous KPC pancreatic tumors in PD-L1 expression and in the infiltration of suppressive leukocytes such as TAMs (Figures 3,4,7,11-13) (Bayne et al., 2012). It is nevertheless possible, and even likely, that certain facets of the KPC tumor microenvironment are not completely recapitulated in the cell line derived subcutaneous tumors. In order to test this hypothesis, spontaneous tumors explanted from KPC mice were re-implanted subcutaneously onto the flanks of other KPC mice harboring ultrasound diagnosed pancreatic tumors (Figure 36). Explanted tumors grew readily under the skin of KPC mice; mice with two tumors were treated 13 days after implantation with αCD40/gemcitabine (Figure 36). Half of treated mice demonstrated regression of the explanted tumors, and treated mice demonstrated a greater influx of both $CD4^+$ and $CD8^+$ T cells compared to untreated controls, indicating that T cells can traffic into the desmoplastic stroma of pancreatic tumors if effectively activated (Figure 37).

Given the strong T cell infiltration into the stromal explanted tumors, I examined the primary pancreatic tumors of these KPC mice bearing two tumors. Histologic

analyses of primary pancreatic tumors determined that "two tumor" mice treated with αCD40/gemcitabine had increased infiltration of T cells compared to untreated "two tumor" controls (Figure 38). Unlike the explanted tumors, the primary tumors showed a significant increase in the influx of $CD4^+$ T cells while $CD8^+$ T cells were not significantly increased in these tumors (Figure 38). This robust T cell infiltrate in primary pancreatic tumors was notable, and suggests that priming T cell responses outside the pancreatic TME may allow for the induction of antitumor T cells that can traffic into these tumors. These data indicate that the desmoplastic stroma of PDA is not the only barrier to T cell infiltration. Furthermore, the pancreatic location of the tumors seems to play a role in preventing an influx of T cells as the explanted tumors saw an increase in the $CD8^+$ T cell infiltrate while the primary tumors did not.

APCs in KPC peripancreatic lymph nodes express higher levels of PD-L1 than the same populations in inguinal lymph nodes draining subcutaneous PDA tumors

One immunologic distinction between tumors in the pancreas and under the skin is lymphatic drainage. KPC tumors arising in the pancreas are drained by peripancreatic lymph nodes (PPLN) which are often enlarged compared to normal during tumor outgrowth. As pancreatic tumors develop they often engulf the PPLNs; PPLNs are increasingly difficult to microdisect from the tumors as they grow, and this process is observable by histology (Figure 39A). Tumors implanted subcutaneously on the flanks of mice are drained by the inguinal lymph node which can be visibly enlarged during tumor growth and especially after immunotherapy. The draining lymph nodes (DLN) of implanted tumors, however, remain spatially separated from the sucbcutaneous tumors

throughout tumor development (Figure 39B). This anatomic reality dictates that any tumor secreted factors, or factors produced by immune or mesenchymal populations in the tumor microenvironment, can directly affect the immune populations in PPLNs whereas that would be less likely (although still possible) for the spatially separate DLN of subcutaneous PDA tumors. This may provide an explanation for the fact that αCD40/chemotherapy can induce a T cell response against subcutaneous tumors but not primary pancreatic lesions.

When I analyzed the immune populations of these lymph nodes the most striking distinction was in the expression of PD-L1 on the APC populations. While the DCs and macrophages of the inguinal lymph nodes draining subcutaneous tumors exhibited a bimodal PD-L1 expression, as is seen in normal lymphoid tissue, APCs in PPLNs uniformly expressed high PD-L1, just as these same populations do in KPC and subcutaneous tumors (Figures 40, 41, 4, and 13). There was a significant difference in the percent of PD-L1 positive DCs (Figure 40) and macrophages (Figure 41) between PPLNs and inguinal LNs draining subcutaneous tumors, suggesting that APCs in PPLNs may be less able to prime antitumor T cell responses.

CONCLUSIONS AND DISCUSSION

Evasion of the immune system is necessary for malignant cells to develop into clinically apparent tumors. This evasion can be mediated through tumor cell intrinsic or extrinsic mechanisms. The process of immunoediting is the tumor cell intrinsic loss of antigenicity (through loss of the antigen or the ability to present it on MHC class I) or the loss of immunogenicity by the acquisition of immunosuppressive properties such as

overexpression of inhibitor receptors such as PD-L1 (Schreiber et al., 2011). Tumor cell extrinsic mechanisms have increasingly become appreciated as regulators of immune evasion; the recruitment of immunosuppressive leukocytic and mesenchymal populations to the tumor microenvironment has been shown to orchestrate networks of interacting cellular populations acting to both promote tumorigenesis through proangiogenic and tissue remodeling properties as well as locally suppress antitumor immune responses (Gabrilovich et al., 2012). The end result of these processes can be the establishment of localized 'immune privileged' sites in the tumor microenvironment, mechanistically similar to the intrinsic immune privilege of certain anatomic sites (Mellor and Munn, 2008). In PDA there is ample evidence that the desmoplastic stromal reaction acts to suppress immune responses; TAMs, MDSCs, Tregs, and fibroblasts have been shown to inhibit antitumor T cell responses in studies of murine and human PDA (Bayne et al., 2012; Beatty et al., 2011; Clark et al., 2007; Feig et al., 2013; Hiraoka et al., 2006). In the studies reported here I use two PDA models, the KPC GEMM and a subcutaneous PDA cell line model, to interrogate the role of the tumor microenvironment as a barrier to T cell immunotherapy. I show that αCD40 and chemotherapy, used as an antitumor vaccine, can induce a T cell response against subcutaneous PDA tumors, while the same treatment does not drive a T cell response against spontaneous KPC tumors, as Dr. Vonderheide has previously published (Beatty et al., 2011). This phenotype is not due to systemic immune suppression in KPC mice; T cell responses against subcutaneous PDA tumors in KPC mice bearing pancreatic tumors were achieved. Furthermore, when explanted from the pancreas and implanted subcutaneously, desmoplastic KPC tumors are likewise susceptible to T cell infiltration upon vaccination. A striking difference in

the PD-L1 expression in the lymph nodes draining pancreatic and subcutaneous tumors may explain the distinct effects of vaccination in these two models. Lastly, induction of antitumor T cells against a subcutaneous lesion leads to a T cell infiltration into primary pancreatic tumors in KPC mice, suggesting the possibility that T cell immunotherapy may yet be achievable for patients with PDA.

There is little evidence that PDA tumor cells undergo immunoediting in the classical sense. The presence of immunosuppressive MDSCs and Tregs at the PanIN stages suggests that these cells do not encounter the selective pressure of effector T cells (which are in fact rare, even in preneopalstic lesions) and hence are not required to lose antigenicity to grow out (Clark et al., 2007; Hiraoka et al., 2006). Analysis of 8 distinct early passage KPC derived PDA cell lines also indicates that while MHC class I is not highly expressed on these cells at baseline, it is readily upregulated in the presence of IFN-γ, indicating that the genetic locus and the regulation of MHC class I is intact in these cells (Figure 42). Furthermore, PD-L1 expression on tumor cells themselves is moderate *in vivo*, and is not affected by IFN-γ *in vivo* despite the fact that it is readily upregulated on pancreatic tumor cells *in vitro* in response to IFN-γ (Figures 3, 16, and 14). This indicates that despite having the capability of upregulating PD-L1 in the setting of immune pressure (a function of normal epithelial and endothelial tissues), there does not seem to be immune pressure on the pancreatic tumor cells *in vivo* (Keir et al., 2008). Implantation of subcutaneous PDA cell lines in KPC mice bearing pancreatic tumors led to normal tumor outgrowth, suggesting that there is no strong systemic antitumor T cell response that has developed in these KPC mice (Figure 34). I demonstrate that these subcutaneous tumors are susceptible to T cell mediated clearance, further indication of

their antigenicity. These data suggest that pancreatic tumor cells are not subject to immune mediated selective pressure directly, lending more credence to the notion that these tumors develop immune privileged sites.

As these tumors are antigenic and maintain MHC class I expression, it seems likely that the desmoplastic microenvironment mediates local immunosuppression. When we explanted pancreatic tumors and implanted them subcutaneously, however, we found that T cell responses could now be induced against these tumors with our vaccine (Figure 37). Even more striking was the infiltration of T cells into the primary pancreatic tumors of these KPC mice bearing the subcutaneous explants after treatment (Figure 38). These data suggest that the inability to drive T cell responses against pancreatic tumors is not solely due to intra-pancreatic immune privilege. The fact that PPLNs are often engulfed by pancreatic tumors (Figure 39), and the remarkable upregulation of PD-L1 on APCs in these lymph nodes compared to the corresponding populations in lymph nodes draining subcutaneous tumors, suggests that disruption of normal immune function of lymph nodes plays a role in PDA's resistance to immune therapy (Figures 40 and 41) (Mellor and Munn, 2008).

In summary, I have demonstrated that PDA tumor cells maintain the ability to express and regulate MHC class I, and that they are antigenic, as these tumors can be rejected in a T cell dependent manner after vaccination with αCD40 and gemcitabine. This same treatment does not induce T cell responses against pancreatic tumors in KPC mice, but these mice are not systemically immunosuppressed as subcutaneous PDA tumors can be rejected in a "two tumor" setting. The desmoplastic stroma of KPC tumors is not the only factor preventing T cell antitumor immunity as explanted pancreatic

tumors become susceptible to the vaccine when implanted under the skin. Dysfunction of PPLNs may contribute to the inability to induce T cells against pancreatic tumors. Lastly, I demonstrated that T cell trafficking into pancreatic tumors can be achieved when T cells can first be induced against a subcutaneous PDA tumor, offering hope for immunotherapeutic approaches in PDA patients.

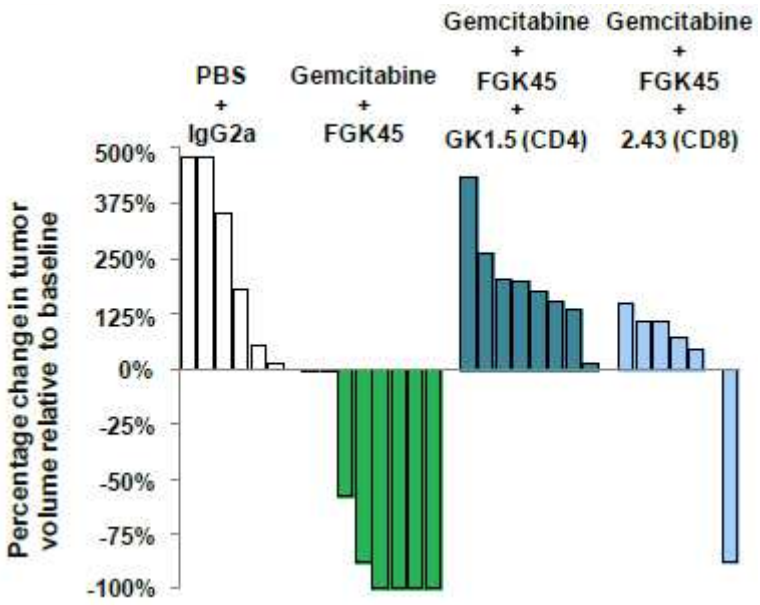


Figure 31. αCD40 and gemcitabine regress subcutaneous PDA tumors in a T cell dependent manner. Normal littermates were implanted subcutaneously on day 0 with a KPC-derived tumor cell line. On day 13, mice were treated with gemcitabine or PBS followed 48 hours later by intraperitoneal injection of αCD40 (FGK45) or control IgG2a with cohorts of mice also receiving depleting antibodies for CD4 (GK1.5) and CD8 (2.43) (as described further in Material and Methods). Waterfall plot of tumor response for each animal ($n \geq 6$ per group) determined by change in tumor volume measured 14 days after treatment. Fisher's exact test: Gemcitabine/FGK45 vs Gemcitabine/FGK45/GK1.5, p = 0.001; Gemcitabine/FGK45 vs Gemcitabine/FGK45/2.43, p = 0.015.

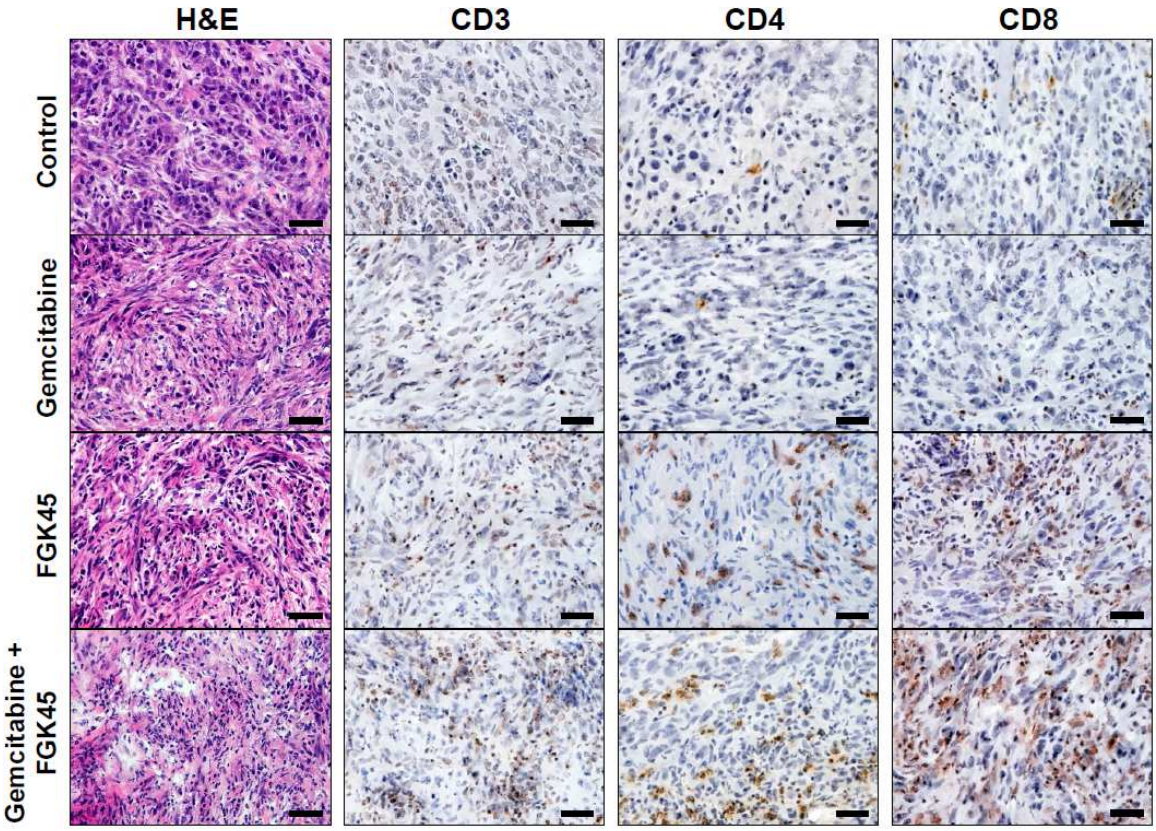


Figure 32. T cells infiltrate subcutaneous PDA tumors after αCD40/chemotherapy treatment. Representative images from mice bearing subcutaneous tumors treated as indicated. Hematoxylin & eosin (H&E) staining and immunohistochemistry for CD3, CD4, and CD8 expressing cells. Scale bars: 50 μm.

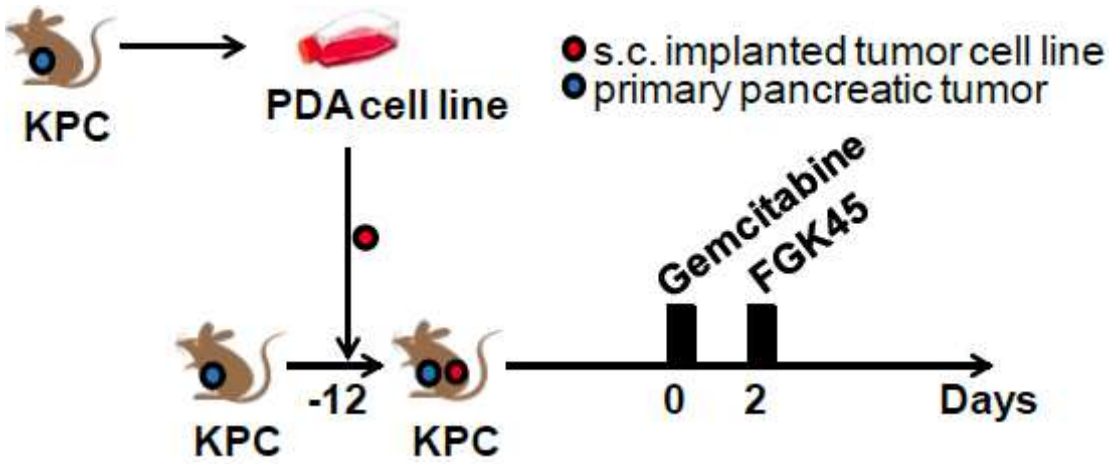


Figure 33. Experimental design for 'two-tumor' model using PDA cell line. KPC mice diagnosed with pancreatic tumors by ultrasonography were implanted with a KPC derived PDA cell line. 12 days later mice were treated with gemcitabine, followed by αCD40 (FGK45) two days later.

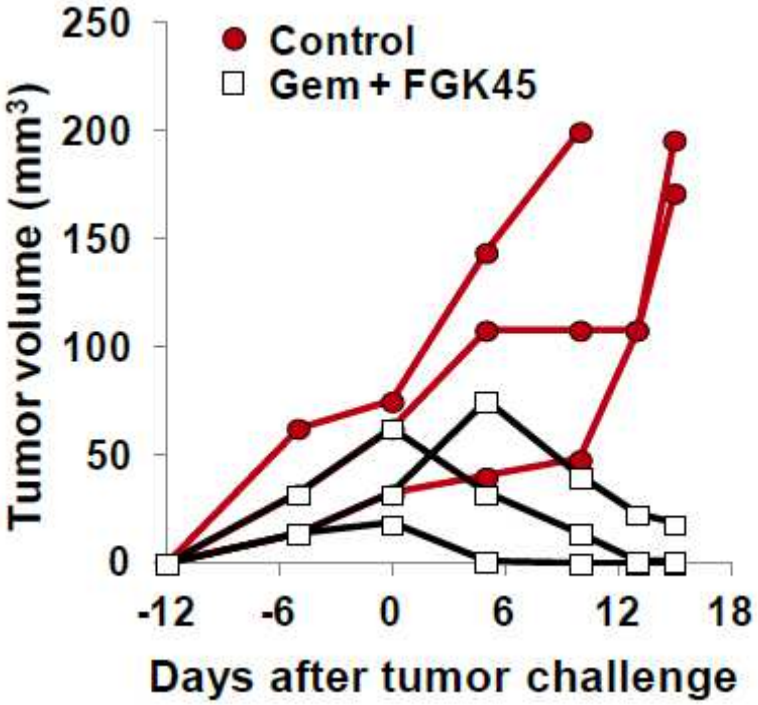


Figure 34. Subcutaneous tumors in KPC mice bearing pancreatic tumors regress after αCD40/gemcitabine treatment. Tumor growth curves of subcutaneously implanted tumors in mice (n = 3 per group) treated with control versus gemcitabine (Gem) plus αCD40 (FGK45).

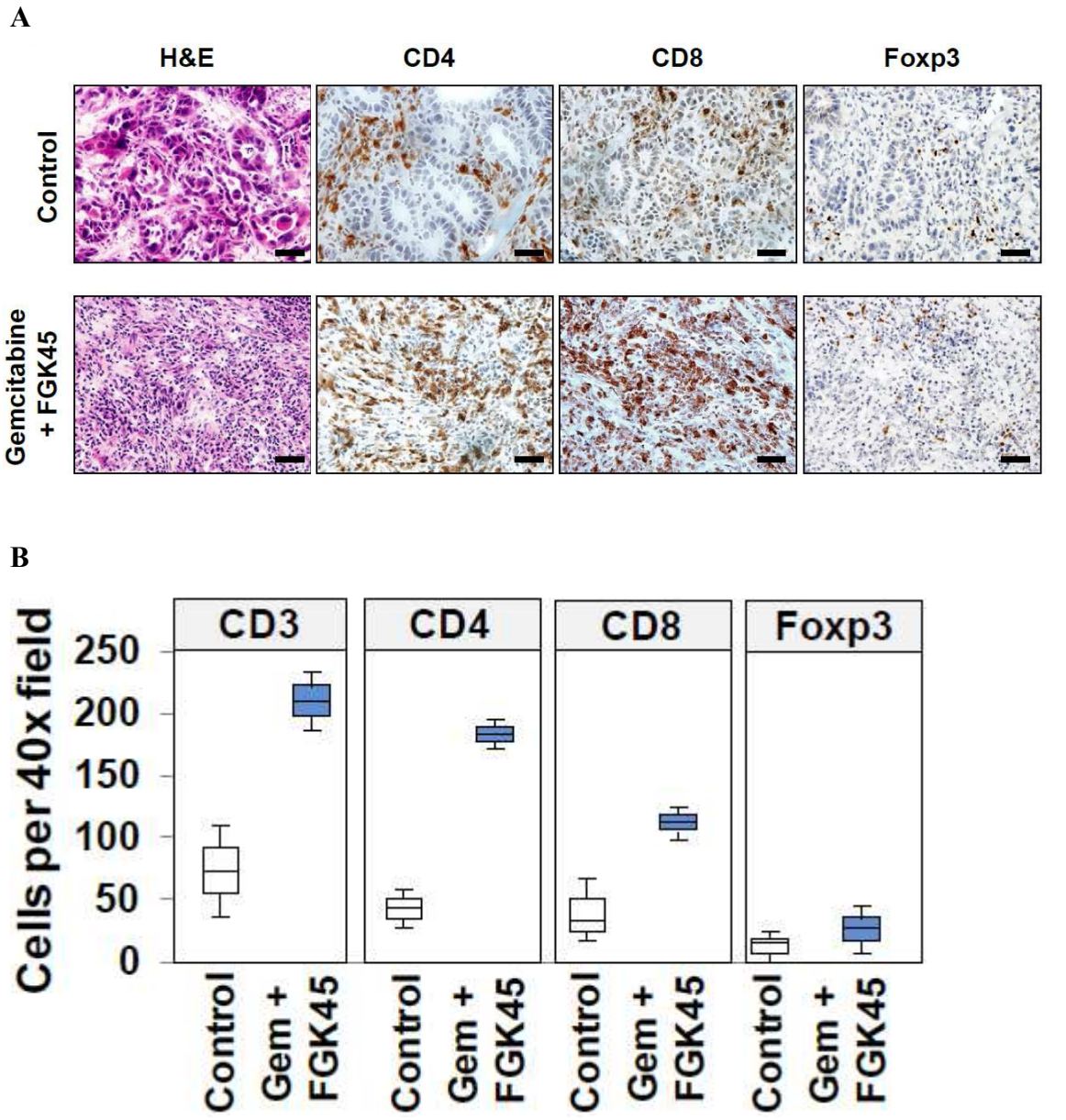


Figure 35. T cell infiltration into subcutaneous PDA tumor in pancreatic tumor bearing KPC mice after αCD40/gemcitabine. (A) Representative images showing H&E staining and immunohistochemistry for CD4, CD8, and Foxp3 expressing cells in subcutaneously growing tumors from mice 14 days after the indicated treatment. Scale bars: 100 μm. **(B)** Quantification (courtesy of Dr. Beatty, as are quantification in Figures 37, 38) of cellular infiltrates into tumors detected by immunohistochemistry 14 days after the indicated treatment ($n = 4$ per group). Student's t test: CD4, $p = 0.002$; CD8, $p = 0.043$; Foxp3, $p = 0.482$.

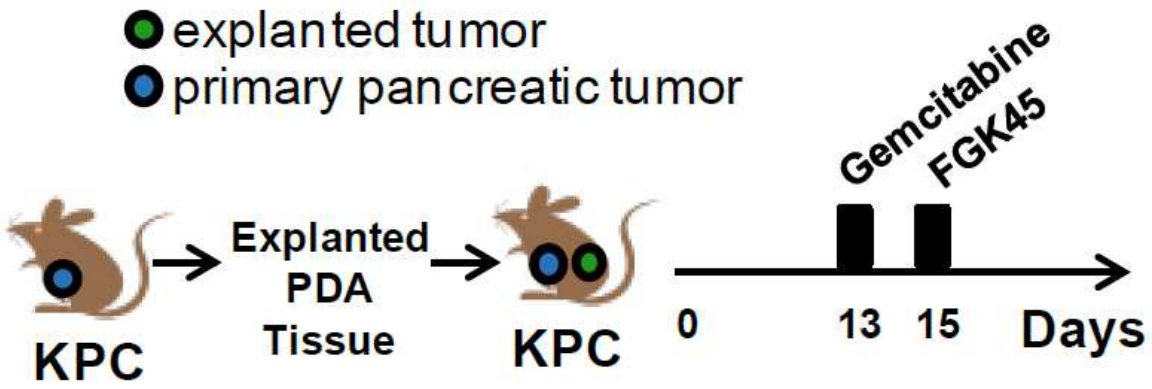


Figure 36. Experimental design for "two tumor" model using explanted KPC tumor. On day 0, explanted PDA tissue was re-implanted subcutaneously into KPC mice with ultrasound confirmed spontaneous pancreatic tumors. Mice were treated on day 13 with gemcitabine or PBS followed 48 hours later by intraperitoneal injection of αCD40 (FGK45) or control IgG2a.

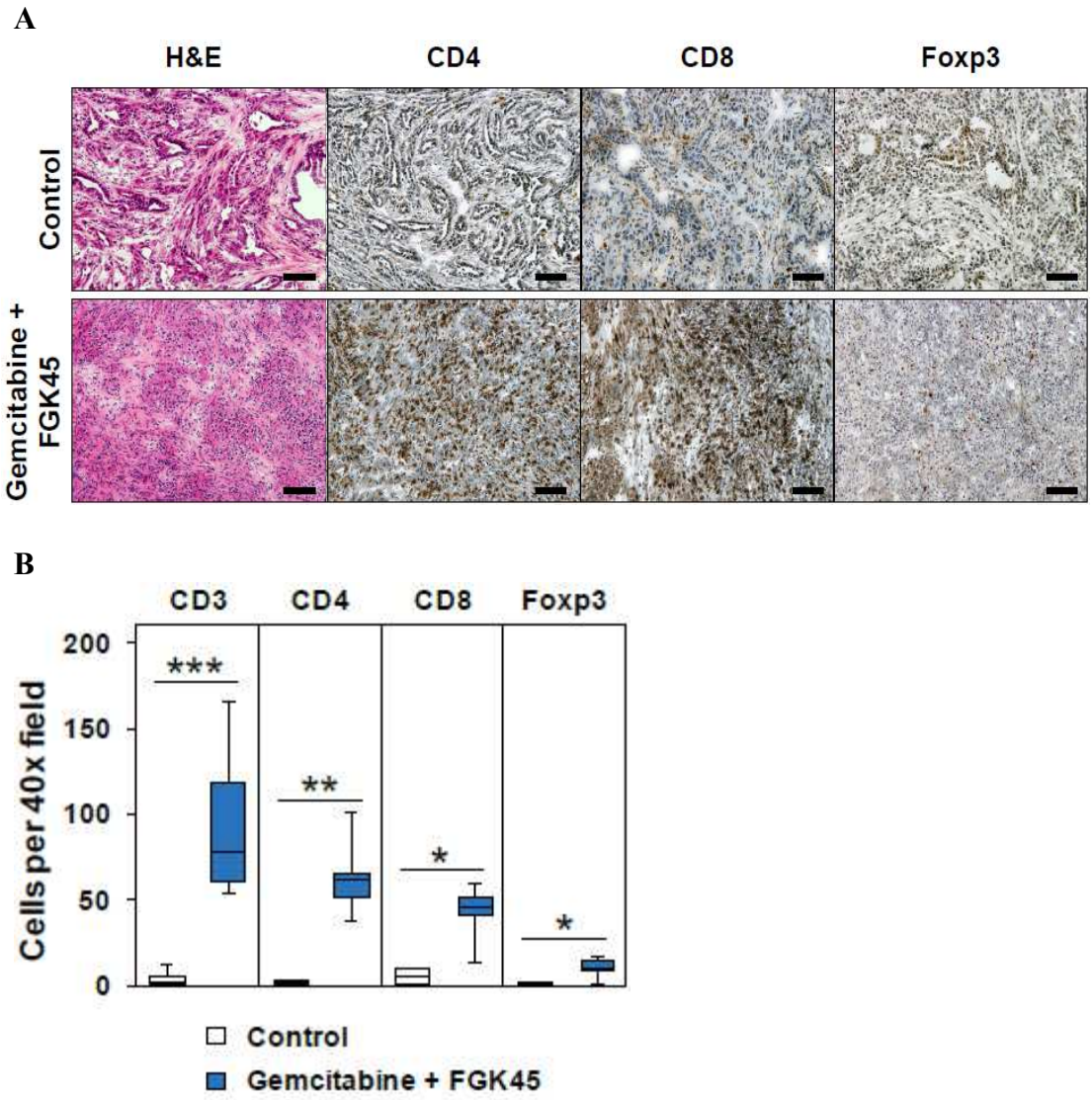


Figure 37. Robust T cell infiltration into explanted KPC tumors after treatment with αCD40/gemcitabine. (A) Representative images showing H&E staining and immunohistochemistry for CD4, CD8, and Foxp3 expressing cells in subcutaneously growing explanted KPC tumors from mice 14 days after the indicated treatment. Scale bars: 50μm. **(B)** Quantification of cellular infiltrates into responding explant tumors detected by immunohistochemistry 14 days after the indicated treatment ($n = 4$ per group). Student's t test: *p< 0.05, **p<0.01, ***p<0.005.

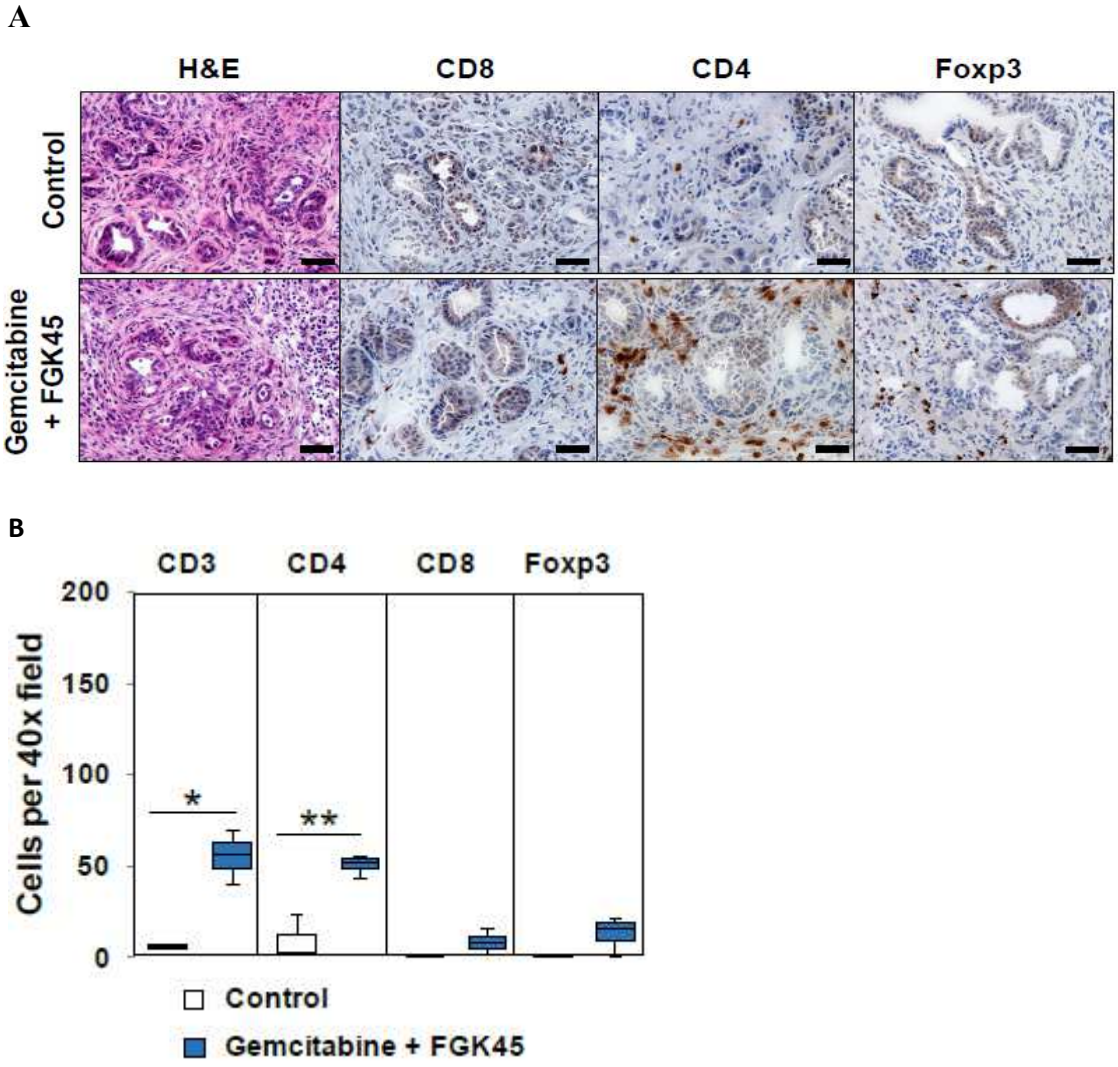
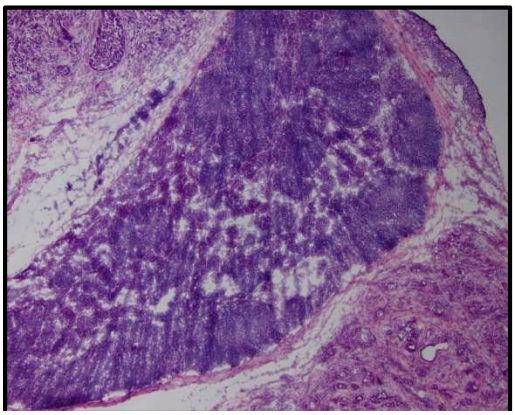
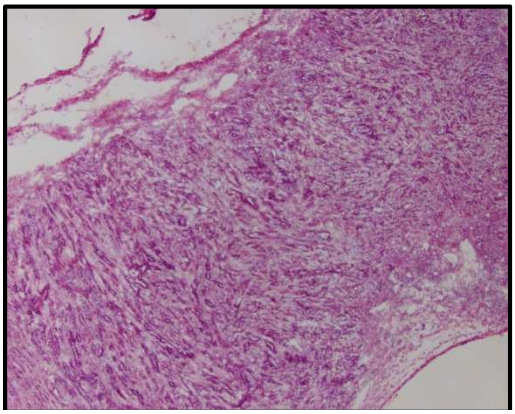


Figure 38. T cell infiltration in primary pancreatic KPC tumors in mice bearing two tumors after αCD40/gemcitabine. (A) Representative images showing H&E staining and immunohistochemistry for CD4, CD8, and Foxp3 expressing cells in primary pancreatic KPC tumors in mice also bearing an explanted KPC tumor. Scale bars: 50μm. **(B)** Quantification of cellular infiltrates into spontaneous primary pancreatic tumors detected by immunohistochemistry 14 days after the indicated treatment ($n = 4$ per group). Student's t test: *$p < 0.05$, **$p < 0.01$.

A



B



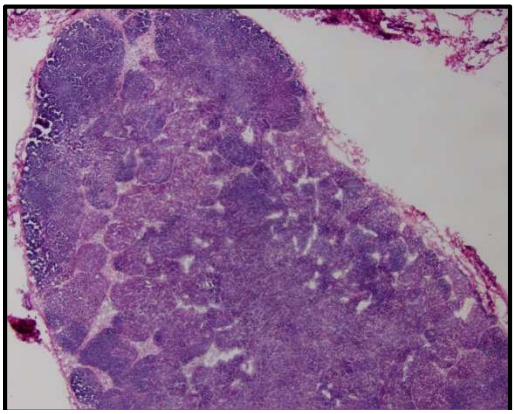


Figure 39. Peripancreatic lymph nodes are often engulfed by pancreatic tumors in KPC mice. (A) H&E image of involved peripancreatic lymph node surrounded by pancreatic tumor. 40x magnification. **(B)** Left- H&E image of subcutaneous PDA tumor; Right- H&E image of inguinal lymph node draining subcutaneous PDA tumor. 40x magnification.

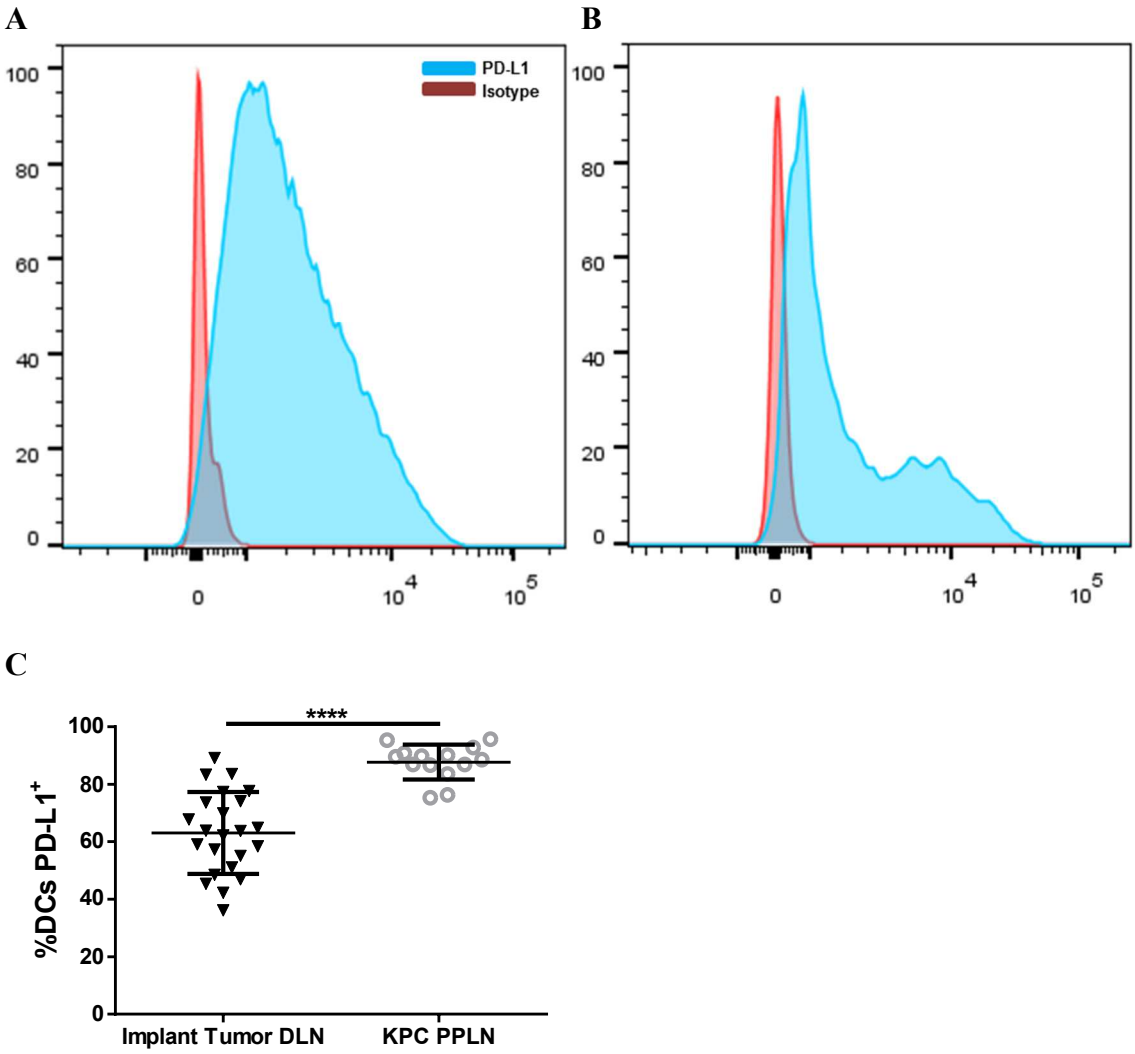


Figure 40. Significantly more DCs in peripancreatic LNs express PD-L1 compared to DCs in inguinal LNs draining subcutaneous tumors. Representative histogram of PD-L1 expression on DCs (gated as above) from **(A)** KPC peripancreatic lymph nodes (PPLN; n=14) or **(B)** inguinal lymph node draining implanted subcutaneous tumor (n=23). **(C)** Quantification of %DCs PD-L1$^+$. Student's *t* test: ****p<0.0001.

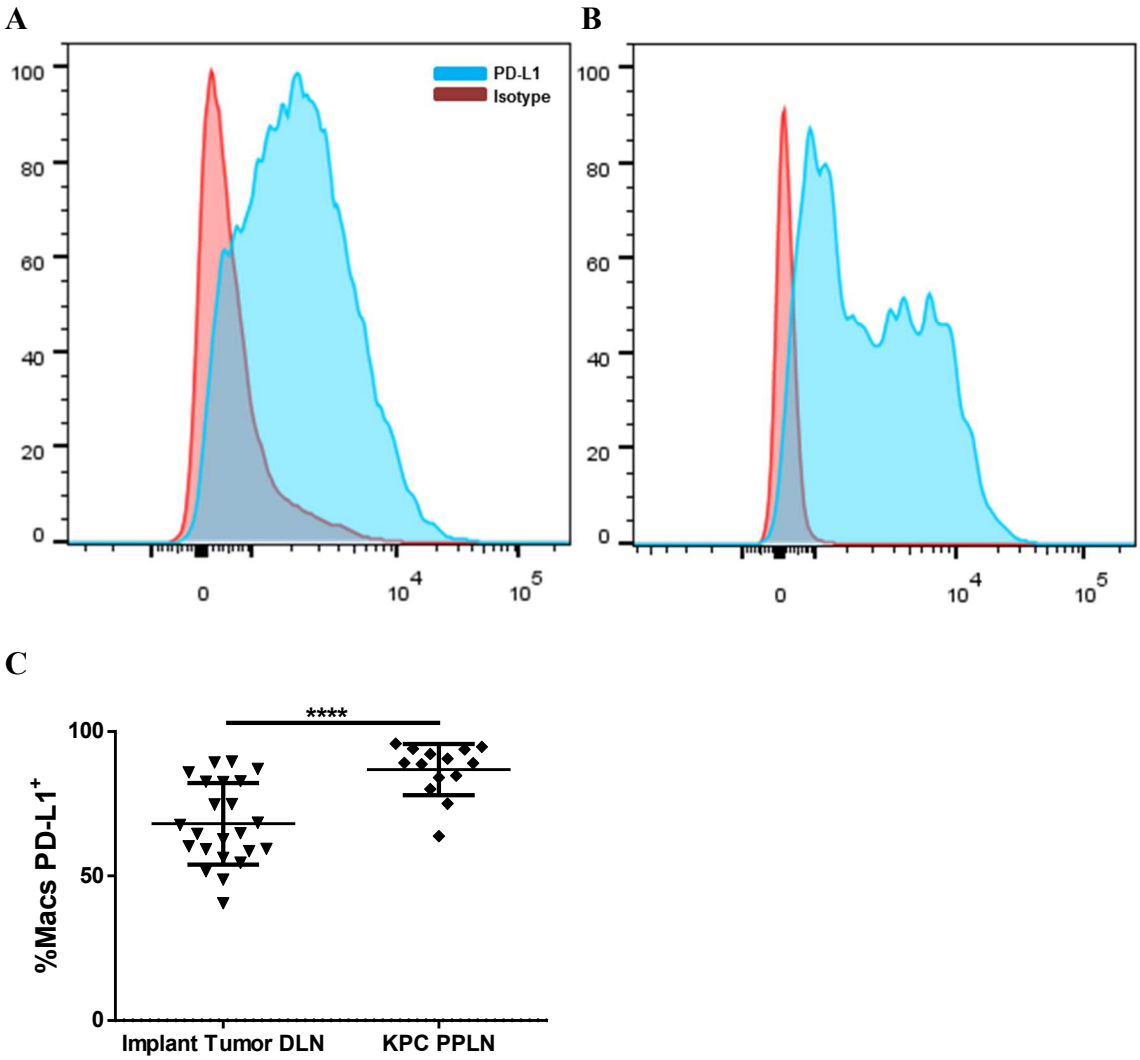


Figure 41. Significantly more macrophages in peripancreatic LNs express PD-L1 compared to macrophages in inguinal LNs draining subcutaneous tumors. Representative histogram of PD-L1 expression on Macs (gated as above) from **(A)** KPC peripancreatic lymph nodes (n=14) or **(B)** inguinal lymph node draining implanted subcutaneous tumor (n-23). **(C)** Quantification of %Macs PD-L1$^+$. Student's *t* test: ****p<0.0001.

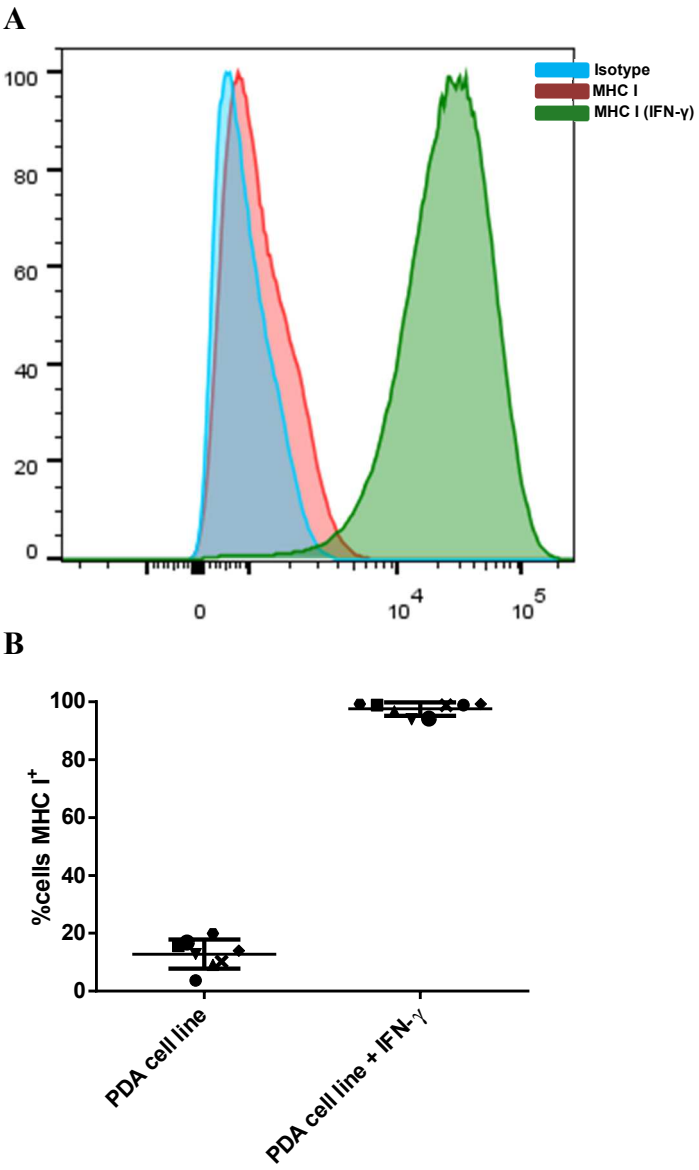


Figure 42. MHC I (H-2Kb) expression on PDA cell lines can be upregulated by IFN-γ *in vitro*. (A) Representative histogram of a KPC-derived PDA cell line interrogated for MHC I expression *in vitro* with or without IFN-γ in the culture, and **(B)** quantification of 8 distinct KPC-derived PDA cell lines interrogated for MHC I expression *in vitro* with or without IFN-γ.

CHAPTER 5 – Discussion and Future Directions

The remarkable clinical successes of mAbs blocking the negative immune checkpoint proteins PD-1/PD-L1 and CTLA-4 has prompted investigations to replicate these responses in other malignancies. Paramount to expanding the populations that can benefit from these therapies is an improved understanding of resistance mechanisms. Some tumor types appear to be completely resistant; for example, patients with pancreatic cancer have been treated with these agents but no responses have been reported (Brahmer et al., 2012; Le et al., 2013; Royal et al., 2010). In this thesis project I studied a genetic model of pancreatic carcinoma which recapitulates the salient molecular, histopathologic, and clinical features of the human disease in order to understand the resistance of this tumor to checkpoint inhibitors and interrogate approaches to overcome this resistance. In Chapter 3 I describe the expression of PD-1 and its ligand PD-L1 in the KPC model of PDA and confirm the relevance of our work by analysis of human pancreatic tumors. I developed a subcutaneous model of PDA which is resistant to single agent (and, in fact, combinatorial) checkpoint inhibitors mimicking the clinical results to date. I hypothesized that the dearth of effector T cells in pancreatic tumors precluded clinical efficacy of mAbs blocking negative checkpoints, and that induction of a T cell response would overcome resistance in this model. Using an agonist αCD40 mAb, combined with chemotherapy to induce immunogenic cell death, I induced a T cell response against PDA tumors and potentiated antitumor effects of checkpoint inhibition; large proportions of tumors were rejected in a T cell dependent manner. Combination treatment also improved survival of KPC mice, highlighting the clinical relevance of my work. In

Chapter 4 I interrogated the immunologic differences between the subcutaneous and pancreatic PDA tumors models. Working with Dr. Beatty, we demonstrated that although the desmoplastic tumor microenvironment of KPC tumors plays a role in inhibiting antitumor T cell responses, explanting pancreatic tumors and implanting them subcutaneously allows for the induction of antitumor T cells. These data suggest that lymph nodes draining pancreatic tumors may preclude proper T cell activation; I found that significantly more APCs in the PPLNs express PD-L1 than APCs in lymph nodes draining subcutaneous tumors. Importantly, the induction of an antitumor T cell response in a "two tumor" model led to a T cell infiltrate in the primary pancreatic tumors, providing insight on immunotherapeutic approaches for patients with PDA. In the pages below I will discuss the strengths and limitations of the data presented here, analyze them within their context in the literature, and offer thoughts on future directions for these projects; some of the finer points of the data are analyzed in more detail in the concluding sections of Chapters 3 and 4 above.

Experimental mouse models of PDA

Genetic mouse models have facilitated the study of the tumor microenvironment. Driven by the same oncogenes that initiate carcinogenesis in human malignancy, murine cancers in GEMMs replicate the tumorigenic processes of acquiring further genetic hits, and overcoming immune surveillance before becoming clinically apparent lesions. These processes, occurring stochastically over time in immune competent hosts lead to the development of lesions that differ greatly from the human transplanted tumors grown in nude mice which had long dominated oncologic investigations. The KPC GEMM used in

the Vonderheide lab, as well as many others, recapitulates human PDA in the development of desmoplastic tumors harboring robust immune and mesenchymal infiltrates which affect tumor initiation and development. Crucially, successful preclinical studies in this model have been translated to the clinic where early results indicate a high degree of fidelity in the types of responses achieved in patients (Beatty et al., 2011; Provenzano et al., 2012; Rhim et al., 2014).

The great strengths of the KPC model are unfortunately counteracted by the slow breeding process and the stochasticity of the model which necessitates regular palpation and ultrasound monitoring in order to diagnose nascent tumors. The pancreatic transcription factor Pdx-1 comes on at embryonic day 9.5 in these mice, meaning that oncogenes are activated *in utero*, which differs from human PDA, and could potentially affect the immune response to these tumors (Hingorani et al., 2003). These factors preclude large scale studies and the repeating of experiments as enrollment for any study can take months. In the studies described in this thesis, I used the KPC model to identify the prominence of the PD-1/PD-L1 axis in pancreatic cancer. After determining that this pathway is overexpressed in the KPC model, I established a subcutaneous tumor model in immune competent mice which recapitulates this phenotype in order to greatly accelerate the pace and scope of my studies. As described above, the subcutaneous tumors that developed were able to form desmoplastic tumors with similar immune infiltrates to the KPC tumors, and, fundamental to my studies, established subcutaneous PDA tumors were resistant to therapeutic mAbs against PD-1 and CTLA-4. This resistance recapitulated the clinical results to date of single agent checkpoint inhibitors in pancreatic cancer, and distinguishes this subcutaneous model from other murine studies which found

significant tumor rejection upon single agent treatment with αPD-1 or αCTLA-4 (Sandin et al., 2014; Soares et al., 2014). After determining that a vaccine can potentiate checkpoint inhibitors in this model, I returned to the KPC model to validate these findings. Despite an almost doubling of overall survival, a significant finding comparable to other successful studies in KPC mice, I did not observe complete tumor eradication in any KPC mice (Olive et al., 2009). While there is no published report of a tumor bearing KPC mouse cured of its disease, the gulf between the responses in the two models warrants further investigation. The differences in the lymphatic drainage of the two tumor systems, and the marked increase in PD-L1 expression on APCs of PPLNs draining KPC tumors suggests that this treatment may fail to overcome the immune privileged microenvironment established by KPC tumors. A slight but statistically significant increase in the percentage of DCs in subcutaneous PDA tumors vs KPC tumors might also facilitate the induction of T cell responses in that model (Figure 7). Further studies to understand these differences and elucidate the mechanisms which govern PD-L1 expression in the KPC microenvironment would potentially provide additional targets and improve rational design of therapeutic interventions in this disease.

IFN-γ and adaptive resistance

Although PD-L1 expression remains responsive to IFN-γ in PDA cell lines *in vitro*, I found that the regulation of PD-L1 expression *in vivo* in the PDA tumor microenvironment does not require IFN-γ. Genetic loss of IFN-γ or depletion of T cells did not affect tumor cell PD-L1, and there was only minimally decreased PD-L1 on tumor APCs in IFN-$\gamma^{-/-}$ mice. These findings differ from the prevailing notions of PD-L1

expression in cancer. Immunohistochemical studies of various human malignancies (melanoma, NSCLC, RCC, HNSCC, and bladder cancer) have all demonstrated that tumor associated PD-L1 expression often co-localizes with lymphocytic immune infiltrates, specifically $CD8^+$ TILs (Lyford-Pike et al., 2013; Powles et al., 2014; Taube et al., 2012; 2014; Velcheti et al., 2013). These data suggest that in many malignancies the upregulation of PD-L1 (on tumor cells or tumor associated immune cells) functions to locally dampen antitumor T cell responses, a concept termed adaptive immune resistance (Taube et al., 2012; Tumeh et al., 2014). Mechanistically, this upregulation is thought to occur upon exposure to IFN-γ secreted by activated infiltrating $CD8^+$ T cells, a pathway that regulates PD-L1 expression on normal epithelial tissues (Keir et al., 2008). In a recent study of murine melanoma, tumor cell PD-L1 expression was demonstrated to be dependent on $CD8^+$ T cells and the secretion of IFN-γ, validating this hypothesis (Spranger et al., 2013). In contrast, both of the PDA mouse models I studied expressed high PD-L1 in the tumor microenvironment despite harboring minimal intratumoral T cells, indicating that despite the prevalence of this pathway in the PDA microenvironment, PD-L1expression does not appear to be an adaptive response to immune pressure. These findings in the KPC model are corroborated by the observations in human PDA, in which there was no spatial correlation between tumor PD-L1 expression and the presence of intratumoral $CD8^+$ T cells. Tumor PD-L1 expression has been reported to be regulated by oncogenes such as EGFR, but oncogenic Kras (at least in lung carcinoma) does not induce PD-L1 expression (Akbay et al., 2013), suggesting that the regulation of PD-L1 in PDA may differ from other solid malignancies. As such, it is important to note that intratumoral PD-L1 does not necessarily serve as a biomarker

of an ongoing antitumor immune response. Investigations in other tumor types that harbor few TILs would illuminate whether PD-L1 upregulation in the absence of $CD8^+$ T cells and IFN-γ is specific to PDA or whether it is a common feature of immune privileged tumors.

Understanding resistance to αPD-1 and αCTLA-4 mAbs

Recently published studies have determined that pretreatment intratumoral PD-L1 expression is in fact predictive of patient responses to αPD-1/αPD-L1 (Herbst et al., 2014; Powles et al., 2014; Tumeh et al., 2014; Taube et al., 2014). However, unlike targeted therapies like trastuzumab or vemurafenib, the presence of PD-L1 in the tumor does not seem to be important for its presence, per se, but rather for the fact that it is indicative of an ongoing antitumor T cell response which is being suppressed by PD-L1 expression. The presence of TILs or $CD8^+$ T cells, or the intratumoral expression of IFN-γ or CTLA-4 in pretreatment biopsies also independently predict response to αPD-1 or αPD-L1 in these same studies (Herbst et al., 2014; Taube et al., 2012; Kvistborg et al., 2014)**.** Furthermore, in bladder and lung cancers tumor cell PD-L1 does not correlate with response, whereas PD-L1 expression on infiltrating immune cells *does* correlate with response to αPD-L1 (Herbst et al., 2014; Powles et al., 2014). If PD-L1 expression were, in fact, primarily important in preventing the targeting of malignant cells by $PD\text{-}1^+$ $CD8^+$ T cells then tumor cell PD-L1 should be the metric which correlates with response, not the PD-L1 on other cells in the TME. Two papers offer first in human evidence of the T cell effects of αPD-1 and αCTLA-4; patients treated with ipilimumab had increased levels of antigen specific antitumor $CD8^+$ T cells, and responders to pembrolizumab

showed increased intratumoral $CD8^+$ T cells compared to nonresponders (Kvistborg et al., 2014; Tumeh et al., 2014). In the context of these data, the lack of response to checkpoint inhibitors in PDA, despite expression of PD-1 and PD-L1 in the TME, makes sense, as few effector T cells are present in these tumors at baseline, indicating the lack of a response to be rescued (Figure 43).

While large subsets of some malignancies present with robust T cell infiltrates or have inflammatory gene signatures, PDA does not commonly present with a robust tumor infiltration of $CD8^+$ T cells (Bernstorff et al., 2001; De Monte et al., 2011; Fukunaga et al., 2004; Gajewski et al., 2010; Galon et al., 2006; Hiraoka et al., 2006; Zhang et al., 2003). PDA is associated with multiple immunosuppressive populations starting at the premalignant stage, likely preventing effector T cells from sculpting these tumors, as evidenced by the retention of MHC class I regulation in KPC derived cell lines (Bayne et al., 2012; Beatty et al., 2011; Clark et al., 2007; 2008; Zhang et al., 2014). TILs in PDA tumors express more PD-1 than T cells in mice chronically infected with LCMV (in which αPD-1 can rescue T cell function (Barber et al., 2006)); yet αPD-1 treatment has no effect on tumor growth in PDA, suggesting that the TILs in PDA are not prevented from targeting the tumor simply by encountering negative checkpoint molecules (Figure 43). These data suggest that unlike other malignancies, the PDA microenvironment may act as an immune privileged site, a paradigm which has implications for immunotherapeutic approaches (Vonderheide et al., 2013). I propose that response to αPD-1 and αCTLA-4 in these PDA models is unlikely, and that both the induction of antitumor T cells with a vaccine, and the blockade of negative immune checkpoints are necessary for successful immune therapy in these kinds of tumors. In fact, one clinical

study combined ipilimumab with a PDA cellular vaccine and an improvement in median overall survival (5.7 months vs. 3.6 months; p=0.072) was observed in comparison to patients treated with ipilimumab alone, supporting my findings in mice (Le et al., 2013).

The observation that αCD40/chemotherapy converts PDA from a tumor that is fully refractory to checkpoint inhibition to one that is highly sensitive, is important in the context of prior efforts to extend the therapeutic range of αPD-1 and αCTLA-4. My goal was to use a murine model that reproduces the lack of clinical effect observed to date with αPD-1 and αCTLA-4 in PDA, in contrast to many tumor models that exhibit baseline levels of responsiveness to αCTLA-4, αPD-1, αPD-L1 or combinations of these agents. For example, in models of colon carcinoma, melanoma, ovarian cancer, bladder cancer, and neuroblastoma, checkpoint inhibitor therapy alone inhibits tumor growth, improves survival, and occasionally mediates complete rejection (Curran et al., 2010; Duraiswamy et al., 2013; Leach et al., 1996; Mangsbo et al., 2010; Williams et al., 2013). In a recent study using the Panc02 subcutaneous PDA model, tumor rejection was observed in 50% of mice after treatment with αCTLA-4 (Sandin et al., 2014), a finding not representative of the clinical record of αCTLA-4 in patients with PDA (Le et al., 2013; Royal et al., 2010) and possibly related to Panc02 being a carcinogen-induced and likely hypermutated tumor (whereas human PDA is not a hypermutated tumor (Jones et al., 2008)). In another study of the Panc02 model, αPD-1 treatment significantly slowed tumor growth and led to complete tumor rejection in 22% of mice (Soares et al., 2014). Previous work in these types of models demonstrates that T cell stimulatory therapies including vaccines, peritumoral poly(I:C), and intratumoral oncolytic virus can improve baseline effects of checkpoint blockade (Bald et al., 2014; Duraiswamy et al., 2013;

Zamarin et al., 2014; Zhou et al., 2010). In PDA, the stimulation of a T cell response using αCD40/chemotherapy is able to fundamentally transform a tumor that is refractory to checkpoint inhibition into a highly sensitive one, rather than only improving upon baseline activity of checkpoint mAb (Figure 43). I demonstrated that αCD40 rather than chemotherapy is crucial for potentiating the effects of checkpoint inhibitors in this PDA model; the role and sequencing of chemotherapy in potentiating or inhibiting immunotherapy will require significant further investigation. It is likely that alternate approaches to induce T cell responses, including the use of Toll like receptor (TLR) agonists such as CpG or polyI:C, among other vaccine approaches, would also synergize with αPD-1 or αCTLA-4 in PDA (Buhtoiarov et al., 2010; Cho and Celis, 2009; Davila et al., 2003; Scarlett et al., 2009).

Summary and closing remarks

With the clinical success of mAbs blocking negative immune checkpoints in malignancies ranging from melanoma to bladder cancer, efforts are underway to replicate these results in other tumor types. In patients with PDA these agents have failed to date, and I propose here that these results are indicative of an immune privileged tumor microenvironment which precludes antitumor T cells (Figure 43). Using two models of pancreatic cancer, I demonstrate that despite high expression of PD-L1 and PD-1 in pancreatic tumors, treatment with αPD-1 fails to slow tumor growth or improve survival. Unlike other tumors, where PD-L1 expression is indicative of an adaptive resistance to immune pressure, PD-L1 in PDA is not dependent on IFN-γ or $CD8^+$ T cells, indicating that regulation of this pathway differs between tumor types. Using a vaccine approach, I

induced an antitumor T cell response which was significantly augmented with the addition of checkpoint inhibitors, leading to rejection of tumors in a large subset of mice. Significant survival improvement in KPC mice treated with vaccine and αPD-1 suggests this approach may prove effective in patients with PDA as this model has previously predicted clinical responses. These results may be more broadly applicable to non-immunogenic malignancies, providing a template for expanding the reach of immunotherapeutic approaches.

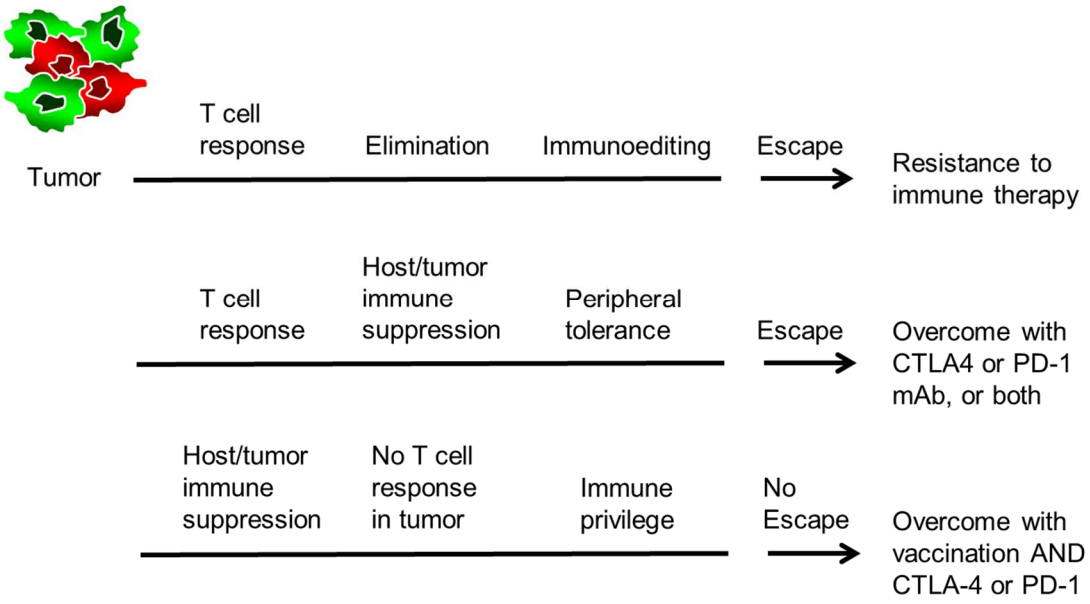


Figure 43. Proposed model describing types of immune responses to cancer and implications for immunotherapy.

REFERENCES

Aguirre, A.J., Bardeesy, N., Sinha, M., Lopez, L., Tuveson, D.A., Horner, J., Redston, M.S., and DePinho, R.A. (2003). Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev. 17, 3112-26.

Akbay, E.A., Koyama, S., Carretero, J., Altabef, A., Tchaicha, J.H., Christensen, C.L., Mikse, O.R., Cherniack, A.D., Beauchamp, E.M., Pugh, T.J., et al. (2013). Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer Discov 3, 1355-63.

Amakye, D., Jagani, Z., and Dorsch, M. (2013). Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nat. Med. 19, 1410-22.

Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., Mignot, G., Maiuri, M.C., Ullrich, E., Saulnier, P., et al. (2007). Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat. Med. 13, 1050-9.

Bald, T., Landsberg, J., Lopez-Ramos, D., Renn, M., Glodde, N., Jansen, P., Gaffal, E., Steitz, J., Tolba, R., Kalinke, U., et al. (2014). Immune cell-poor melanomas benefit from PD-1 blockade after targeted type I IFN activation. Cancer Discov 4, 674-87.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439, 682-687.

Bayne, L., Beatty, G., Jhala, N., Clark, C., Rhim, A., Stanger, B., and Vonderheide, R. (2012). Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. Cancer Cell 21, 822-35.

Beatty, G.L., Chiorean, E.G., Fishman, M.P., Saboury, B., Teitelbaum, U.R., Sun, W., Huhn, R.D., Song, W., Li, D., Sharp, L.L., et al. (2011). CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. Science 331, 1612-6.

Beatty, G.L., Torigian, D.A., Chiorean, E.G., Saboury, B., Brothers, A., Alava, A., Troxel, A.B., Weijing, S., Teitelbaum, U.R., Vonderheide, R.H., and O'Dwyer, P.J. (2013). A phase I study of an agonist CD40 monoclonal antibody (CP-870,893) in

combination with gemcitabine in patients with advanced pancreatic ductal adenocarcinoma. Clin Cancer Res 19, 6286-95.

Bernstorff, von, W., Voss, M., Freichel, S., Schmid, A., Vogel, I., Jöhnk, C., Henne-Bruns, D., Kremer, B., and Kalthoff, H. (2001). Systemic and local immunosuppression in pancreatic cancer patients. Clin Cancer Res 7, 925s-932s.

Blackburn, S.D., Shin, H., Freeman, G.J., and Wherry, E.J. (2008). Selective expansion of a subset of exhausted CD8 T cells by PD-L1 blockade. Proc Natl Acad Sci 105, 15016-15021.

Blackburn, S.D., Shin, H., Haining, W.N., Zou, T., Workman, C.J., Polley, A., Betts, M.R., Freeman, G.J., Vignali, D.A.A., and Wherry, E.J. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 10, 29-37.

Brahmer, J.R., Tykodi, S.S., Chow, L.Q.M., Hwu, W., Topalian, S.L., Hwu, P., Drake, C.G., Camacho, L.H., Kauh, J., Odunsi, K., et al. (2012). Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N. Engl. J. Med. 366, 2455-65.

Buhtoiarov, I.N., Sondel, P.M., Wigginton, J.M., Buhtoiarova, T.N., Yanke, E.M., Mahvi, D.A., and Rakhmilevich, A.L. (2010). Anti-tumour synergy of cytotoxic chemotherapy and anti-CD40 plus CpG-ODN immunotherapy through repolarization of tumour-associated macrophages. Immunology 132, 226-39.

Burnet, F.M. (1970). The concept of immunological surveillance. Prog. Exp. Tumor Res. 13, 1-27.

Burnet, F.M. (1957). Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J 1, 841-7.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., and Riley, J.L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J. Immunol. 173, 945-54.

Cho, H., and Celis, E. (2009). Optimized peptide vaccines eliciting extensive CD8 T-cell responses with therapeutic antitumor effects. Cancer Res 69, 9012-9.

Clark, C.E., Beatty, G.L., and Vonderheide, R.H. (2008). Immunosurveillance of pancreatic adenocarcinoma: insights from genetically engineered mouse models of cancer. Cancer Lett. 279, 1-7.

Clark, C.E., Hingorani, S.R., Mick, R., Combs, C., Tuveson, D.A., and Vonderheide, R.H., (2007). Dynamics of the immune reaction to pancreatic cancer from inception to invasion. Cancer Res 67, 9518-27.

Clemente, C.G., Mihm, M.C., Bufalino, R., Zurrida, S., Collini, P., and Cascinelli, N. (1996). Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 77, 1303-10.

Condamine, T., Kumar, V., Ramachandran, I.R., Youn, J., Celis, E., Finnberg, N., El-Deiry, W.S., Winograd, R., Vonderheide, R.H., English, N.R., et al. (2014). ER stress regulates myeloid-derived suppressor cell fate through TRAIL-R-mediated apoptosis. J. Clin. Invest. 124, 2626-39.

Conroy, T., Desseigne, F., Ychou, M., Bouché, O., Guimbaud, R., Bécouarn, Y., Adenis, A., Raoul, J., Gourgou-Bourgade, S., La Fouchardière, de, C., et al. (2011).

FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N. Engl. J. Med. 364, 1817-25.

Crawford, A., Angelosanto, J., Kao, C., Doering, T., Odorizzi, P., Barnett, B., and Wherry, E.J. (2014). Molecular and transcriptional basis of CD4+ T cell dysfunction during chronic infection. Immunity 40, 289-302.

Curiel, T.J., Wei, S., Dong, H., Alvarez, X., Cheng, P., Mottram, P., Krzysiek, R., Knutson, K.L., Zimmermann, M.C., David, O., et al. (2003). Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat. Med. 9, 562-7.

Curran, M.A., Montalvo, W., Yagita, H., and Allison, J.P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci 107, 4275-4280.

Davila, E., Kennedy, R., and Celis, E. (2003). Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade. Cancer Res 63, 3281-8.

De Monte, L., Reni, M., Tassi, E., Clavenna, D., Papa, I., Recalde, H., Braga, M., Di Carlo, V., Doglioni, C., and Protti, M.P. (2011). Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. J. Exp. Med. 208, 469-78.

Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N., and Raulet, D.H. (2001). Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat. Immunol. 1, 119-26.

Diehl, L., Boer, den, A.T., Schoenberger, S.P., van der Voort, E.I., Schumacher, T.N., Melief, C.J., Offringa, R., and Toes, R.E. (1999). CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat. Med. 5, 774-9.

Doering, T.A., Crawford, A., Angelosanto, J.M., Paley, M.A., Ziegler, C.G., and Wherry, E.J. (2012). Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity 37, 1130-1144.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., et al. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. 8, 793-800.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreiber, R.D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. Nat. Immunol. 3, 991-8.

Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004). The three Es of cancer immunoediting. Annu. Rev. Immunol. 22, 329-60.

Duraiswamy, J., Kaluza, K.M., Freeman, G.J., and Coukos, G. (2013). Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res 73, 3591-603.

Ehrlich, P. (1909). Ueber den jetzigen stand der Karzinomforschung. Ned. Tijdschr. Geneeskd. 5, 273-90

Elgueta, R., Benson, M.J., de Vries, V.C., Wasiuk, A., Guo, Y., and Noelle, R.J. (2009). Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev 229, 152-72.

Feig, C., Jones, J.O., Kraman, M., Wells, R.J.B., Deonarine, A., Chan, D.S., Connell, C.M., Roberts, E.W., Zhao, Q., Caballero, O.L., et al. (2013). Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc. Natl. Acad. Sci. U.S.A. 110, 20212-7.

Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C., et al. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. 192, 1027-34.

French, R.R., Chan, H.T., Tutt, A.L., and Glennie, M.J. (1999). CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nat. Med. 5, 548-53.

Fukunaga, A., Miyamoto, M., Cho, Y., Murakami, S., Kawarada, Y., Oshikiri, T., Kato, K., Kurokawa, T., Suzuoki, M., Nakakubo, Y., et al. (2004). CD8+ tumor-infiltrating

lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. Pancreas 28, e26-31.

Gabrilovich, D.I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 12, 253-68.

Gajewski, T.F., Louahed, J., and Brichard, V.G. (2010). Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. Cancer J 16, 399-403.

Gajewski, T.F., Schreiber, H., and Fu, Y. (2013). Innate and adaptive immune cells in the tumor microenvironment. Nat. Immunol. 14, 1014-22.

Galon, J., Angell, H., Bedognetti, D., and Marincola, F. (2013). The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 39, 11-26.

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pagès, C., Tosolini, M., Camus, M., Berger, A., Wind, P., et al. (2006). Type, density, and location

of immune cells within human colorectal tumors predict clinical outcome. Science 313, 1960-4.

Gubin, M.M., Zhang, X., Schuster, H., Caron, E., Ward, J.P., Noguchi, T., Ivanova, Y., Hundal, J., Arthur, C.D., Krebber, W., et al. (2014). Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature 515, 577-81.

Hamid, O., Robert, C., Daud, A., Hodi, F.S., Hwu, W., Kefford, R., Wolchok, J.D., Hersey, P., Joseph, R.W., Weber, J.S., et al. (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N. Engl. J. Med. 369, 134-44.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell 144, 646-74.

Herbst, R.S., Soria, J., Kowanetz, M., Fine, G.D., Hamid, O., Gordon, M.S., Sosman, J.A., McDermott, D.F., Powderly, J.D., Gettinger, S.N., et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515, 563-7.

Hidalgo, M. (2010). Pancreatic cancer. N. Engl. J. Med. 362, 1605-17.

Hingorani, S.R., Petricoin, E.F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M.A., Ross, S., Conrads, T.P., Veenstra, T.D., Ben Hitt, A., et al. (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4, 437-50.

Hingorani, S.R., Wang, L., Multani, A.S., Combs, C., Deramaudt, T.B., Hruban, R.H., Rustgi, A.K., Chang, S., and Tuveson, D.A. (2005). Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 7, 469-83.

Hiraoka, N., Onozato, K., Kosuge, T., and Hirohashi, S. (2006). Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res 12, 5423-34.

Ho, P., Meeth, K.M., Tsui, Y., Srivastava, B., Bosenberg, M.W., and Kaech, S.M. (2014). Immune-based antitumor effects of BRAF inhibitors rely on signaling by CD40L and IFNγ. Cancer Res 74, 3205-3217.

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., et al. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. N. Engl. J. Med. 363, 711-23.

Howlader N., Noone A.M., Krapcho M., Garshell J., Neyman N., Altekruse S.F., Kosary C.L., Yu M., Ruhl J., Tatalovich Z., Cho H., Mariotto A., Lewis D.R., Chen H.S., Feuer E.J., Cronin K.A. (eds). SEER Cancer Statistics Review, 1975-2010, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site, April 2013.

Huang, A.Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., Levitsky, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science 264, 961-5.

Huang, R.R., Jalil, J., Economou, J.S., Chmielowski, B., Koya, R.C., Mok, S., Sazegar, H., Seja, E., Villanueva, A., Gomez-Navarro, J., et al. (2011). CTLA4 blockade induces frequent tumor infiltration by activated lymphocytes regardless of clinical responses in humans. Clin Cancer Res 17, 4101-9.

Iwama, S., De Remigis, A., Callahan, M.K., Slovin, S.F., Wolchok, J.D., and Caturegli, P. (2014). Pituitary expression of CTLA-4 mediates hypophysitis secondary to administration of CTLA-4 blocking antibody. Sci Transl Med 6, 230ra45.

Ji, R., Chasalow, S.D., Wang, L., Hamid, O., Schmidt, H., Cogswell, J., Alaparthy, S., Berman, D., Jure-Kunkel, M., Siemers, N.O., et al. (2011). An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol. Immunother. 61, 1019-31.

Jones, S., Zhang, X., Parsons, D.W., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A., Hong, S., et al. (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321, 1801-6.

Josefowicz, S.Z., Lu, L., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. Annu. Rev. Immunol. 30, 531-64.

Keir, M.E., Butte, M.J., Freeman, G.J., and Sharpe, A.H. (2008). PD-1 and its ligands in tolerance and immunity. Annu. Rev. Immunol. 26, 677-704.

Khong, H.T., and Restifo, N.P. (2002). Natural selection of tumor variants in the generation of “tumor escape” phenotypes. Nat. Immunol. 3, 999-1005.

Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J., and Schreiber, R.D. (2007). Adaptive immunity maintains occult cancer in an equilibrium state. Nature 450, 903-7.

Kopp, J., von Figura, G., Mayes, E., Liu, F., Dubois, C., Morris, J., Pan, F., Akiyama, H., Wright, C.E., Jensen, K., et al. (2012). Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma. Cancer Cell 22, 737-50.

Kvistborg, P., Philips, D., Kelderman, S., Hageman, L., Ottensmeier, C., Joseph-Pietras, D., Welters, M.J.P., van der Burg, S., Kapiteijn, E., Michielin, O., et al. (2014). Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. Sci Transl Med 6, 254ra128.

Le, D.T., Lutz, E., Uram, J.N., Sugar, E.A., Onners, B., Solt, S., Zheng, L., Donehower, R.C., Jaffee, E.M., Laheru, D.A., et al. (2013). Evaluation of ipilimumab in combination with allogeneic pancreatic tumor cells transfected with a GM-CSF gene in previously treated pancreatic cancer. J. Immunother. 36, 382-9.

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

Lyford-Pike, S., Peng, S., Young, G.D., Taube, J.M., Westra, W.H., Akpeng, B., Bruno, T.C., Richmon, J.D., Wang, H., Bishop, J.A., et al. (2013). Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res 73, 1733-41.

Mangsbo, S.M., Sandin, L.C., Anger, K., Korman, A.J., Loskog, A., and Tötterman, T.H. (2010). Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. J. Immunother. 33, 225-35.

Mellor, A.L., and Munn, D.H. (2008). Creating immune privilege: active local suppression that benefits friends, but protects foes. Nature Reviews. Immunology 8, 74-80.

Naito, Y., Saito, K., Shiiba, K., Ohuchi, A., Saigenji, K., Nagura, H., and Ohtani, H. (1998). CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 58, 3491-4.

Nishimura, H., Okazaki, T., Tanaka, Y., Nakatani, K., Hara, M., Matsumori, A., Sasayama, S., Mizoguchi, A., Hiai, H., Minato, N., et al. (2001). Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 291, 319-22.

Nowak, A.K., Robinson, B.W.S., and Lake, R.A. (2003). Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. Cancer Res 63, 4490-6.

Odorizzi, P.M., and Wherry, E.J. (2012). Inhibitory receptors on lymphocytes: insights from infections. J Immunol 188, 2957-2965.

Olive, K.P., Jacobetz, M.A., Davidson, C.J., Gopinathan, A., McIntyre, D., Honess, D., Madhu, B., Goldgraben, M.A., Caldwell, M.E., Allard, D., et al. (2009). Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 324, 1457-61.

Özdemir, B., Pentcheva-Hoang, T., Carstens, J., Zheng, X., Wu, C., Simpson, T., Laklai, H., Sugimoto, H., Kahlert, C., Novitskiy, S., et al. (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell 25, 719-34.

Page, D.B., Postow, M.A., Callahan, M.K., Allison, J.P., and Wolchok, J.D. (2013). Immune Modulation in Cancer with Antibodies. Annu Rev Med 65, 185-202.

Pagès, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R., Mlecnik, B., Kirilovsky, A., Nilsson, M., Damotte, D., et al. (2005). Effector memory T cells, early metastasis, and survival in colorectal cancer. N. Engl. J. Med. 353, 2654-66.

Paley, M.A., Kroy, D.C., Odorizzi, P.M., Johnnidis, J.B., Dolfi, D.V., Barnett, B.E., Bikoff, E.K., Robertson, E.J., Lauer, G.M., Reiner, S.L., et al. (2012). Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science 338, 1220-1225.

Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12, 252-64.

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S.V., Linsley, P.S., Thompson, C.B., and Riley, J.L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol. Cell. Biol. 25, 9543-53.

Parsa, A.T., Waldron, J.S., Panner, A., Crane, C.A., Parney, I.F., Barry, J.J., Cachola, K.E., Murray, J.C., Tihan, T., Jensen, M.C., et al. (2006). Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. Nat. Med. 13, 84-8.

Peggs, K.S., Quezada, S.A., Chambers, C.A., Korman, A.J., and Allison, J.P. (2009). Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J. Exp. Med. 206, 1717-25.

Phan, G.Q., Yang, J.C., Sherry, R.M., Hwu, P., Topalian, S.L., Schwartzentruber, D.J., Restifo, N.P., Haworth, L.R., Seipp, C.A., Freezer, L.J., et al. (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc. Natl. Acad. Sci. U.S.A. 100, 8372-7.

Powles, T., Eder, J.P., Fine, G.D., Braiteh, F.S., Loriot, Y., Cruz, C., Bellmunt, J., Burris, H.A., Petrylak, D.P., Teng, S., et al. (2014). MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 515, 558-62.

Provenzano, P., Cuevas, C., Chang, A., Goel, V., Von Hoff, D., and Hingorani, S. (2012). Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. Cancer Cell 21, 418-29.

Pylayeva-Gupta, Y., Lee, K., Hajdu, C., Miller, G., and Bar-Sagi, D. (2012). Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. Cancer Cell 21, 836-47.

Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., et al. (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 332, 600-3.

Rahib, L., Smith, B.D., Aizenberg, R., Rosenzweig, A.B., Fleshman, J.M., and Matrisian, L.M. (2014). Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 74, 2913-21.

Reichert, M., Takano, S., Burstin, von, J., Kim, S., Lee, J., Ihida-Stansbury, K., Hahn, C., Heeg, S., Schneider, G., Rhim, A.D., et al. (2013). The Prrx1 homeodomain transcription factor plays a central role in pancreatic regeneration and carcinogenesis. Genes Dev. 27, 288-300.

Rhim, A.D., Mirek, E.T., Aiello, N.M., Maitra, A., Bailey, J.M., McAllister, F., Reichert, M., Beatty, G.L., Rustgi, A.K., Vonderheide, R.H., et al. (2012). EMT and dissemination precede pancreatic tumor formation. Cell 148, 349-61.

Rhim, A., Oberstein, P., Thomas, D., Mirek, E., Palermo, C., Sastra, S., Dekleva, E., Saunders, T., Becerra, C., Tattersall, I., et al. (2014). Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer Cell 25, 735-47.

Robert, C., Ribas, A., Wolchok, J.D., Hodi, F.S., Hamid, O., Kefford, R., Weber, J.S., Joshua, A.M., Hwu, W., Gangadhar, T.C., et al. (2014). Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet 384, 1109-17.

Robert, C., Thomas, L., Bondarenko, I., O'Day, S., Weber, J., Garbe, C., Lebbe, C., Baurain, J., Testori, A., Grob, J., et al. (2011). Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N. Engl. J. Med. 364, 2517-26.

Royal, R.E., Levy, C., Turner, K., Mathur, A., Hughes, M., Kammula, U.S., Sherry, R.M., Topalian, S.L., Yang, J.C., Lowy, I., et al. (2010). Phase 2 trial of single agent

Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. J. Immunother. 33, 828-33.

Rustgi, A.K. (2006). The molecular pathogenesis of pancreatic cancer: clarifying a complex circuitry. Genes Dev. 20, 3049-53.

Sandin, L.C., Eriksson, F., Ellmark, P., Loskog, A.S., Tötterman, T.H., and Mangsbo, S.M. (2014). Local CTLA4 blockade effectively restrains experimental pancreatic adenocarcinoma growth in vivo. Oncoimmunology 3, e27614.

Scarlett, U.K., Cubillos-Ruiz, J.R., Nesbeth, Y.C., Martinez, D.G., Engle, X., Gewirtz, A.T., Ahonen, C.L., and Conejo-Garcia, J.R. (2009). In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. Cancer Res 69, 7329-37.

Schreiber, R.D., Old, L.J., and Smyth, M.J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 331, 1565-1570.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J., and Schreiber, R.D. (2001). IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410, 1107-11.

Shepard, H.M., Jacobetz, M.A., Chan, D.S., Neesse, A., Bapiro, T.E., Cook, N., Frese, K.K., Feig, C., Nakagawa, T., Caldwell, M.E., et al. (2012). Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. Gut 62, 112-20.

Sherman, M.H., Yu, R.T., Engle, D.D., Ding, N., Atkins, A.R., Tiriac, H., Collisson, E.A., Connor, F., Van Dyke, T., Kozlov, S., et al. (2014). Vitamin d receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. Cell 159, 80-93.

Simpson, T.R., Li, F., Montalvo-Ortiz, W., Sepulveda, M.A., Bergerhoff, K., Arce, F., Roddie, C., Henry, J.Y., Yagita, H., Wolchok, J.D., et al. (2013). Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J. Exp. Med. 210, 1695-710.

Soares, K.C., Rucki, A.A., Wu, A.A., Olino, K., Xiao, Q., Chai, Y., Wamwea, A., Bigelow, E., Lutz, E., Liu, L., et al. (2014). PD-1/PD-L1 Blockade Together With

Vaccine Therapy Facilitates Effector T-Cell Infiltration Into Pancreatic Tumors. J. Immunother. 38, 1-11.

Spranger, S., Spaapen, R.M., Zha, Y., Williams, J., Meng, Y., Ha, T.T., and Gajewski, T.F. (2013). Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med 5, 200ra116.

Street, S.E., Cretney, E., and Smyth, M.J. (2001). Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood 97, 192-7.

Sznol, M., and Chen, L. (2013). Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. Clin Cancer Res 19, 1021-1024.

Taube, J.M., Anders, R.A., Young, G.D., Xu, H., Sharma, R., McMiller, T.L., Chen, S., Klein, A.P., Pardoll, D.M., Topalian, S.L., et al. (2012). Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 4, 127ra37.

Taube, J.M., Klein, A., Brahmer, J.R., Xu, H., Xiaoyu, P., Kim, J.H., (2014). Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 20, 5064-74.

Thomas, L. (1959) in Cellular and Humoral Aspects of the Hypersensitive States, ed. Lawrence, H.S. 529-32. Hoeber-Harper, New York.

Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3, 541-7.

Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., Atkins, M.B., et al. (2012). Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N. Engl. J. Med. 366, 2443-54.

Tumeh, P.C., Harview, C.L., Yearley, J.H., Shintaku, I.P., Taylor, E.J.M., Robert, L., Chmielowski, B., Spasic, M., Henry, G., Ciobanu, V., et al. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 515, 568-71.

Velcheti, V., Schalper, K.A., Carvajal, D.E., Anagnostou, V.K., Syrigos, K.N., Sznol, M., Herbst, R.S., Gettinger, S.N., Chen, L., and Rimm, D.L. (2013). Programmed death ligand-1 expression in non-small cell lung cancer. Lab. Invest. 94, 107-16.

Vesely, M.D., Kershaw, M.H., Schreiber, R.D., and Smyth, M.J. (2011). Natural innate and adaptive immunity to cancer. Annu. Rev. Immunol. 29, 235-71.

Von Hoff, D.D., Ervin, T., Arena, F.P., Chiorean, E.G., Infante, J., Moore, M., Seay, T., Tjulandin, S.A., Ma, W.W., Saleh, M.N., et al. (2013). Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N. Engl. J. Med. 369, 1691-703.

Vonderheide, R.H., and Bayne, L.J. (2013). Inflammatory networks and immune surveillance of pancreatic carcinoma. Curr. Opin. Immunol. 25, 200-5.

Vonderheide, R.H., Bajor, D.L., Winograd, R., Evans, R.A., Bayne, L.J., and Beatty, G.L. (2013). CD40 immunotherapy for pancreatic cancer. Cancer Immunol. Immunother. 62, 949-54.

Wherry, E.J., Ha, S., Kaech, S.M., Haining, W.N., Sarkar, S., Kalia, V., Subramaniam, S., Blattman, J.N., Barber, D.L., and Ahmed, R. (2007). Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity 27, 670-684.

Williams, E.L., Dunn, S.N., James, S., Johnson, P.W., Cragg, M.S., Glennie, M.J., and Gray, J.C. (2013). Immunomodulatory monoclonal antibodies combined with peptide vaccination provide potent immunotherapy in an aggressive murine neuroblastoma model. Clin Cancer Res 19, 3545-3555.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science 322, 271-5.

Wolchok, J.D., Kluger, H., Callahan, M.K., Postow, M.A., Rizvi, N.A., Lesokhin, A.M., Segal, N.H., Ariyan, C.E., Gordon, R., Reed, K., et al. (2013). Nivolumab plus ipilimumab in advanced melanoma. N. Engl. J. Med. 369, 122-33.

Yu, P., Spiotto, M.T., Lee, Y., Schreiber, H., and Fu, Y. (2003). Complementary role of CD4+ T cells and secondary lymphoid tissues for cross-presentation of tumor antigen to CD8+ T cells. J. Exp. Med. 197, 985-95.

Zamarin, D., Holmgaard, R.B., Subudhi, S.K., Park, J.S., Mansour, M., Palese, P., Merghoub, T., Wolchok, J.D., and Allison, J.P. (2014). Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. Sci Transl Med 6, 226ra32.

Zhang, L., Conejo-Garcia, J.R., Katsaros, D., Gimotty, P.A., Massobrio, M., Regnani, G., Makrigiannakis, A., Gray, H., Schlienger, K., Liebman, M.N., et al. (2003). Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N. Engl. J. Med. 348, 203-13.

Zhang, Y., Yan, W., Mathew, E., Bednar, F., Wan, S., Collins, M.A., Evans, R.A., Welling, T.H., Vonderheide, R.H., and di Magliano, M.P. (2014). CD4+ T lymphocyte ablation prevents pancreatic carcinogenesis in mice. Cancer Immunol Res 2, 423-35.

Zhou, Q., Xiao, H., Liu, Y., Peng, Y., Hong, Y., Yagita, H., Chandler, P., Munn, D.H., Mellor, A., Fu, N., et al. (2010). Blockade of programmed death-1 pathway rescues the effector function of tumor-infiltrating T cells and enhances the antitumor efficacy of lentivector immunization. J. Immunol. 185, 5082-92.

Zhu, Y., Knolhoff, B.L., Meyer, M.A., Nywening, T.M., West, B.L., Luo, J., Wang-Gillam, A., Goedegebuure, S.P., Linehan, D.C., and DeNardo, D.G. (2014). CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. Cancer Res 74, 5057-69.