T CELL IMMUNOSURVEILLANCE IN PANCREATIC DUCTAL ADENOCARCINOMA

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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

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ACKNOWLEDGEMENTS

I would like to express gratitude to my thesis advisor, Dr. Robert Vonderheide, for his exceptional mentorship throughout this rigorous and demanding educational endeavor. His advice and support have been invaluable during the course of my PhD. I would also like to thank Dr. Gregory Beatty for his supervision and guidance during my rotation in Dr. Vonderheide's laboratory, which helped develop my foundations as a graduate student both technically at the bench and professionally in the field. Additionally, I thank Dr. Michael Atchison for his superb leadership in the combined degree VMD/PhD program and his help in navigating my graduate school career.

ABSTRACT

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The prevailing theory of cancer immune surveillance, as understood from carcinogen-driven mouse models of highly mutated tumors, states that T cell-mediated immune pressure drives a continuum of tumor elimination, equilibrium, and escape. This "Triple E Hypothesis" reflects host-tumor interactions in a subset of human cancers that have responded well to cancer immunotherapy, including melanoma, small cell lung carcinoma, and bladder carcinoma; yet, many tumors remain refractory to such interventions. These clinical failures suggest that immunosurveillance in other cancers manifests with a fundamentally different biology than previously described.

Here, using a genetic mouse model of spontaneous pancreatic carcinoma that features an immunosuppressive microenvironment and few non-synonymous mutations, we report that the natural history of such cancers is T cell-independent. Furthermore, tumor escape from T cell surveillance is not required for cancer progression, as tumor cells arising in T cell-depleted genetic mice grow unchecked in immune-competent hosts upon implantation. Checkpoint blockade with CTLA-4 and PD-1 antibodies does not expose mutant epitopes strong enough to elicit therapeutic responses; combined with

whole exome sequencing of PDA-derived murine cell lines, these findings confirm that that PDA does not harbor a mutational burden commensurate with the current hypothesis of immunosurveillance. However, ectopic expression of a neo-antigen in PDA tumor cells is sufficient to restore immunosurveillance, override its immunosuppressive microenvironment, and establish T cell memory against "quiescent" endogenous antigens. Thus, cardinal features of tumor immunosurveillance are elicited by an antigen of sufficient strength irrespective of microenvironmental immunosuppression. The finding that antigen strength itself is a critical determinant of cancer immunosurveillance informs future clinical approaches for the majority of human tumors that are "immunologically cold" and refractory to current immunotherapies.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

MANUSCRIPTS

The following manuscripts are discussed in this thesis:

Evans, R.A., Diamond, M.S., Rech, A.J., Chao, T., Richardson, M.W., Lin, J.H., Bajor, D.L., Byrne, K.T., Stanger, B.Z., Riley, J.L., Markosyan, N., Winograd, R., and Vonderheide, R.H. (2015). Neo-antigen overrides tumor microenvironment in determining cancer immunosurveillance.

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. (2015). Induction of T cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma. Cancer Immunol Res. *3,* 399-411.

Beatty, G.L., Winograd, R., **Evans, R.A**., Long, K.B., Luque, S.L., Lee, J.W., Clendenin, C., Gladney, W.L., Knoblock, D.M., Guirnalda, P.D., and Vonderheide, R.H. (2015). Exclusion of T Cells From Pancreatic Carcinomas in Mice is Regulated by Ly6C(low) F4/80(+) Extratumoral Macrophages. Gastroenterology, *149,* 201-10.

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

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:

Markosyan N., Chen E.P., **Evans R.A**., Ndong V., Vonderheide R.H., Smyth EM. (2013). Mammary Carcinoma Cell Derived Cyclooxygenase 2 suppresses Tumor Immune Surveillance by Enhancing Intratumoral Immune Checkpoint Activity. Breast Cancer Res. *15*, R75

CHAPTER 1 – Introduction

Cancer immune surveillance

Anti-tumor immunity is now widely acknowledged as a significant impediment to cancer formation, akin to an extrinsic tumor suppressor system; currently, immune evasion is defined in the field as an "emerging hallmark" of cancer progression (Hanahan and Weinberg, 2011). For decades, however, the field of tumor immunology was tangential to cancer biology despite the supposition that immunity might target malignancies with the same potency observed against bacteria, viruses, and parasites. The earliest scientific foray into tumor immunology harnessed the known antibacterial function of the immune system in the hands of Dr. William Coley, who observed a remarkable case of recurrent sarcoma that was eradicated upon accidental contraction of erysipelas (Coley, 1891). In a series of trials in patients with recurrent and inoperable sarcomas, Coley intentionally inoculated the patients' tumors with various strains of erysipelas; in a number of cases, notably those where patients suffered a full onset of erysipelas infection, the tumors either shrank or dissolved completely. Thus, the field of tumor immunology in its nascent form was born in 1891, and "Coley's toxins" have since been described as the first clinical trial in this field.

In 1909, Paul Ehrlich inferred from these clinical observations that the immune system might function as a sentinel-like presence against tumor formation, and only in its occasional failures would tumors become clinically evident (Dunn et al., 2002). A more

formal hypothesis was stated by Burnet and Thomas in 1957, where the term "immune surveillance" was first coined to describe the immune system's putative protective role against cancer development. Their hypothesis incorporated the likely genetic basis for immune recognition of these tumors, stating "inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step towards malignancy" and that such cells have "new antigenic potentialities" (Burnet, 1957, 1964; Burnet, 1970). This postulation was unfortunately confounded by mounting evidence that tumor engraftment and rejection studies in mice merely represented an allograft rejection, not true tumor surveillance against tumor antigens (Billingham et al., 1956). Further studies would clearly require syngeneic hosts for any conclusive answers.

The first rigorous scientific assessments of immunosurveillance involved various methods of inducing immune incompetence in mice followed by observation for increased susceptibility to tumor formation (Grant and Miller, 1965; Balner and Dersjant, 1966; Burstein and Law, 1971; Trutin-Ostovic et al., 1986). Not surprisingly, an increase in virally-induced tumors and lymphomas was the result of this early work; in mice subjected to thymectomy or pharmacological means of immune suppression, this was primarily attributed to the well-known function of the immune system to protect against viruses that are potentially transformative. In the instance of increased lymphomas, this was attributed to chronic inflammation that was a byproduct of bacterial and/or viral susceptibility. The work was otherwise inconclusive regarding carcinogen-induced cancers and the development of spontaneous non-viral tumors; if anything, these studies suggested no effect by the immune system on tumor development.

The advent of genetic models of immune incompetence, namely the athymic nude mouse, instilled new hope for conclusive evidence of an anti-tumor surveillance function for the immune system. In the seminal studies performed by Stutman *et al.*, the theory of immunosurveillance seemed to be definitively discredited; athymic nude mice did not demonstrate a decreased latency in tumor onset or increased incidence post-injection of methylcholanthrene, despite an inability to reject allografts (Stutman, 1974, 1979b, a). The work by Stutman and colleagues led to the temporary dissolution of the immunosurveillance hypothesis. Later work, however, revealed key limitations in the nude mouse as a model of immunodeficiency; these mice were shown to harbor detectable levels of αβ T cells in addition to extra-thymic production of γδ T cells (Ikehara et al., 1984; Maleckar and Sherman, 1987). NK cells are also known to be intact in this model, and their contribution to surveillance was not assessed in the Stutman study. Moreover, the CBA/H background for the nude mice used by Stutman *et al* is now known to have hyper-transformative properties due to a particular enzyme involved in methylcholanthrene biotransformation to the carcinogenic form (Dunn et al., 2002).

The supposedly defunct immunosurveillance hypothesis experienced a revival with the discovery of carcinogenic susceptibility in IFNγ and perforin-deficient mice, suggestive of two immunological pathways involved in surveillance (Dighe et al., 1994; Street et al., 2001). In a series of landmark studies by Schreiber and colleagues beginning in 2001, the immunosurveillance hypothesis was virtually resurrected and redefined in a new context: the hypothesis of immunoediting. In the first of these seminal studies, Schreiber and colleagues induced tumor formation by injection of methylcholanthrene into the flank of immunocompetent or *RAG2*-null mice that lack

mature T and B cells (Shankaran et al., 2001). Immunodeficiency was shown to augment tumorigenesis in *RAG2*-null mice, suggesting an *elimination* phase of immunosurveillance whereby adaptive immunity can recognize neoplastic cells and destroy them. This elimination of tumor cells recalled the former suggestion of a sentinel function by the immune system as stated by Burnet and Thomas. However, the study revealed new complexity regarding the interplay between the immune system and tumors; clearly, this was not an "all or nothing" interaction. 40% of tumors derived from the *RAG2-/-* mice were rejected in immune-competent hosts following transplantation, whereas tumors derived from wild-type mice grew without any seeming protection from the immune system. These findings suggested the adaptive immune system's potential for "sculpting" a tumor's behavior; these tumors have to *escape* immune pressure in order to progress, and this is accomplished by selection of stochastic clones that are not susceptible to T cell recognition or killing. Additionally, they crossed the *RAG2-/-* mouse to *STAT1-/-* mice (RkSk mice) to assess the overlap between lymphocytic control and IFNγ-mediated immunity, known to function through STAT1 signaling (Bach et al., 1997). Only a marginal increase in tumor incidence was observed with administration of methylcholanthrene in the double knockout compared to either individual knockout model, indicative of significant overlap between these two anti-tumor systems.

A third state of host-tumor interactions was defined in a subsequent study, referred to as "equilibrium" (Koebel et al., 2007). Schreiber and colleagues subjected C57BL/6 wildtype mice to low doses of methylcholanthrene, and monitored any tumor development for 200 days. Those mice that developed progressive tumors were removed from the study, whereas those with stable tumors (or lack thereof) were further subjected

to a T-cell depleting antibody cocktail or an IgG isotype control. Those mice in the immune-compromised cohort developed rapid outgrowth of tumors in comparison to the control cohort. Delayed *de novo* onset of tumors was ruled out by comparing the time to tumor formation in *RAG2-/-* mice post-MCA with time to formation in wild-type mice subjected to T cell depletion at day 200; the rapidity of measurable tumor growth after day 200 in T cell-depleted mice suggested outgrowth of occult tumor cells rather than the *de novo* formation seen in *RAG2-/-* mice. Thus, Schreiber and colleagues hypothesized that fully transformed cells were being restrained in a static state, and were released to the escape phase upon interference with T cell-mediated immunity. This study led to the current prevailing theory of immunosurveillance, known as the "Triple E hypothesis." This theory encompassed not only the Burnet and Thomas concept of *elimination*, but also incorporated *equilibrium* and *escape* phases as extensions of their original hypothesis for a more comprehensive description of host-tumor interactions.

The first E: Elimination

As first suggested by Paul Ehrlich and confirmed by Schreiber, clinically apparent cancers in immune competent hosts represent a mere fraction of the transformed cells that are eradicated throughout an individual's lifetime, due to complete control in a majority of instances and truncation of the full immunoediting process (Dunn et al., 2004). Thus, the immune system represents an extrinsic line of defense when the cell intrinsic mechanisms of DNA damage repair and apoptosis have failed. This eradication of nascent, transformed cells by the coordinated response of innate and adaptive immunity

is indeed demonstrated in numerous clinical examples. The presence of CD3+ tumor-infiltrating lymphocytes (TILs) has been correlated with better prognosis for both ovarian and colorectal cancer, among many others (Zhang et al., 2003; Galon et al., 2006). The increased incidence of cancer in immunodeficient human populations corroborates this protective role (Frisch et al., 2001; MacKie et al., 2003). Therefore, clinical evidence supports a model whereby the immune system can eradicate cancerous cells, which may be an ongoing process even in cancers that have continued to progress.

The mechanisms by which the immune system recognizes and destroys transformed cells are still being elucidated, although significant strides have been made in understanding this complex process. Innate immunity and adaptive immunity coalesce to provide the integrated control required for recognition and eradication of diseased cells. Integral to this process appears to be the production of the cytokine IFNγ, as demonstrated by the early knockout studies (Dighe et al., 1994; Shankaran et al., 2001), but this cascade requires numerous preliminary steps. Cancerous cells instigate a local inflammatory reaction through the production of various "danger signals," such as the secretion of uric acid (Shi et al., 2003), or by triggering inflammatory signaling pathways through heat shock proteins or extracellular matrix products such as hyaluronic acid and heparan sulfate (Dunn et al., 2004). Transformed cells experience metabolic stress by virtue of their hyperproliferative state, leading to the upregulation of certain MHC-I-related molecules on the surface of these cells, notably MIC-A, MIC-B, and Rae1 (Groh et al., 1999; Diefenbach et al., 2001). These molecules function as ligands for the activating NK cell receptor, NKG2D, which stimulates the PI 3-kinase pathway and ultimately generates IFNγ production by the cell (Khong and Restifo, 2002).

Innate-like lymphocytes, known as γδ T cells, likewise induce IFNγ at early timepoints in response to NKG2D ligation or direct recognition of antigens (Girardi et al., 2001; Gao et al., 2003). Not only are these cells shown to be recruited as early as day 3 following MCA induction of sarcomas, but moreover bone marrow chimeras where mice were reconstituted with IFNγ-deficient γδ cells and functional αβ cells led to an increased susceptibility to MCA-induced carcinogenesis and B16 melanoma implants (Gao et al., 2003). NKT cells similarly demonstrate robust and early production of IFNγ; their invariant receptor recognizes aberrant glycolipids.

The overarching effect of these early responders is to secrete a critical mass of the IFNγ cytokine at the site of tumorigenesis, which provides the essential link between innate and adaptive anti-tumor immunity (Dighe et al., 1994; Bromberg et al., 1996; Kumar et al., 1997; Kaplan et al., 1998; Street et al., 2002). The effects of IFNγ are manifold; in addition to recruiting antigen presenting cells (APCs) such as macrophages and dendritic cells, IFNγ itself can induce a degree of tumor cell death directly or through inhibition of neovascularization and subsequent ischemic necrosis. Such immunogenic cell death releases tumor-associated antigens, in the context of pro-inflammatory "danger-associated molecular patterns" (DAMPs), which can be taken up by the APCs through phagocytosis or macropinocytosis (Zitvogel et al., 2010). These cells traffic to local lymph nodes, where they encounter their cognate CD4+ (helper) and CD8+ (effector) T cells. Activated helper CD4+ T cells "license" APC's via ligation of CD40, which upregulates various costimulatory molecules on the surface of the APC, such that cross-presentation of antigen on MHC class I ("signal 1") in the context of co-stimulation ("signal 2") activates the cognate CD8+ T cell receptor. Subsequently, these

lymphocytes are induced to proliferate and mature, resulting in an effector phenotype capable of trafficking to the site of tumorigenesis.

Once the adaptive immune response has been engaged in this manner, innate and adaptive immunity synergize to eliminate the tumor cells. A predominant component of effector function in CD8+ T cells is perforin, a cytotoxic protein secreted via degranulation (van den Broek et al., 1996; Smyth et al., 2000). Experimentally, mice lacking perforin or IFNγ demonstrated a similar increase in susceptibility to carcinogenesis that was not significantly enhanced in the double knockout, suggesting significant overlap of these two pathways (Street et al., 2001). Serine proteases called granzymes are also present in these granules; the two proteins function concomitantly in a calcium-dependent manner to kill the target cell. When the peptide-MHC class I complex is bound by the cognate TCR of an activated T cell, degranulation occurs. Perforin forms a pore in the target cell membrane, through which the granzyme proteins enter the cell and initiate cleavage of caspase 3, triggering a cascade of events that results in DNA fragmentation and cell death (Khong and Restifo, 2002). This granular release is highly polarized at the immunological synapse, allowing for selective killing of the target cell. Meanwhile, an activated CD8 T cell will provide a new source of IFNγ, which induces an M-1 (anti-tumor) fate in macrophages (Mills et al., 1992). A tumoricidal phenotype in peritoneal macrophages has been shown to depend on CD40 ligation and stimulation by IFNγ, resulting in reduced proliferation of B16 melanoma cells in vitro, as well as an increase in apoptosis and nitrous oxide production (Buhtoiarov et al., 2005). In turn, such M1-skewed macrophages enhance the T cell anti-tumor response and create

a positive feedback loop that integrates both innate and adaptive immunity (Duluc et al., 2009).

CD4+ T cells have also demonstrated efficacy in the elimination phase, although they are often considered a secondary means of clearance compared to their CD8+ cytotoxic counterparts. Due to their dependence on MHC class II presentation, CD4 cells usually cannot recognize tumor cells directly; however, one example of a CD4-mediated anti-tumor response involves clearance of pre-malignant cells harboring a senescence-associated secretory phenotype (SASP) (Kang et al., 2011). Thus, cells that would otherwise remain and ultimately develop into malignancies are eliminated at early stages due to an aberrant SASP program.

The second E: Equilibrium

The most elusive stage of immunoediting can occur if the elimination process does not eradicate the tumor, but neither has full escape of immune control been achieved by the transformed cells – resulting in the covert presence of a sub-clinical tumor. Hypothetically, this period may be short or protracted depending on the degree of immune pressure and the opposing ability of the tumor to mutate and adapt. Indeed, this may be likened to a state of tumor dormancy or latency. Conclusive evidence of this intermediate "static" state remained underwhelming until an interesting case study published in the *New England Journal of Medicine* (MacKie et al., 2003). An organ

donor at Glasgow University, who had successfully undergone treatment for melanoma, died 15 years later of unrelated causes. Two patients received the donated kidneys; within two years of successful engraftment, however, each recipient developed secondary melanoma. Immunosuppressive therapy was ceased in both patients, one of whom succumbed to metastatic melanoma. The other rejected the diseased kidney following IFNγ treatment, and went on to survive without spread of the disease. This clinical evidence of an immune-mediated suppression of melanoma in the donor that subsequently progressed in immune-compromised patients suggests a state of static dormancy; the melanoma cells were never fully eliminated in the original donor, but they had been diminished to a state of clinical irrelevance prior to outgrowth in the recipients. This interesting case study prompted Schreiber and colleagues to interrogate the presence of such a state in a controlled mouse model, as previously described, and the term "equilibrium" was coined for this intermediate state (Koebel et al., 2007).

From the initial MCA-induced carcinoma study, it was apparent that equilibrium was mediated by CD4+ and CD8+ T cells as well as IFNγ. A follow-up study determined two further immune components critical to this stasis, which help define it as a discrete (and potentially prolonged) stage in the immunoediting process (Teng et al., 2012). The MCA model of carcinogenesis was again utilized to assess the interplay between the heterodimeric cytokines IL-23 and IL-12, which share a p40 subunit. Study of tumor-free mice 200, 300, or 400 days post-MCA induction revealed that equilibrium could be markedly prolonged until disruption of immunity with antibodies targeting T cells and IFNγ. Additionally, neutralizing antibodies targeting IL-23 and IL-12 in MCA-treated, disease-free mice resulted in decreased or increased tumor escape, respectively.

Administration of agonistic CD40 antibodies to promote IL-12 production by dendritic cells confirmed these findings; this treatment conferred a protective effect, as measured by a decrease in tumor escape upon disruption of T cell immunity. Overall, the data suggested a role for IL-23 in maintenance of occult tumor cells, whereas IL-12 inhibits progression to escape. Other cytokines important in elimination were shown to be irrelevant at this stage, distinguishing it as a unique step in the immunoediting process with a discrete set of regulating factors (Muller-Hermelink et al., 2008; Kang et al., 2011).

Equilibrium can also be achieved by simply preventing tumor outgrowth without actual elimination of tumor cells. As shown using the RIP-TAG mouse, in which Tag-driven carcinoma is specific to all pancreatic islet cells, adoptive transfer of Tag-specific CD4+ T cells did not cause autoimmune diabetes (Muller-Hermelink et al., 2008). At an age where untreated RIP-TAG mice typically developed multiple carcinoma lesions with robust vasculature, the same mice treated with the T-cell therapy had only small, hypovascular, pre-neoplastic lesions. Using immunohistology to detect $\alpha_v\beta_3$-expressing vessels as a measure of angiogenesis, the study concluded that such vessels were rare in mice given the adoptive transfer procedure. Likewise, proliferation was decreased in the islet cells of adoptively transferred TAG mice, as determined by BrdU labeling. Interestingly, arrest was *not* CD8-dependent in this study, indicating that CD4+ cells may frequently be involved with the equilibrium state since they only indirectly inhibit tumor growth rather than directly recognizing and killing MHC class I+ tumor cells. In the same model, another study demonstrated that Th-1 helper cytokines IFNγ and TNF were

shown to induce cell cycle arrest in G1/G0; the effect was lost in *Tnfr1*-null mice (Braumuller et al., 2013).

The third E: Escape

If tumors escape immune control, they are described as having been "immunoedited" by their interaction with the immune system. These escaped tumor populations have been selected for cell-autonomous properties that permit them to grow in the presence of a functional immune system. Schreiber and colleagues have recently used the MCA mouse model to describe a principal mechanism of escape: downregulation of a particular antigenic epitope as determined by exome sequencing of parent lines and escaped daughter clones (Matsushita et al., 2012). Similarly, downregulation of MHC class I molecules by tumor cells has been implicated in many instances of immune escape. In a landmark melanoma study of human biopsy samples, known tumor antigens like Melan A/MART-1 and tyrosinase were not lost in all cases of progressive melanoma; 9/20 tumors had in fact lost expression of MHC class I, thereby rendering the antigens invisible to the immune system (Jager et al., 1997). Even within a single individual, a combination of these escape mechanisms may be seen in recurrent tumors or metastases, some of which lose antigen expression, while some lesions may lose MHC class I expression (Khong et al., 2004). Other malfunctions in the antigen processing pathway are likewise implicated in cancer progression. The peptide transporters, TAP-1 and TAP-2, have been downregulated in cases of small lung cell carcinoma, non-small cell lung

cancer, prostate carcinoma, and renal cell carcinoma (Restifo et al., 1993; Sanda et al., 1995; Korkolopoulou et al., 1996). IL-10 production by tumor cells has been implicated in this downregulation, representing an autocrine means of escape.

Cancer cells may also develop mutations that disrupt the apoptotic pathway and render them resistant to T cell killing. Downregulation of Fas expression may occur directly, or downstream signaling by cleaved caspase may be disrupted upon upregulation of cFLIP, a protein that inhibits caspase-8 (Khong and Restifo, 2002). Even perforin and granzyme B-induced killing can be subverted in the presence of the serine protease inhibitor PI-9 (Medema et al., 2001). Tumors may also exert negative influences on otherwise reactive T cells, sometimes directly by death receptor ligation. FAS-L upregulation on various tumor types has been reported clinically in lung carcinoma, melanoma, colon carcinoma, and hepatocellular carcinoma (Khong and Restifo, 2002); presumably, ligation of any FAS+ T cell would be susceptible to apoptosis. Despite this interesting observation, its implications are still unclear and these data remain mostly correlative. In other studies, FAS-L expression was not observed at either the mRNA or protein levels; moreover, in vitro exposure of melanoma cells to FAS+ T cells did not induce killing (Chappell et al., 1999). A more relevant and likely scenario for apoptosis of tumor-reactive T cells is the upregulation of FAS-L upon antigen recognition within the tumor, a phenomenon known as activation-induced cell death (AICD), resulting in "suicide" of the cell itself or "fratricide" of bystander FAS+ T cells (Zaks et al., 1999). Each of these examples represents the conventional understanding of immunoediting in which edited tumors possess *direct* means of escaping immunity, either by avoiding recognition or immune-mediated killing.

Tumors may also *indirectly* hamper T cell efficacy through manipulating the tumor microenvironment. As critical regulators of T cell priming, dendritic cells are frequently co-opted by tumors to disrupt T cell surveillance. IL-10 produced by tumor cells can inhibit dendritic cell maturation and function, leading to sub-optimal priming (Rabinovich et al., 2007). Tumor-derived cytokines like TGF-β1 have been shown to promote dysfunctional antigen presentation via the upregulation of tolerogenic PDL-1 on dendritic cells (Scarlett et al., 2012).

In numerous cancers, tumor-associated myeloid cells have been extensively shown to disrupt T cell function through a variety of mechanisms. These cells may induce T cell apoptosis by secretion of nitric oxide (Rabinovich et al., 2007) or arginase-1, which depletes the microenvironment of critical T cell nutrients like L-arginine (Albina and Henry, 1991; Rodriguez et al., 2004). Tumor-derived GM-CSF has recently been observed to recruit myeloid-derived suppressor cells (MDSCs) in the setting of pancreatic ductal adenocarcinoma, resulting in abrogation of perforin-dependent T cell function (Bayne et al., 2012). The hypoxic microenvironment typical of many solid tumors can also upregulate HIF-1α in tumor-associated myeloid cells, which induces an immunosuppressive effect on lymphocytes. In the MMTV-PyMT model of mammary carcinogenesis, the generation of myeloid *HIF-1α*-null transgenics slowed tumor progression in a T cell-dependent manner (Doedens et al., 2010).

Macrophages can likewise be harnessed by tumors; in a process known as polarization, otherwise anti-tumor "M1" cells are redirected to a tumor-promoting "M2" fate by a specific cytokine milieu. M1 macrophages arise in the context of an

environment rich in IFNγ and bacterial products, whereas M2 macrophages develop under conditions of IL-4, IL-10, and IL-13 (Gabrilovich et al., 2012). In fact, Th2 cells within the tumor have been shown to be a source of IL-4 and alternative activation of macrophages; in the MMTV–PyMT model of mammary carcinogenesis, CD4-deficient mice demonstrated an abundance of type-1 cytokines and decreased M2 markers (DeNardo et al., 2009). The alternatively activated macrophages demonstrate anti-immune effects similar to their myeloid precursors, as described above. Additionally, they provide a source of CCL22 to attract another inhibitory population of cells known as regulatory T cells (Tregs) (Curiel et al., 2004). Tregs, in turn, have been shown to drive alternative skewing of macrophages by secreting IL-10 and preventing their sensitivity to LPS (Tiemessen et al., 2007), creating a positive feedback loop whereby various components of the tumor microenvironment synergize to construct a complex immunosuppressive network.

Such crosstalk between tumors and the stromal cells has thus been proposed as an alternative form of immunoediting, whereby the tumor retains its antigenicity and instead mediates immunosuppression in order to permit outgrowth. Directly or indirectly, escape mechanisms can permit paradoxical tumor progression within immunocompetent individuals. Similar to the antibiotic resistance observed in microbial strains, an immune response that incompletely suppresses tumor growth can inadvertently select for clones that have escaped this pressure. However, the term "tumor escape" is actually a misnomer; it implies an active and intentional behavior by tumor cells (Khong and Restifo, 2002). Rather, failure of T cell immunosurveillance most likely involves a passive process, whereby tumors evade T cells through inherent genomic instability,

subsequent heterogeneity, and ultimately natural selection of clones that can survive in a setting of immune-based pressure.

Although considered the prevailing view of immunosurveillance, the immunoediting hypothesis has been subject to various criticisms. Blankenstein and colleagues have proposed that a majority of immunogenic cancers will induce peripheral T cell tolerance rather than undergo tumor immunoediting; thus, he suggests that the failure of surveillance may ultimately be T cell intrinsic (Willimsky and Blankenstein, 2005; Willimsky et al., 2008). Another example of T cell dysfunction was suggested in a study of the gene expression profiles of various metastatic melanoma samples, where a subset of T cell-rich tumors paradoxically demonstrated increases in inhibitory molecules like IDO, PD-LI, and CCR4-binding chemokines that cause an influx of Tregs (Spranger et al., 2013). Interestingly, those tumors that were T cell-poor lacked the expression of these factors, suggesting a T cell-mediated immunosuppression. This finding was corroborated in mouse models of B16.SIY melanoma; in CD8-depleted mice, upregulation of inhibitory molecules failed to occur, and Treg trafficking was greatly reduced. Proponents of the immunoediting hypothesis, however, might point to the upregulation of such inhibitory molecules in the presence of T cells as "adaptive immune resistance" – essentially, a form of tumor escape. This "chicken-egg" question is still debated within the field of tumor immunology (Tumeh et al., 2014; Winograd et al., 2015).

Indeed, the constitutive expression of inhibitory molecules on the surface of T cells, including PD-1 and CTLA-4, can account for failed T cell responses in many

cancers; although beneficial for preventing auto-immunity and resolving inflammation, these molecules serve as a double-edged sword in the context of cancer. This counter-productive expression has formed the basis of checkpoint blockade therapy, which has led to dramatic clinical responses in certain cancers such as melanoma (Tumeh et al., 2014). Schreiber and colleagues utilized checkpoint inhibitors to prompt inert T cells to respond to "silent" neo-antigens in a progressor MCA-induced cell line (Gubin et al., 2014). Following checkpoint blockade of this cell line in host mice, Gubin *et al.* used RNA-seq of tumor-infiltrating lymphocytes to reveal that these cells had decreased markers of exhaustion, such as LAG-3 and TIM3. Without checkpoint intervention, expression of these molecules was similar to the levels observed during chronic viral infection. Thus, features inherent to T cell immunobiology – albeit protective of the organism in other settings – become counter-productive in certain cancers and account for tumor progression in the absence of tumor-derived "escape" mechanisms.

Pancreatic ductal adenocarcinoma and the KPC model

Pancreatic ductal adenocarcinoma is a notoriously devastating disease. It currently ranks 4th amongst cancer-associated deaths in the United States despite being only the 10th most-commonly diagnosed cancer, indicative of its highly aggressive nature (Siegel et al., 2012); however, it is predicted to surpass all but lung carcinoma by the year 2020 in its lethality (Rahib et al., 2014). Most patients present with a vague array of symptoms, which may be limited to abdominal pain, nausea and weight loss. Often the results of

bloodwork are similarly non-specific. A combination of jaundice, dysglycemia, and pancreatitis is more overtly suggestive of PDA. Regardless of the presenting complaints, most patients are diagnosed with advanced malignancy; even those few patients deemed to have resectable tumors usually succumb to metastatic disease, resulting in a 5-year survival rate of only 6% (Hidalgo, 2010; Siegel et al., 2012).

The treatment options available for PDA are indeed limited and insufficient. The previous standard-of-care chemotherapy, gemcitabine, extends lifespan by only a few months beyond palliative care, which remains the ultimate recourse for a majority of patients with advanced disease (Tempero et al., 2003). A combined chemotherapy regimen, FOLFIRINOX, was recently demonstrated to increase median overall survival to 11.1 months in patients with metastatic disease, but toxicity limits its use to candidates with a good performance status (Conroy et al., 2011). Similarly, the 2013 FDA approval of Nab-paclitaxel reflects the underwhelming advances in PDA treatment; a phase 3 clinical trial combining Nab-paclitaxel with gemcitabine extended median overall survival to 8.5 months in patients with metastatic disease, compared to 6.7 months in the gemcitabine monotherapy cohort (Von Hoff et al., 2013). Such minimal improvements underscore the need for alternative treatments, especially in light of an aging population and the striking lethality of this disease. Development of future therapies will require understanding what drives this uniquely aggressive biology.

Tuveson and colleagues recently developed a genetically engineered mouse model (GEMM) that will facilitate investigation of such concerns; this model closely recapitulates the molecular, histopathologic and clinical features of human PDA

(Hingorani et al., 2005). Human PDA is driven by the cumulative effect of successive driver and supporting mutations, beginning with a histological precursor known as pancreatic intraepithelial neoplasia (PanIN) (Hidalgo, 2010). Activation of the mutant *KRAS* oncogene is the most notable genetic aberration, occurring in greater than 95% of patients; this mutation causes a constitutively active GTPase, encouraging cellular proliferation. Subsequent mutations frequently include inactivation of the tumor suppressor gene *CDKN2A* (encoding INK4A), as well as two other tumor suppressor genes, *TP53* and *SMAD4* (Hidalgo, 2010). Accordingly, pancreatic cancer in the mouse model is driven by the targeted expression of activating $Kras^{G12D}$ and dominant negative $Trp53^{R172H}$, which are introduced embryonically by Cre-mediated recombination of LSL-cassettes in the pancreas, under control of the pancreas-specific promoter Pdx-1. At day 8 of embryonic development, the Pdx-1 promoter becomes active, and subsequent production of the Cre protein unlocks the transgenes and their production of the mutant proteins. The resulting "KPC" mouse thus reflects two of the most commonly mutated alleles in human PDA (Rustgi, 2006). Concomitant expression of these mutations in the KPC mouse leads to 100% penetrance of a highly invasive, genomically unstable carcinoma that parallels human PDA at all stages, including the robust desmoplastic reaction seen by histology (Hingorani et al., 2005).

Desmoplasia within PDA is driven in part by pancreatic stellate cells; in the context of growth factors, these cells upregulate α-smooth muscle actin and deposit an abundance of collagen fibers (Hidalgo, 2010). This hallmark reaction has garnered attention in various studies but remains a controversial topic. Previously, the dense stroma was shown to physically impede gemcitabine delivery (Olive et al., 2009;

Provenzano et al., 2012). Disruption of the stroma-promoting Hedgehog signaling pathway in the KPC model synergized with gemcitabine in a perfusion-dependent manner. Similarly, depletion of hyaluronic acid increased intratumoral vessel density and perfusion in this model, facilitating distribution of chemotherapy and improving survival.

More recently, however, two studies challenged this paradigm and demonstrated that the stromal compartment of PDA may function, at least in part, to limit tumor growth and invasion. Sonic hedgehog-null mice, when crossed with the KPC model, exhibited a more undifferentiated, aggressive phenotype and enhanced angiogenesis (Rhim et al., 2014). In a similar mouse model of PDA, known as the PKT model (Cre-mediated recombination of floxed TGFβ, in addition to KrasG12D), Ozdemir *et al*. crossed these mice with a ganciclovir-sensitive αSMA-tk transgenic mouse to temporally regulate the levels of αSMA+ myofibroblasts (Ozdemir et al., 2014). Depletion of the myofibroblasts in mice at early or late stages of tumor development decreased overall survival, which correlated with increased invasiveness and poor cellular differentiation. Thus, the complexity of the tumor microenvironment in PDA suggests that targeting the stroma may be more nuanced than initially thought.

The KPC model has been further manipulated by inducing expression of the YFP protein at the Rosa locus (Rhim et al., 2012). The lineage tracing permitted by this model has provided new insights into metastasis and epithelial-to-mesenchymal transition (EMT) in murine PDA. Cells of pancreatic epithelial origin were effectively traced regardless of their phenotype; surprisingly, these circulating cells were found in abundance throughout the blood as well as in the liver – a common site of metastasis –

even at the pre-malignant phase of pancreatic intraepithelial neoplasia (PanIN) in the pancreas. Contrary to the previous belief that metastasis occurs late in cancer progression, this study revealed that the lethality of PDA may be associated with early dissemination of pre-malignant cells.

Another variation of this model allows for the doxycycline-dependent induction of Kras (or de-induction), permitting temporal as well as spatial control in the expression of this activating mutation (Ying et al., 2012). This triple transgenic model revealed an absolute dependence of PDA maintenance upon the Kras mutation, even once PDA has been established with all the classic stromal features that typically define it. Within 24 hours of doxycycline withdrawal, the stromal elements dissolved, tumor cells regressed, and FGD-uptake was lost upon PET/CT scanning. Thus, the KPC model and its subsequent derivatives strongly mimic human PDA and reveal unique features of the pathobiology of this aggressive disease.

Human PDA has recently been subjected to broad-scale analysis on a genomic level, revealing further insights into the "mutational landscape" of this disease (Waddell et al., 2015). In the most recent study, whole genome sequencing of 100 patients' tumors reiterated the significance of known driver mutations like Kras, P53, SMAD4 and CDkN2, as well as a plethora of less frequent contributors. Importantly, the study accentuated the significance of chromosomal (structural) variations, such as deletion, rearrangement, amplification, or gene fusions. Certain genetic signatures defined four subtypes of PDA: stable, scattered, unstable, or locally rearranged. These categories were then correlated with responsiveness to platinum-based therapy; interestingly, the

"unstable" genotype was the most responsive. Selective therapeutics based on these signatures may be on the horizon, although the heterogeneity in these tumors is still a daunting impediment to therapeutic efficacy. A similar study of Kras-driven lung cancer revealed that the source of the Kras mutation is important in determining the scope and type of genomic aberrations (Westcott et al., 2015). Carcinogen-induced Kras expression by methyl-nitrosourea (MNU) resulted in 192 non-synonymous somatic single-nucleotide variants, in stark contrast to the 6 induced by genetic induction of Kras. Moreover, the abnormalities observed in the genetic Kras model validated the findings of the previous PDA study; a majority harbored structural abnormalities like aneuploidy and copy number alteration.

PDA and the immune system

The inflammatory infiltrate that co-exists within the PDA fibrous network is intimately associated with the initiation and progression of this disease, as demonstrated by the strong correlation between the incidence of chronic pancreatitis and PDA (Chu et al., 2007). Both PDA and chronic pancreatitis elicit constitutive NF-κB activity, leading to subsequent production of pro-invasive and pro-angiogenic factors that can contribute to tumorigenesis and progression (Chu et al., 2007). Likewise, mutant Kras itself has been shown to be inflammatory. In a mouse model of Kras-driven lung adenocarcinoma, mice die early from a robust immune infiltrate consisting of macrophages and neutrophils in the alveolar airspace, rather than from tumor burden itself (Ji et al., 2006).

Likewise, Kras establishes a pro-inflammatory microenvironment early in the development of PDA in KPC mice. Histologic analysis of KPC tumors reveals an abundance of leukocytes but a scarcity of effector CD8+ cells, even at the pre-invasive stage of disease known as pancreatic intraepithelial neoplasia (PanIN) (Clark et al., 2007; Clark et al., 2009). Instead, immunosuppressive populations including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells dominate this leukocytic infiltrate. As described above, each of these populations has been shown to interfere with T cell function through an assortment of mechanisms that ultimately disrupt priming, inhibit cytokine secretion by effector T cells, or induce T cell tolerance and even apoptosis (Rabinovich et al., 2007).

Despite the refractory nature of PDA to chemotherapy, targeting the immunosuppressive microenvironment has demonstrated some promise in overcoming this lethal disease. CD40, an activating receptor on antigen presenting cells, can be ligated with an agonistic antibody to drive T cell priming; initially, our lab hypothesized that such treatment might enhance T cell-mediated surveillance of PDA in combination with gemcitabine. Agonistic CD40 antibodies indeed drove tumor regression in both humans and mice; however, we were surprised to discover that this mechanism was T cell-independent (Beatty et al., 2011). Agonistic CD40 in fact re-educated macrophages to become tumoricidal, resulting in macrophage-dependent ablation of the PDA stromal compartment in mice.

I assisted in a parallel study while a rotating student in the Vonderheide laboratory, where the function of macrophages in PDA proved more complex than we

anticipated (Beatty et al., 2015). We initially sought to determine whether immunosuppression in the KPC model was systemic, and employed a "two tumor" model to address this question. KPC mice bearing autochthonous pancreatic tumors were simultaneously implanted with a KPC-derived cell line under the skin, resulting in T cell-dependent regression of the implanted tumors upon administration of gem/CD40 ("FGK"). This finding indicated that the immunosuppressive effect was compartmentalized to the pancreas, akin to a site of immune privilege. In surprising contrast to the prior study, we discovered that a subset of Ly6C(low) macrophages mediated this local suppression, as their depletion by clodronate-encapsulated liposomes (CEL) in combination with gem/CD40 therapy was required for CD8+ T cell trafficking into the pancreas (Fig 1), which coincided with increased levels of T cell-associated cytokines in the serum. This study highlights the daunting complexity of the tumor microenvironment in PDA, where innate immune cells demonstrate highly contextualized roles.

In support of this finding, our lab and colleagues at New York University recently demonstrated that tumor-derived GM-CSF promotes intratumoral infiltration of myeloid cells in KPC mice, in which their production of arginase and iNOS can diminish T cell infiltration, proliferation and IFNγ production (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). Thus, it appears that PDA is a prime example of how cancers may usurp the innate compartment of the immune system and harness its ability to regulate T cell function.

We more recently demonstrated that T cell-mediated effects can also be induced in the KPC model through checkpoint blockade of PD-1 and CTLA-4, in combination with a vaccination cocktail of the CD40 agonist and chemotherapy (Winograd et al., 2015). Together, these studies reveal the presence of endogenous antigens in PDA; albeit weak enough to be overpowered by immunosuppressive factors at baseline, these antigens can elicit a T-cell mediated response when immune therapies enhance T cell function or disrupt the tumor microenvironment.

Goals and key findings of this thesis project

PDA is lethal in 100% of untreated KPC mice, reflective of the dismal survival statistics in humans. This implies an ultimate failure of T cell immunosurveillance, but previous studies of PDA have not interrogated the extent to which T cell function is abrogated, or whether immunosuppression permits wholesale exclusion of T cell immunity throughout tumor inception and invasion. Addressing this gap in our understanding of the immunobiology of PDA is critical for directing future approaches to immunotherapy.

I hypothesized that the ultimate failure of T cell immunosurveillance in PDA was not due to classical immunoediting and escape; rather, I believed that the immunosuppressive microenvironment would exclude surveillance at the outset, permitting paradoxical outgrowth of tumors with antigenic profiles. I therefore postulated that the tumor microenvironment principally dictates the course of this disease.

To begin interrogating this hypothesis, I depleted juvenile KPC mice of their various T cell subsets to determine their effect on the overall outcome of this PDA. As I anticipated, T cells did not alter the course of tumor progression in KPC mice. However, upon implantation of the 4662 KPC cell line in an immune-competent host, tumors were not rejected and outgrowth was not delayed. Moreover, generating cell lines from T-cell depleted KPC mice did not render them susceptible to rejection. I then considered the possibility that KPC tumors are actually immunologically "cold," contrary to my original hypothesis, and that immunosuppression may not dictate the outcome of this disease.

Whole exome sequencing of the KPC cell lines revealed that these tumors, regardless of the immune competence of the donor, harbored very few non-synonymous mutations. Moreover, these rare mutations were not predicted to form neo-epitopes. To determine if greater immunogenicity could supersede the immunosuppression in this model and drive classical editing, I introduced ovalbumin as a "neo-antigen" to the 4662 cell line. These tumors were rapidly rejected in a CD8 T cell-dependent manner. Moreover, mixed tumor implants of Ova-positive and Ova-negative 4662 revealed that this initial CD8-mediated response was highly specific, allowing outgrowth of Ova-negative cells and restoration of immunosurveillance. I further determined that priming by Ova4662 permitted a T cell response upon re-challenge with parental 4662 cells despite the immunosuppressive microenvironment, indicative of epitope spread to otherwise quiescent antigens. Thus, PDA is immunologically "cold," but a strong antigen restores immunosurveillance even in the presence of an immunosuppressive microenvironment. Therefore, antigen strength rather than immunosuppression principally determines the immunological fate of a tumor.

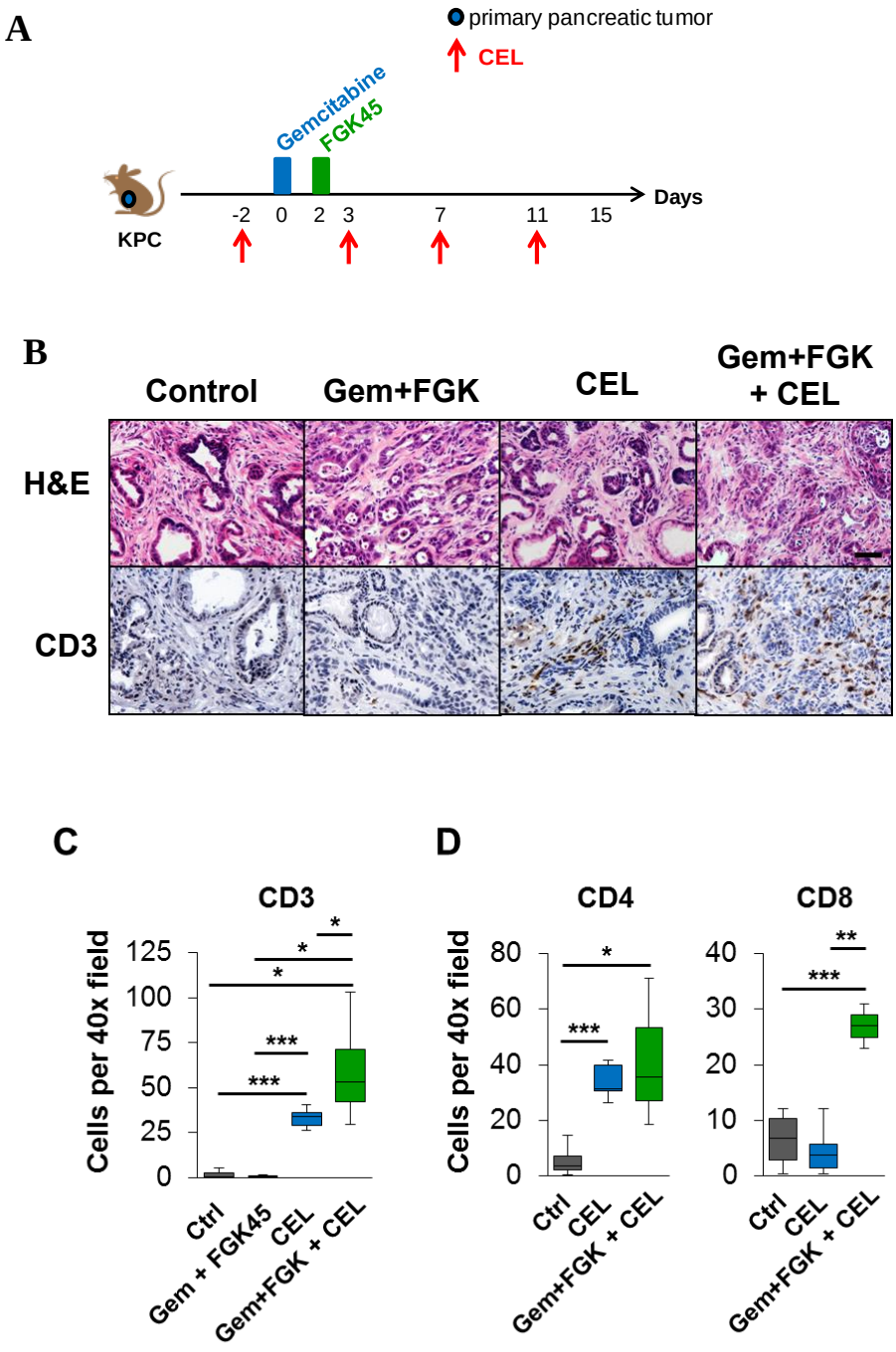


Fig 1. Intratumoral T cell infiltration in Gem/CD40-treated KPC mice is dependent upon ablation of macrophages. (A) Experimental schematic of treatments administered in KPC tumor-bearing mice. CEL, clodronate-encapsulated liposomes. (B) H&E (top panel) and CD3 immunohistochemistry (bottom panel) of tumor-bearing KPC mice treated with various combinations of gemcitabine, CD40 ("FGK"), and CEL. (C,D,) Quantification of tumor-infiltrating T cell subsets across treatment groups according to immunohistochemistry.

Chapter 2: Materials and Methods

Human RNA-Seq Gene Expression Data

Gene expression for 6 human tumors (PDA, melanoma, squamous carcinoma of the lung, adenocarcinoma of the lung, kidney clear cell carcinoma, and low-grade glioma) in normalized gene abundance estimates (rsem.genes.normalized_results) were downloaded from the NIH TCGA Research Network through GDAC Firehose (http://gdac.broadinstitute.org/) and included all available Illumina HiSeq 2000 Level 3 gene-level data. Normal tissue samples were excluded from the analysis. A cytolytic signature was calculated as the log-average of granzyme A (GZMA) and perforin 1 (PRF1) expression per sample as previously described (Rooney et al., 2015). Similarly, a CD8 signature was calculated as the log-average of CD8A and CD8B expression. To compare gene signature expression among different cancer cohorts, one-way ANOVA was performed and a Dunnett's multiple comparisons test was used to calculate significant differences from PDA.

Animals

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Survival data and primary cell lines were generated from *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{LSL\text{-}R172H/+}$, *Pdx1-Cre* (KPC) mice (Hingorani et al., 2005) bred in-house, backcrossed more than ten generations with syngeneic mice, and found to be congenic to the syngeneic background as assessed at the DartMouse™ Speed Congenic

Core Facility at the Geisel School of Medicine at Dartmouth. DartMouse uses the Illumina GoldenGate Genotyping Assay to interrogate 1449 SNPs spread throughout the genome. The raw SNP data were analyzed using DartMouse's SNaP-Map™ and Map-Synth™ software, allowing the determination for each mouse of the genetic background at each SNP location. Tumor implant studies were performed using eight- to ten-week-old female C57BL/6 mice purchased from Jackson Laboratories.

Collection of Tissue Samples from Mice

Whole pancreata of KPC mice or subcutaneous tumors were dissected and processed as previously described (Bayne et al., 2012). Tissues were extensively minced with a razor and incubated in collagenase IV solution (1mg/ml in RPMI media) for 45 minutes at 37°C. The dissociation reaction was stopped by 1:1 dilution with cold FCS, followed by passing cells through a 70μm cell strainer. The cell suspension was carefully passed through a 27g needle, washed twice in DMEM, and passed through a second strainer prior to use for flow cytometry or cell culture.

Spleens were homogenized through a 70μm strainer and carefully passed through a 27g needle to create a single-cell suspension. Following centrifugation, pelleted cells were incubated with ACK lysis buffer (BioWhittaker) at a 1:10 ratio for 8 minutes to induce red blood cell lysis. Cells were washed twice in RPMI prior to use for flow cytometry.

Peripheral blood was collected by tail vein into heparinized microhemtocrit capillary tubes. Blood was removed from the tubes by centrifugation and similarly prepared for flow cytometry with ACK lysis buffer and serial washes in RPMI media.

Preparation of Cell Lines

Tumors from the pancreata of KPC mice were excised with sterile scissors and extensively minced prior to dissociation in 1mg/ml collagenase IV solution at 37°C for 45 minutes. Dissociated cells were passed through a 70um strainer and plated in serum-free DMEM at varying concentrations in a 6-well plate. Cells were maintained in serum-free media for at least two weeks and passaged to higher volume flasks for expansion in DMEM + 10% FCS. Cells were used for implant studies at low (3-5) passages. Cell lines were validated by RT-PCR to assess for Cre-mediated recombination of the mutant *Kras* and *Trp53* alleles. Primer sequences to detect recombined *Kras* and *Trp53* loci are as follows: forward 5'-gtc ttt ccc cag cac agt gc-3' and reverse 5'-ctc ttg cct acg cca cca gct c-3', and forward 5'-agc ctg cct agc ttc ctc agg-3' and reverse 5'-ctt gga gac ata gcc aca ctg-3', respectively.

In vivo mouse studies

T cell depletion of either KPC or C57BL/6 mice was achieved by intraperitoneal injection of 0.2 mg of αCD8 (2.43), αCD4 (Gk1.5), or an IgG2b isotype control (LTF-2) diluted in 100ul sterile PBS. All antibodies were purchased from BioXcell. For long-

term depletion in KPC mice, pups were administered a first dose at 4 weeks of age and every 4 days thereafter until euthanasia. The initial depletion was verified by flow cytometry of peripheral blood, and then reassessed every 2 or 3 weeks thereafter for the duration of the study; blood was drawn prior to re-administration of the antibody to assess peak T cell levels. For subcutaneous implant studies, mice were pre-treated with T cell-depleting antibodies 2 days prior to tumor challenge and then every 4 days thereafter. T cell depletion was confirmed by peripheral blood samples and end-of-study flow cytometry. NK cell depletion was achieved by intraperitoneal injection of 0.2mg PK136 mAb (BioXCell) on days -1, 0, +1, and every 5 days thereafter. NK cell depletion was confirmed by end-of-study flow cytometry on splenocytes.

KPC mice were enrolled in survival studies on a rolling basis, as described (Beatty et al., 2011). Genotyped mice were allocated to treatment groups in a randomized fashion, and monitored for development of tumor-associated morbidities including ascites, lethargy, depression, or a tumor volume >1000mm^3. Mice were censored for unrelated morbidities, including prolapsed rectum or penis, non-PDA tumors (thoracic and submandibular), and malocclusion.

Diagnosis of PDA in KPC mice was performed by abdominal ultrasound beginning at 7-8 weeks of age and every other week thereafter; between scheduled ultrasounds, mice were additionally monitored by palpation and imaged when indicated. Ultrasound was performed with a Vevo 2100 imaging system with a 55MHz MicroScan transducer (Visual Sonics). Tumors were visualized and reconstructed using the integrated Vevo

Workstation software package to assess tumor volume. Mice were censored from the diagnostic study if euthanized for non-PDA morbidity prior to a definitive diagnosis.

Subcutaneous implant studies were performed using PDA cell lines titrated to grow with similar kinetics over 21-24 days, at a relatively low dose ($<10^6$ cells) to avoid ulceration and permit any relevant immune response (4662: $5x10^5$ cells; 1638: $7.5x10^5$ cells; 1262: $2x10^5$ cells; 1493: $3x10^5$ cells). Cells were harvested at 80-90% confluence, washed twice in sterile DMEM, and administered subcutaneously into the right flank in 100ul sterile DMEM. Caliper measurements were obtained of the longest tumor dimension (length) and the perpendicular dimension (width) every 3 to 4 days. Tumor volume was calculated as $(L \times w^2)/2$.

Antibodies

The following mAb were used for flow cytometry: from BD Biosciences, mIgG2a, κ isotype control (MOPC-173, FITC), αCD3e (145-2c11, FITC), αCD31 (MEC 13.3, FITC), αCD44 (IM7, FITC), αCD45 (30-F11, FITC), αCD45 (30-F11, PE), αCD3 (145-2C11, PerCP), αCD45 (30-F11, PercP), αCD8a (53-6.7, PE-Cy7), αCD45 (30-F11, PE-Cy7), αCD45 (30-F11, APC), αCD11b (M1/70, APC-Cy7), αCD45 (30-F11, APC-Cy7), αCD11c, (HL3, V450), and αCD4 (RM4-5, V450); from BD Horizon, Streptavidin-V450; from eBiosciences, αCD8a (53-6.7, PE); from Biolegend, αH2-D^b-Biotin (KH95), αKi-67 (16A8, FITC), αH2-K^b (AF6-88.5, FITC), αOVA-H2-K^b (25-D1.16, APC), αCD90.2 (53-2.1, PerCP), αIFN-γ (XMG1.2, PE-Cy7), αI-A/I-E (M5/114.15.2, PE-Cy7), αPD-L1 (10F.9G2, APC), αGranzyme B (GB11, AlexaFLuor 647), and αTbet (4B10, Brilliant Violet 421). H2-K^b Ova Tetramer

(SIINFEKL–PE) and the MHC class I negative tetramer (APC) were purchased from MBL International Corporation (Beckman Coulter). Viability of cells was determined by staining with either 7-aminoactinomycin D (7-AAD; BD Biosciences) or Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies).

Neo-epitope prediction pipeline

Genomic DNA from KPC mouse tumor cell lines or mechanically digested control tissue (KPC spleen) was extracted using the PureLink Genomic DNA minikit (Invitrogen) and assessed for purity and yield using a NanoDrop 2000c spectrophotometer. DNA integrity was further assessed by electrophoresis on 1% agarose gels and DNA concentration was determined using a Qubit dsDNA BR Assay Kit (ThermoFisher Scientific). Exome enrichment was performed using an Illumina Paired End Sample Prep Kit and library sequencing was performed using a 100 bp paired-end protocol on the Illumina platform (HiSeq2500) (High-Throughput Sequencing Center, Beijing Genomics Institute at Children's Hospital of Philadelphia). Sequence alignment and processing were performed as previously described (Westcott et al., 2015). Single nucleotide variants in tumor samples were identified using MuTect (version 1.1.7) (Cibulskis et al., 2013) with default filters against normal splenocytes from KPC mice (for PDA lines) or C57BL/6 splenocytes (for B16 melanoma). Variants were annotated using SnpEff (version 4.1 L) with default settings and filtered against known SNPs. 8-14 amino acid sequences surrounding surviving missense mutations were then ranked for binding affinity to MHC class I H-2D^{b} and H-2K^{b} molecules using the consensus method provided by the Immune

Epitope Database and Analysis Resource (IEDB) (http://www.iedb.org/). Using two thresholds of potential binding affinity, peptides with a median half-maximum inhibitory concentration (IC50) of less than 50nM or less than 100nM were identified as potential neo-epitopes. The IC50 for each peptide was considered the median value of individual prediction method results provided by the IEDB consensus method.

Retroviral transduction of 4662 cell line with Tdt-Ova

The pMX-Tdt-Ova plasmid containing a pMXs retroviral backbone with full-length ovalbumin fused to Td-Tomato was a kind gift of Dr. Li Wang (Dartmouth University). 70-90% confluent HEK 293T packaging cells were transfected with the plasmid and a packaging mix (pCMV-Gag/Pol and pVSV-G) using lipofectamine to produce viral supernatant. 4662 cells were plated in a 24-well plate until reaching 50% confluence. Cells were washed, and 1ml of a 1:2 virus:DMEM solution was incubated for 36-48 hours (diluted at 24 hours with an additional mL of DMEM). Cells were passaged to larger flasks, and sorted by flow cytometry for the top 15% of Td-Tomato-expressing cells to create an enriched cell line. Cells were then single-cell sorted into a 96-well plate to create single-cell clones of TdT-Ova4662.

IFNγ Stimulation Assay

To assess for processing and presentation of SIINFEKL peptide on MHC class I under conditions of IFNγ stimulation, 4662 parental Tdt-Ova transduced clones (V6, G7, and

G10 single-cell clones) were plated in T25 flasks, and at 50% confluence, 500units/μl of IFNγ (R&D systems) were added to one flask of each cell line and incubated for 24 hours, while a duplicate flask remained unstimulated. As a positive control, SIINFEKL peptide was then added at 10ug/ml for 30 minutes at 37°C to stimulated and unstimulated 4662 cells. Cells were then trypsinized, washed and stained for viability and Ova-H2-Kb by flow cytometry.

Flow Cytometry and Tetramer staining

Following preparation of a single-cell suspension, up to $5x10^6$ cells were plated per well in a 96 well plate, washed, and stained with fluorochrome-labeled antibodies at 4°C for 20 minutes in a buffer of PBS/1%FCS and 0.5mM EDTA. For tetramer stains, prior to surface staining, cells were incubated at 37°C with positive or negative tetramer at 1:100 in FACS buffer, and then diluted with a 2x concentrate of the remaining surface stains. Cell were run on a FACSCanto flow cytometer (BD Biosciences) and analyzed with Flowjo software.

Intracellular Cytokine Stimulation Assay

A single cell suspension was prepared of tumor and spleen and samples were plated in duplicate in a 96-well plate for stimulated and unstimulated treatments. Stimulation was performed using RPMI-1640 media supplemented with L glutamine, gentamicin, 10% FCS, and 0.05mM 2-ME.; for unstimulated samples, GolgiStop (Monensin) was added to

media at 1:150, and stimulated samples were additionally incubated with PMA (1mg/ml stock) at 1:20,000 and ionomycin (1mM stock) at 1:100. Both stimulated and unstimulated samples were incubated for 5 hours at 37°C. Viability was then assessed using Live/Dead Fixable Aqua Dead Cell Stain Kit, followed by surface staining and fixation/permeabilization for intracellular staining.

Statistics Analysis

Variations between two groups were determined by an unpaired two-tailed Student *t* test. Differences between three (or more) groups for one factor were analyzed by one-way ANOVA, with Tukey multiple comparison test used as a *post hoc* test to evaluate differences between any two groups. To study the effect of multiple factors across multiple groups, two-way ANOVA was utilized with the Sidak's multiple comparison test for *post hoc* evaluation of 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 determine differences between any two groups. Survival curves were assessed by the log rank (Mantel–Cox). All statistical analyses were performed using GraphPad Prism 6 (GraphPad) except two-way ANOVA and related *post hoc* testing that were performed on R Statistical Software (R Core Team). $P \leq 0.05$ indicates differences that are statistically significant.

CHAPTER 3 – The natural history and progression of PDA are independent of T cell immunosurveillance.

*The majority of these studies have been described in the manuscript submitted for publication

INTRODUCTION

The prevailing theory of cancer immunosurveillance asserts that the adaptive immune system can recognize and eliminate nascent cancer cells, but tumor clones with a selective advantage may escape recognition by the immune system, leading to unrestrained tumor growth (Schreiber et al., 2011; DuPage et al., 2012). This concept, supported by seminal studies in carcinogen-induced mouse models of tumorigenesis (Shankaran et al., 2001; Matsushita et al., 2012), has led to the immunoediting hypothesis: escaped tumors are understood to be sculpted or "edited" under the Darwinian-like pressure exerted by tumor-specific T cells. Key evidence for immunoediting includes: (i) *in vivo* models in which tumors emerge at a higher frequency and decreased latency in mice lacking T cells (Shankaran et al., 2001; DuPage et al., 2011; DuPage et al., 2012) and (ii) observations that cancer cells isolated from progressively growing tumors in T cell-depleted hosts are rejected upon implantation in a T cell-replete, but not T cell-deficient, host. The primary model that established this hypothesis, however, resulted in a "hypermutated" tumor phenotype, exhibiting a ten-fold higher mutational burden than even smoking-induced lung cancers (Matsushita et al., 2012). Thus, the model that generated the immunoediting hypothesis may not be representative of most human malignancies.

This theory has remained untested across a wide spectrum of mouse models, including spontaneously arising carcinomas driven by oncogenes at the endogenous locus; given that a majority of solid human malignancies develop in this fashion, additional studies are imperative to understanding the full spectrum of host-tumor interactions. Moreover, the implication of variable tumor histologies and microenvironments has not been addressed in the current hypothesis of immunosurveillance.

I reassessed the cardinal features of cancer immunosurveillance using a genetically engineered mouse model of PDA that mimics the human disease with high fidelity and permits inspection of T cell infiltration from inception to invasion of mutant Kras-driven tumors (Hingorani et al., 2003; Clark et al., 2007). In the "KPC" model of PDA, which includes targeted pancreatic expression of mutant Kras and p53, tumors arise in immune competent hosts in the absence of carcinogens and in the setting of an oncogene expressed at the endogenous locus (Hingorani et al., 2005). PDA in both humans and KPC mice is characterized by a dense, desmoplastic stroma, which features a prominent network of immunosuppressive leukocytes driven in part by the tumor itself (Clark et al., 2007; Olive et al., 2009). Tumor-derived GM-CSF, for example, acts in the tumor microenvironment to recruit myeloid-derived suppressor cells that inhibit T cell-dependent, perforin-mediated cytotoxicity (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). These data suggest that the immunosuppressive microenvironment mediated by PDA has the ability to impede or even circumvent immunosurveillance.

In the following studies, I tested the hypothesis that the immunosuppressive microenvironment exhibited by PDA would preclude T cell immunosurveillance at its inception, thereby preventing sculpting of the tumor. I theorized that PDA tumors represent a site of immunological ignorance, which may paradoxically permit development of tumors that retain strong antigens in immune-competent hosts. I utilized both the KPC model as well as implantable studies of KPC-derived cell lines to assess the sculpting effect of the immune system on the natural history of this disease at both an organismal and cellular level. To study changes in the genomic landscape potentially induced by T cells, I utilized whole exome sequencing (WES) of PDA cell lines derived from immune-competent and immune-compromised KPC hosts. Through several collaborative efforts, I further utilized various mechanisms of microenvironmental disruption and thereby determined the degree of immunogenicity shrouded within an immunosuppressive network.

RESULTS

In the KPC model, effector T cell infiltration is modest even at the earliest stages of neoplasia (Clark et al., 2007; Clark et al., 2009; Beatty et al., 2011), similar to the dearth of T cells observed histologically in other oncogene-driven cancer GEMMs (Akbay et al., 2013; Skoulidis et al., 2015). To compare the T cell-poor phenotype of the KPC model to human PDA, I assisted fellow graduate student Tim Chao in analyzing The Cancer Genome Atlas (TCGA) for expression data of 147 cases of human PDA. We observed a statistically lower level of CD8 expression within PDA tumors compared to melanoma, renal cell carcinoma, or

lung cancer – three tumors for which impressive clinical responses to immune checkpoint blockade have been observed (Fig 2). Moreover, PDA tumors exhibit relatively low levels of granzyme A and perforin-1, which together constitute a normalized cytolytic expression signature that distinguishes immunologically "hot" tumors from "cold" tumors (Rooney et al., 2015; Sharma and Allison, 2015). Thus, the low quantity and quality of T cells in both the KPC model and human PDA suggest that immunologically inert cancers may not undergo classical immunoediting driven by potent T cell immunity.

Modeling my approach after the seminal immune studies of MCA-induced tumorigenesis, I evaluated immunosurveillance in KPC mice. For MCA tumors, an increased tumor incidence and decreased survival are observed in T cell-deficient mice; however, these carcinogen-driven cancers have a hypermutator phenotype and strong T cell reactivity (Matsushita et al., 2012). To determine whether T cells play a similar role in PDA, I serially administered CD4- and CD8-depleting antibodies or an isotype control to juvenile (3-5 week-old) KPC mice (Fig 3A). Treated mice were then monitored by ultrasound for the development of PDA and evaluated for signs of morbidity (Fig. 3B). Efficacy of antibody-mediated T cell depletion for the duration of the study was confirmed (Fig. 4). Tumor-free survival (i.e., time to diagnosis) and overall survival were statistically indistinguishable between CD4/CD8-depleted mice and isotype-treated mice (Fig. 5). Likewise, no difference was observed for tumor-free and overall survival in mice exclusively depleted of CD8 T cells (Fig. 5). Tumors from each cohort exhibited similar histology (Fig. 6), and flow cytometry demonstrated the same prominent leukocytic infiltrate (Fig. 7) in isotype control vs. T cell-depleted mice. These results differ from classic mouse sarcomas in which immunodeficient

hosts exhibit a greater frequency of tumors and decreased survival (Shankaran et al., 2001; DuPage et al., 2012).

To test further for T cell immunosurveillance in PDA, I then created cell lines derived from the tumors of immune-competent KPC mice (Fig. 8). Following tumor harvest and elimination of stromal elements in culture, a representative line (4662) was selected and verified to express the recombinant mutant alleles by RT-PCR. This low-passage cell line was then implanted subcutaneously into T cell-depleted or isotype-treated syngeneic mice, and followed tumor growth for 21-24 days by caliper (Fig. 8). Similar to the findings in the autochthonous KPC model, tumor growth of 4662 cells was unaffected by T cell depletion of recipient mice (Fig. 9). Tumor growth featured rapid recapitulation of the dense, stromal microenvironment of the original tumor despite elimination of non-tumor cells from the cell line (Fig. 10). Similarly, the extracellular matrix of 4662 PDA tumors is extensive (Lo et al., 2015).

To exclude the possibility that the initial bolus of $5x10^5$ tumor cells overwhelmed an otherwise relevant immune response, I also tested a lower subcutaneous dose of 10^5 4662 cells. Again, tumors grew with similar kinetics in the presence or absence of T cells (Fig. 11). 4662 tumors also grow with similar kinetics in NOD/SCID/$\gamma c^{-/-}$ immune-incompetent mice compared to wild-type mice (Lo et al., 2015). To assess whether the negative immune checkpoint molecules PD-1 and CTLA-4 were influencing tumor rejection as observed in the MCA model (Gubin et al., 2014), I also repeated these experiments in the presence of mAb blocking PD-1 and CTLA-4 with the assistance of fellow graduate student Rafael Winograd. In our system, this treatment did not enable tumor rejection (Fig. 12). Furthermore, 4662

cells remained responsive to IFNγ in upregulating MHC class I (but not MHC class II) (Fig. 19, see chapter 4), indicating that this cell line is theoretically susceptible to T cell-mediated recognition.

To elucidate the mechanism of this inevitable tumor outgrowth in the KPC model, immunoediting was assessed directly by generating cell lines from tumors arising in chronically T cell-depleted KPC mice (validated by flow cytometry, Fig. 13) and implanting them in immune-competent or T cell-depleted syngeneic hosts (as previously described, Fig. 8). Each cell line derived from a T cell-depleted KPC mouse (1262, 1493, and 1638) grew with similar kinetics regardless of the immune status of the recipient mouse, and tumor regression was not observed in any individual mouse (Fig. 14). These findings differ from similarly designed experiments in the MCA model (Shankaran et al., 2001), implicating a mechanism of tumor outgrowth other than immune editing.

I hypothesized that these divergent manifestations of cancer immune surveillance in the Kras-driven PDA model and the prior MCA studies may reflect differences in the incidence of T cell neo-epitopes arising from tumor missense mutations (Wolfel et al., 1995; Robbins et al., 1996; Dubey et al., 1997; Matsushita et al., 2012). Such mutations drive strong anti-tumor T cell responses in the MCA model, which features a high level of non-synonymous tumor mutations, and lead to antigen loss as a means of escape consistent with immunoediting (Matsushita et al., 2012). In collaboration with fellow graduate student Andrew Rech and the BGI sequencing core at the Children's Hospital of Philadelphia, we therefore performed whole exome sequencing (WES) on 4662 PDA cells as well as the three PDA cell lines derived from T cell-depleted KPC mice. As anticipated from WES analysis of

tumors from other Kras-driven GEMMs (Westcott et al., 2015) and human PDA (Jones et al., 2008; Sausen et al., 2015; Waddell et al., 2015), we found that 4662 PDA cells exhibited a relatively low mutational burden. Among the 10 non-synonymous mutations identified, none were predicted to comprise an 8-14 amino acid epitope with high affinity for MHC class I H2-D^b or H2-K^b molecules based on the previously defined cutoff of 50nM (Fig 15) (Matsushita et al., 2012; Westcott et al., 2015). T cell-depleted PDA cell lines 1262, 1493, and 1638 also exhibited a low mutational burden with 16, 16, and 34 non-synonymous mutations, respectively, with either 0 (1262) or 1 (1493 and 1638) of these mutations predicted to generate a neo-epitope (Fig. 15). Similar findings were obtained using a binding affinity cutoff of 100nM (Fig. 15). As a positive control in our analysis pipeline, we determined that murine B16-F10 melanoma tumors expressed 1077 non-synonymous mutations, 13 of which were predicted to trigger specific T cell reactivity at the 50 nM cutoff, or 21 at the 100nM threshold (Fig. 15). A prior study of B16-F10 melanoma tumors similarly found >500 non-synonymous mutations, and at least 30% of those experimentally tested were predicted to trigger specific T cell reactivity (Castle et al., 2012). In further contrast, MCA tumor cell lines established from immunodeficient *Rag2*$^{-/-}$ mice exhibited >2000 somatic, non-synonymous mutations (Matsushita et al., 2012). My findings, therefore, suggest that the T cell-independent growth properties of KPC-derived PDA cell lines may be a consequence of the low incidence of missense mutations and subsequent lack of neo-epitopes in these tumors.

Even though the human and mouse data suggest an immunologically "cold" tumor, during the course of my research I discovered that otherwise quiescent antigens (or self-antigens) could be unveiled by manipulating the tumor microenvironment. Long-

term depletion of CD4+ T cells in KPC mice surprisingly resulted in prolonged overall survival, an effect I knew to be abrogated in mice depleted of both CD4 and CD8 cells (Fig. 16A). These data suggested a CD4+ cell population that inhibits an otherwise relevant CD8 T cell response, albeit weak. By serial ultrasound, I determined that the survival benefit of anti-CD4 treatment was due to a delayed onset of disease, rather than slowing tumor progression (Fig. 16B and 16C).

In collaboration with the Pasca di Magliano laboratory at the University of Michigan, we validated this early-stage impact of CD4 cells in a genetic model of depletion (Fig. 17) (Zhang et al., 2014). This model provides an excellent system for studying the early stages of tumorigenesis, as PDA is driven solely by doxycycline-inducible KrasG12D in these so-called iKras mice; without concurrent mutation or deletion of p53, tumorigenesis progresses slowly in this model, such that mice develop early PanIN-stage lesions over prolonged periods of time (Hingorani et al., 2003). We discovered that, in this model, CD4 cells were in fact required for progression to PanIN-stage lesions. Even in the context of cerulein-induced pancreatitis to drive carcinogenesis, the tissue damage and inflammation seen in iKras/CD4-/- mice resolved quickly, such that the pancreas was histologically normal at a timepoint when CD4-replete iKras mice demonstrated severe histologic aberrations including acinar-to-ductal metaplasia (ADM) and multiple PanIN lesions. Early timepoints post-induction revealed significant cleaved caspase 3 (CC3) staining in CD4-null mice, whereas the CD4-replete iKras cohort demonstrated rare CC3 staining and significant proliferation by Ki67 staining in the epithelial compartment. In accordance with my own findings, this effect was abrogated upon CD8 depletion, such that iKras/CD4-/- pancreata histologically

resembled the iKras mice following induction of pancreatitis. Thus, two different model systems of CD4 deficiency and tumor induction demonstrate that CD8 T cells are able to inhibit the development of PDA; this suggests that antigens are in fact present and able to be recognized through manipulation of the tumor microenvironment. The sequencing data indicate a dearth of strongly immunogenic neo-antigens in this model, implicating *weak* antigenicity in the failure of T cell surveillance at baseline, but nevertheless providing productive responses upon disrupting the CD4-mediated microenvironment.

We sought to determine if manipulation of T cell function could likewise elicit a response against weak antigens not recognized at baseline. As described above, PD-1 and CLTA-4 blockade were insufficient to induce a response (Fig. 12). However, through collaboration with fellow graduate student Rafi Winograd, we studied the effect of a potential "vaccine" in conjunction with checkpoint inhibition, as described in his thesis work and our published data (Winograd et al., 2015). This cocktail included gemcitabine and abraxane chemotherapy to induce cell death and release of antigens, in combination with CD40 therapy to drive licensing of antigen-presenting cells (Beatty et al., 2011; Vonderheide et al., 2013). In the joint setting of immunogenic cell death, checkpoint inhibition, and improved T cell priming, weaker antigens were able to elicit productive T cell responses, including a CD8-dependent memory response upon re-challenge.

DISCUSSION and CONCLUSIONS

According to these studies of the KPC model and KPC-derived cell lines, T cell immunosurveillance in PDA does not appear to manifest in the classically described immunoediting process. Unlike Schreiber and colleagues, T cell-depleted mice did not demonstrate decreased survival. Although a cell line derived from an immune-competent mouse (4662) mimicked the "escape" phenotype seen in the MCA model, whereby implantation of tumor cells in an immune competent host resulted in tumor outgrowth (suggestive of prior sculpting and immune pressure), concurrent studies with cell lines from T cell-depleted KPC mice suggest a different mechanism of failed surveillance. According to immunoediting standards, a tumor arising in a T cell-depleted environment should maintain immunogenicity and be rejected in an immune-competent recipient; in my data set, tumor regression was never observed upon transfer of any of the three T cell-depleted cell lines.

The collective data herein suggest that the ultimate failure of T cell surveillance in KPC mice must result from a different mechanism than immune-driven sculpting and escape clones. Two explanations could result in these aberrant findings: 1) the immunosuppressive microenvironment arises in tandem with tumor onset, such that the tumor is protected at the outset from any immune pressure (and that this microenvironment is recapitulated by KPC-derived cell lines upon implantation), or 2) these tumors are immunologically "cold" and therefore remain invisible to circulating T cells. These two mechanisms are not mutually exclusive; the degree of "coldness" and the extent of immunosuppression remain unclear based on these studies alone.

To deconstruct the contribution of these two factors in failed T cell surveillance of PDA, I used a genomic approach. Neo-antigens derived from non-synonymous mutations are described as the primary source of immunogenicity in cancer, because they encode novel proteins that are seen as "foreign" by the immune system (Sette et al., 1994; Castle et al., 2012; Matsushita et al., 2012). In contrast, self-antigens expressed by tumors may be subject to central tolerance (Pardoll, 2003; Pradeu and Carosella, 2006). Whole exome sequencing of the 4662 line revealed very few non-synonymous mutations, and a well-established epitope prediction pipeline suggested that none would generate peptides that bind MHC class I with high affinity. Although this lack of immunogenic neo-antigens might be expected in a tumor that arose in the context of immune pressure, tumors derived from T cell-depleted KPC mice could theoretically retain their immunogenic neo-antigens. However, my three KPC cell lines that arose in a T cell-free environment did not have a statistically higher number of mutations or predicted epitopes. Thus, it appears that KPC tumors are indeed immunologically "cold" and represent a sub-type of tumors that lack a blatant "red flag" for immune recognition. Interestingly, a recent study strongly supports this finding. Lung adenocarcinomas driven by oncogenic Kras or carcinogen-induced Kras demonstrated markedly different genomic landscapes. Similar to the contrasting mutational burden in MCA-driven tumors and our oncogene-driven model, this study found a dramatic disparity in mutational burden based on the source of tumorigenesis (Westcott et al., 2015).

Despite the bleak outlook this might suggest for immunotherapy in the context of PDA, I also found that the microenvironment indeed appears to contribute to immune failure, and may provide another avenue for immunotherapy. CD4-depleted KPC mice

demonstrated a delayed onset of tumorigenesis and overall increased survival, a finding corroborated by our collaborators at the University of Michigan (Zhang et al., 2014). In both models of CD4 depletion, CD8 cells conferred protection against PDA development; without CD8 cells, the protective benefit of CD4-depletion was lost. Therefore, some quiescent epitopes must exist in the KPC model of PDA, albeit not as profound as neo-antigens.

It appears that CD4-mediated immunosuppression is capable of restraining an otherwise productive CD8 T cell response against weak antigens. Our lab continues to work on determining the CD4+ cell type responsible for this phenomenon, which could clarify novel targets in pancreatic cancer. The CD4+ cell compartment is very complex, including Th1 or Th2 helper T cells, Th17 cells, regulatory T cells, and even a subset of macrophages; the Gk1.5 antibody used in our study would deplete any of these cell types. Several recent papers help clarify which of these populations may drive the immunosuppressive effect we have observed.

As our lab has previously established, Foxp3+ regulatory T cells (Tregs) are present even at the earliest PanIN stages of PDA in the KC model of tumorigenesis, which strongly implicates this population in mediating a suppressive network. In a histopathologic study of human PDA samples, Hiraoka *et al.* discovered that CD4+ CD25+ Foxp3+ cells indeed increased in tandem with the stages of PDA development, from non-neoplastic inflammatory pancreata to frank carcinoma, and that this increase was statistically significant ($p<0.0001$) (Hiraoka et al., 2006). This observation correlated inversely with the infiltration of CD8+ cytotoxic T cells, which were prevalent

at pre-malignant PanIN stages and tapered during the progression to full-grade adenocarcinoma. Importantly, the degree of Foxp3+ infiltrate correlated strongly with a better prognosis ($p<0.0001$), which mirrors the findings of improved survival in our CD4-depleted KPC mice.

This population may indeed represent the most likely culprit in a CD4-mediated immunosuppressive microenvironment, but it is a difficult population to target. CTLA-4 blockade has been shown to deplete these cells from draining lymph nodes in an FC-dependent manner (Simpson et al., 2013), but this marker is expressed on many other cell types and therefore lacks specificity. A more targeted approach to ablating regulatory T cells involves crossing the KPC model with Foxp3-DTR transgenic mice, and monitoring survival of these mice following administration of diphtheria toxin. This study is currently ongoing in our laboratory.

IL-17-producing CD4+ cells have likewise been implicated in the progression of pre-neoplastic lesions in the pancreas, and represent another potential mechanism behind CD4-mediated immunosuppression. Differentiation of CD4+ helper cells to a Th17 phenotype can be driven by IL-6 and TGF-β signaling within the tumor microenvironment, and this correlates strongly with chronic inflammation. Using KCmist1 mice, the Leach laboratory demonstrated by flow cytometry a high proportion of CD45+ IL-17A+ cells within the pancreata of mice with chronic pancreatitis and oncogenic Kras; moreover, the combination of cerulein-induced pancreatitis and Kras activation increased these IL-17-A levels synergistically (McAllister et al., 2014). Since both CD4+ cells and γδTCR+ cells can produce this cytokine, McCallister *et al.* depleted

mice weekly with the same GK1.5 antibody utilized in our long-term ultrasound study, which resulted in a significant delay in PanIN formation in these mice.

A similar finding was observed in lethally irradiated KCmist mice receiving bone marrow transplants from IL-17KO mice. 8 weeks after induction of Kras, these mice demonstrated fewer ADM and PanIN lesions in their pancreata by histopathology. Pharmacologic inhibition of IL-17 likewise slowed PanIN formation, suggesting this treatment may recapitulate the phenotype we observed in our CD4-depleted KPC mice. However, no dependence on CD8+ cells was observed in this model, so the mechanism of IL-17 may be pro-inflammatory rather than immunosuppressive.

In collaboration with fellow graduate student Rafael Winograd, I also demonstrated that this immunosuppression and lack of immunogenicity can be overcome by stimulating T cells with a multifaceted vaccine approach (Winograd et al., 2015). Combining checkpoint inhibition (which was alone insufficient to drive anti-tumor responses) with chemotherapy and CD40 therapy allowed for long-term tumor regressions and establishment of memory responses in T cells – notably in mice with an intact CD4 compartment. Thus, multiple mechanisms can be used to drive immune responses against weak antigens despite a baseline lack of T cell reactivity, low immunogenicity, and an immunosuppressive microenvironment.

FIGURES

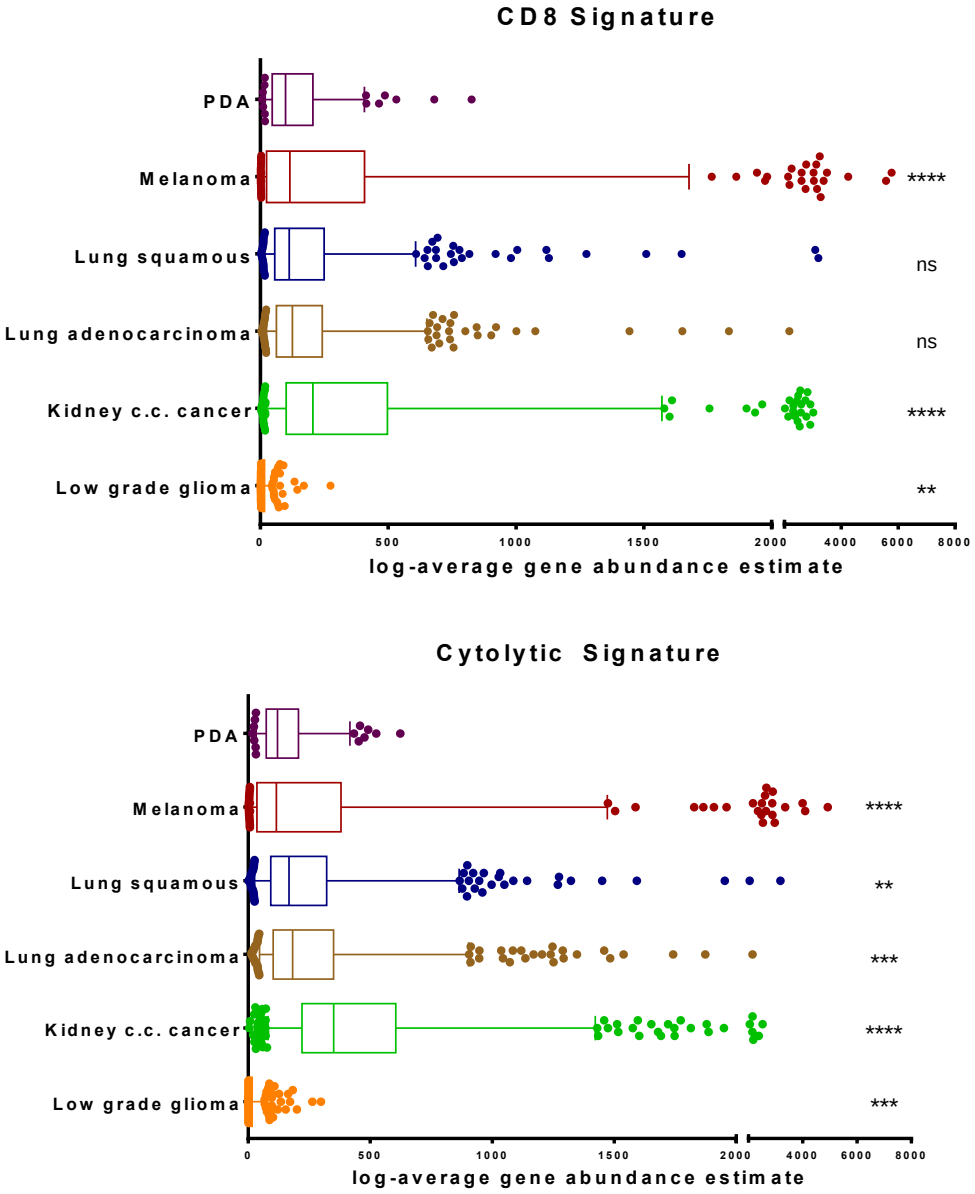
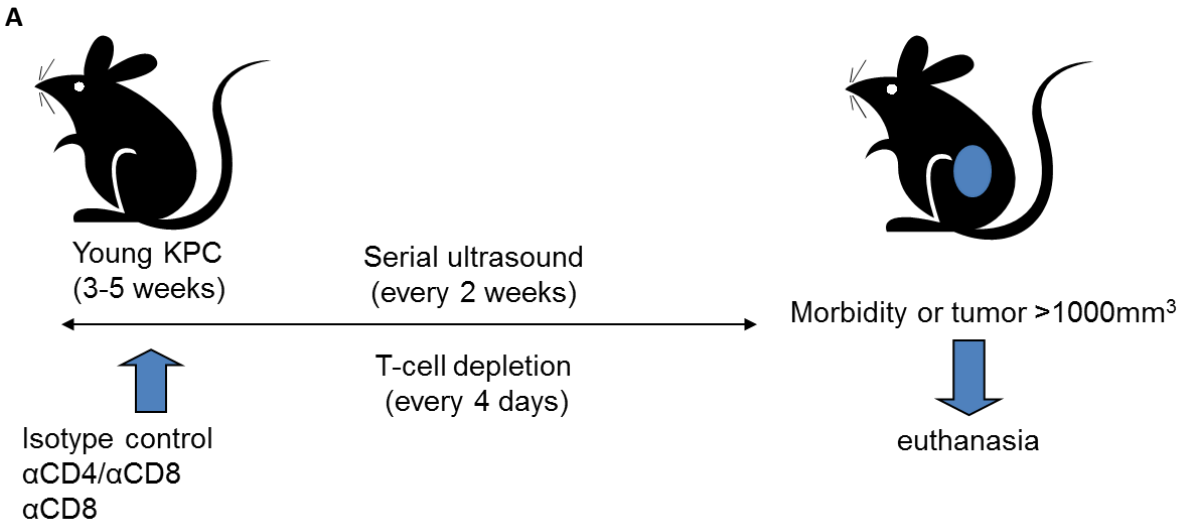


Fig 2. Human PDA does not express high levels of CD8 or markers of cytolytic activity. CD8 (top) and cytolytic (bottom) signature expression for 6 human tumors (PDA, N=147; melanoma, N=471; lung squamous, N=501; lung adenocarcinoma, N=517; renal cell carcinoma, N=534; low-grade glioma, N=530). Box plots represent the interquartile range and the whiskers represent the 5th-95th percentile of the log-averaged gene signature abundance estimates. One-way ANOVA and Dunnett's multiple comparisons test were used to calculate a significant difference from PDA. ** indicates P<0.01, *** represent P<0.001, and **** indicates P<0.0001.



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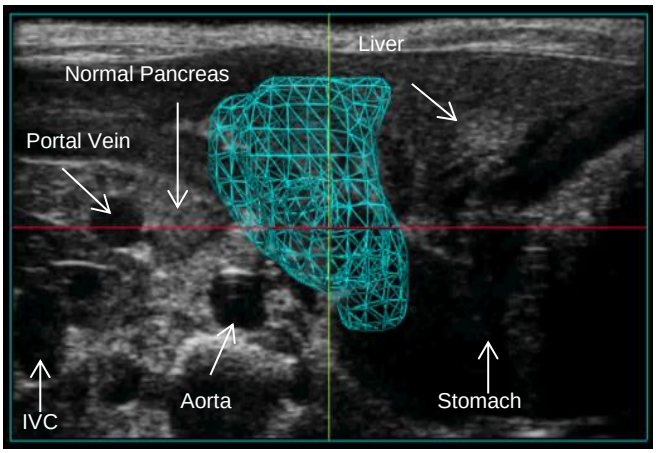
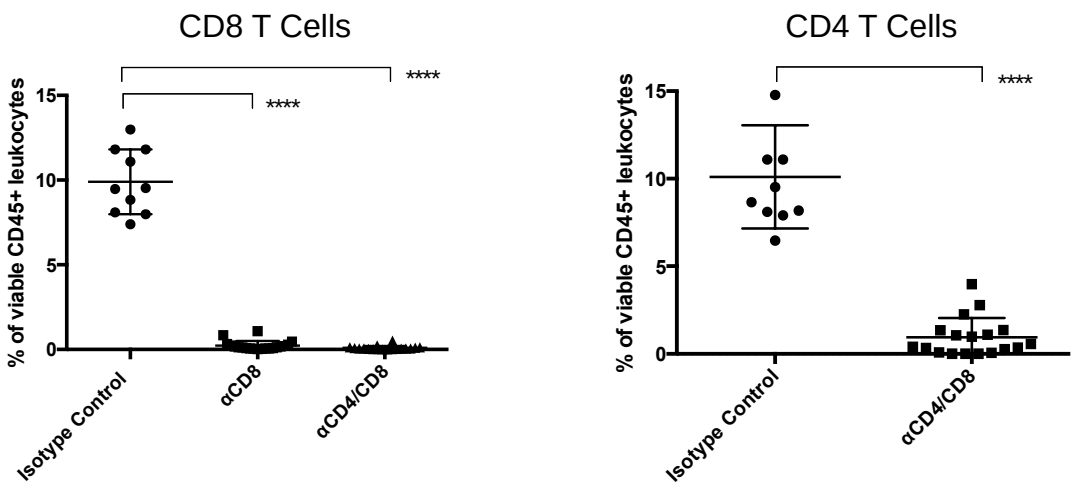


Fig 3. Experimental design for KPC survival studies. A) Syngeneic KPC mice treated with an isotype control antibody, depleted of CD4 and CD8 T cells (αCD4/αCD8), or depleted of CD8 T cells alone (αCD8) beginning at 3-5 weeks of age. N=19-21 mice per cohort. Starting at 7-8 weeks of age, mice were monitored by ultrasound every other week for tumor development and examined daily for morbidity. (B) Representative image of a tumor at the time of diagnosis by abdominal ultrasound (volume=19mm^3).

A



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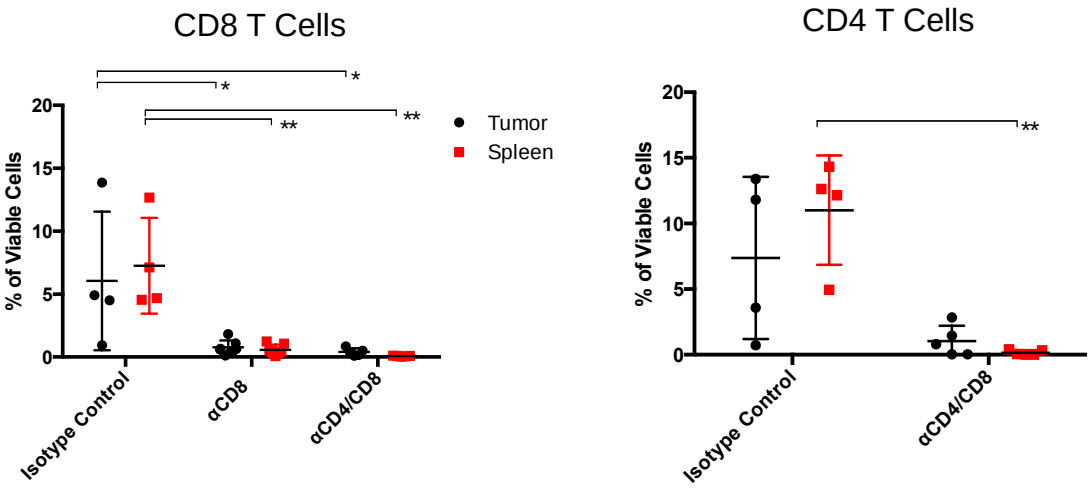
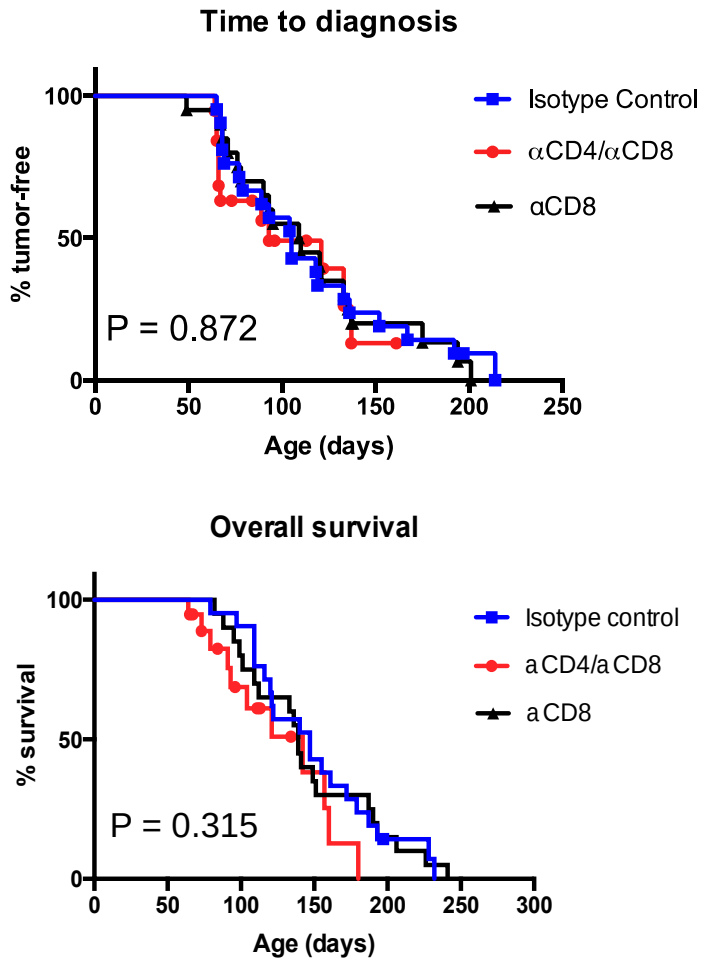


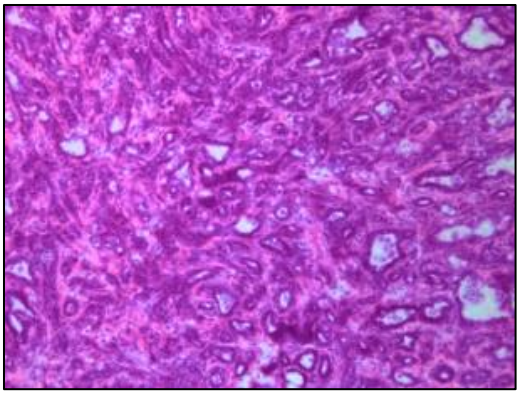
Fig 4. Validation of T cell depletion by antibodies. (A) Peripheral blood of T cell-depleted mice was monitored by flow cytometry beginning at the initial depletion and every 2-3 weeks thereafter. Data for CD8+ T cells (left) and CD4+ T cells (right) for all depleted mice are shown as the mean of the percentage of CD45+ leukocytes, pooling all measurements for each individual mouse. For isotype-treated KPC mice, a randomly selected subset (N=10) was assessed for T cell levels at an interim timepoint. $P<0.0001$ for all depleted cohorts compared to isotype control by one-way ANOVA. (B) T cell levels in pancreatic (tumor) tissue and spleen at the time of harvest from isotype or T cell-depleted mice enrolled in the survival study. CD4 and CD8 cells are expressed as a percentage of viable (7AAD-) cells.



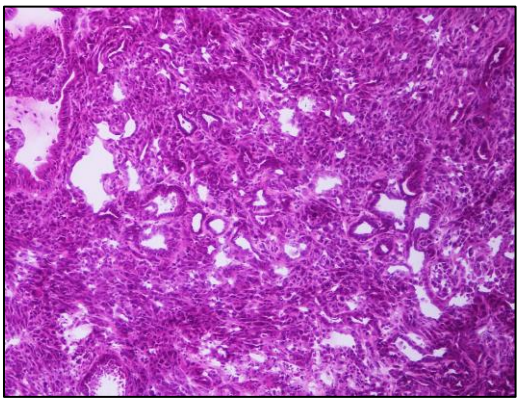
	Isotype	αCD4/αCD8	αCD8
Median time to diagnosis (days)	105	93	110
Median overall survival (days)	147	142	139

Fig 5. Time to diagnosis and overall survival of KPC mice are independent of T cells. Tumor-free survival according to ultrasound monitoring (time to diagnosis, top panel) and overall survival according to daily monitoring (bottom panel) for the three cohorts described in Fig. 3. P-values were determined by Log-rank (Mantel-Cox) analysis. Table (below) shows median survival and median time to diagnosis for each cohort.

Isotype Control



αCD4/αCD8



αCD8

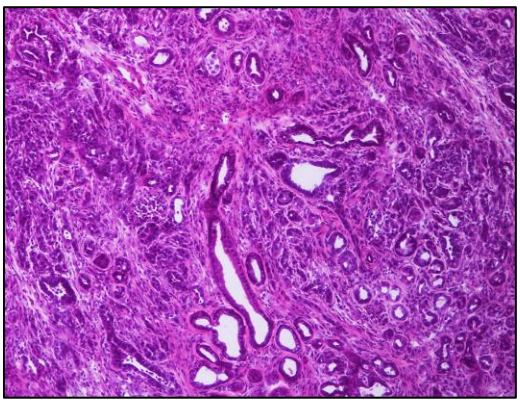


Fig 6. Pancreatic tumors of immune-competent and T cell-depleted KPC mice demonstrate similar histology. Hematoxylin and eosin (H&E) staining of a representative pancreatic tumor from each treatment cohort at the time of sacrifice (10x magnification).

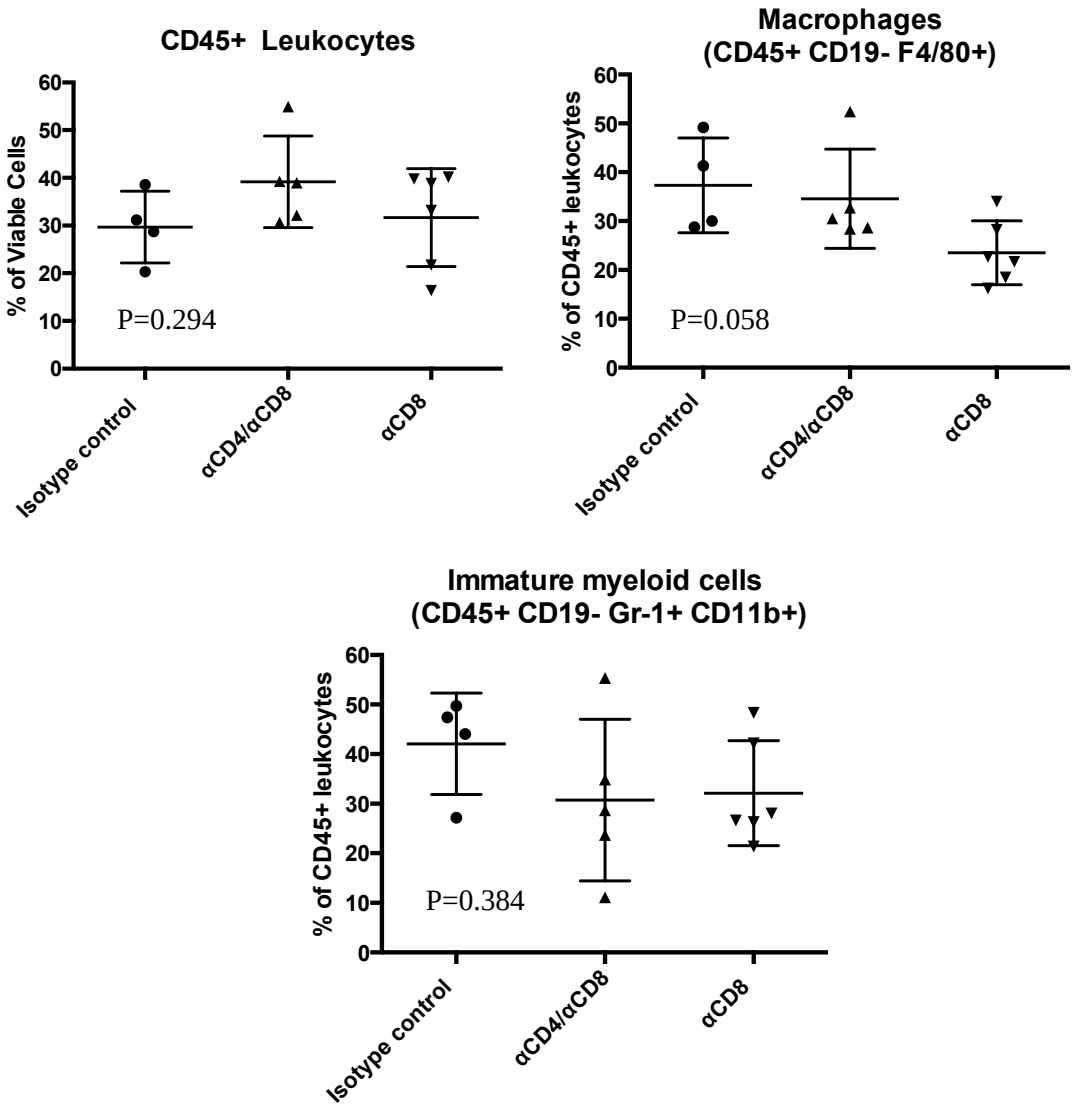


Fig 7. Pancreatic tumors of immune-competent and T cell-depleted KPC mice demonstrate similar leukocytic infiltrates. Flow cytometric analysis of tumors at the time of euthanasia (4-6 mice per cohort) to assess infiltration by leukocytes (top left panel, CD45+ cells as percent of viable cells), macrophages (top right panel, CD45+ CD19- F4/80+ as percent of viable CD45+ cells), and immature myeloid cells (bottom panel, CD45+ CD19- Gr-1+ CD11b+ as percent of viable CD45+ cells). Data are shown as whisker plots, with P-values determined by two-way ANOVA.

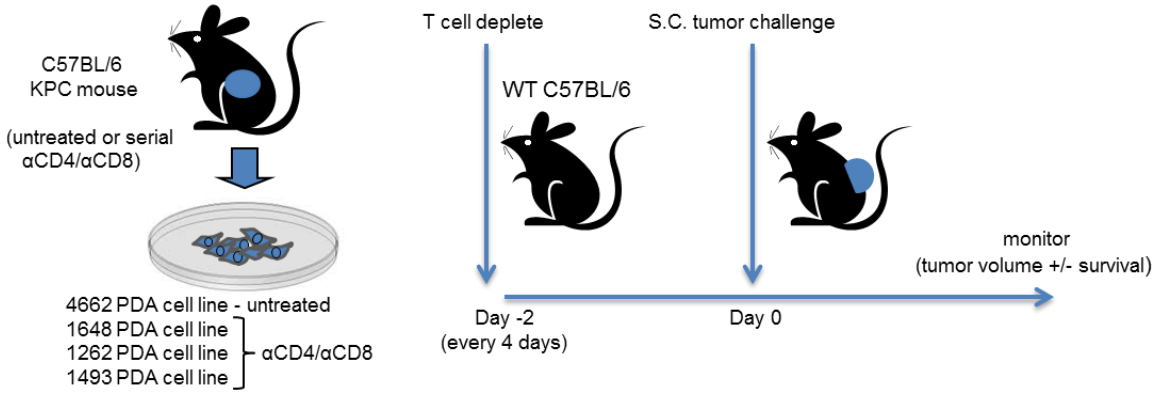


Fig 8. Experimental design for implant studies of syngeneic PDA cell lines. Cell lines were generated from immune-competent KPC mice or KPC mice serially depleted of CD4/CD8 T cells beginning at 3-5 weeks of age as shown in Fig. 3. Cell lines were implanted subcutaneously (s.c.) in syngeneic female mice pre-depleted of CD4 and CD8 T cells or administered an isotype control antibody (N=8-10 mice per cohort). Tumor growth was measured by caliper over time and mice were monitored for overall survival.

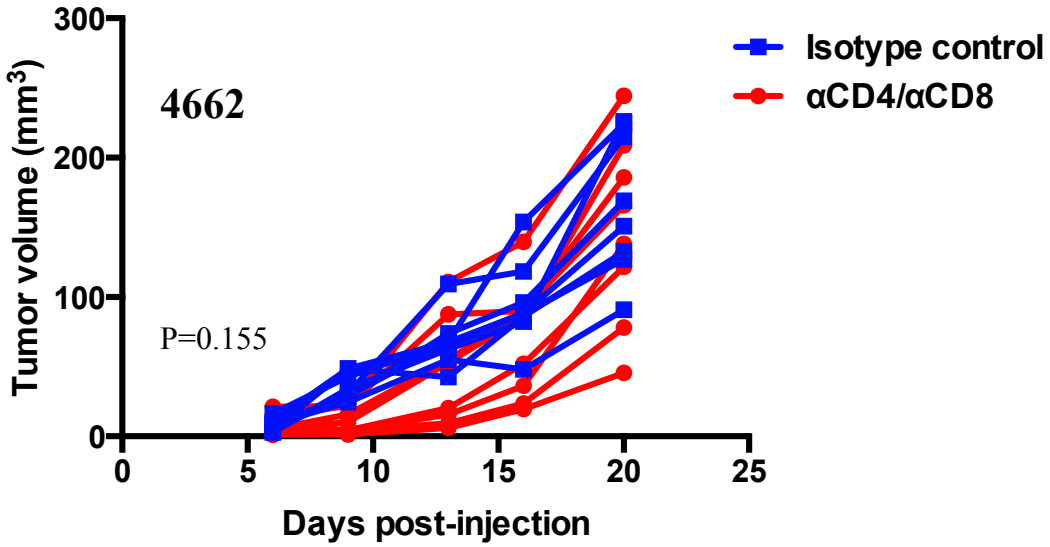


Fig 9. 4662 KPC cell line grows with the same kinetics upon implantation in immune-competent or T cell-depleted mice. Subcutaneous tumor growth of 4662 PDA cells in immune-competent syngeneic hosts (Isotype control) or immune-compromised mice (αCD4/αCD8), shown for high inoculum ($5x10^5$ tumor cells). Growth data are shown as spaghetti plots of individual mice, and P-value indicates analysis by two-way ANOVA.

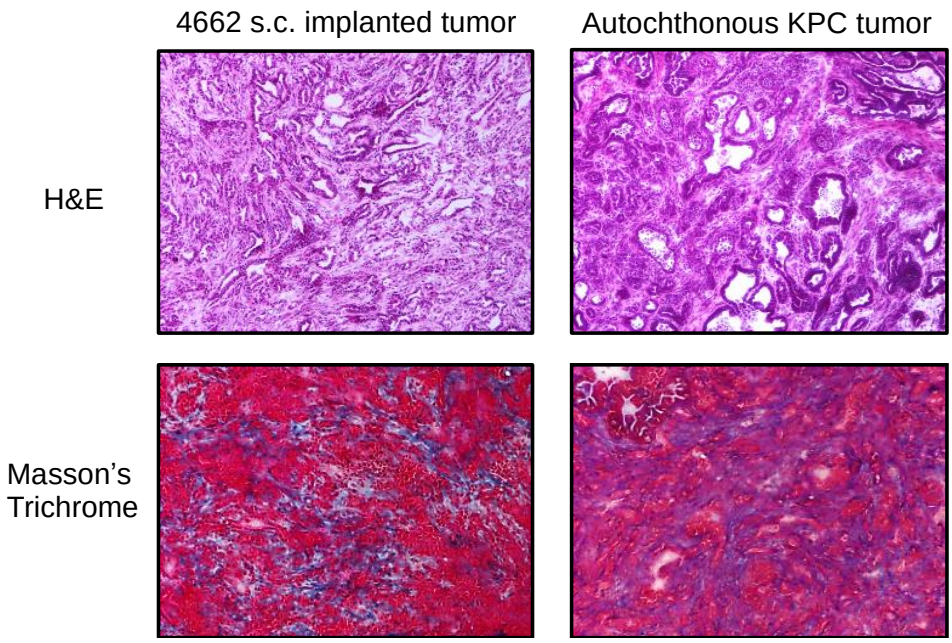


Fig 10. 4662 implanted tumors recapitulate the microenvironment of autochthonous KPC tumors. Histology of 4662 implanted tumors after 3 weeks of growth (left panel) stained by H&E and Masson's trichrome (top and bottom, respectively). Right panels show H&E and trichrome staining of a representative autochthonous KPC tumor. All images, 10x magnification.

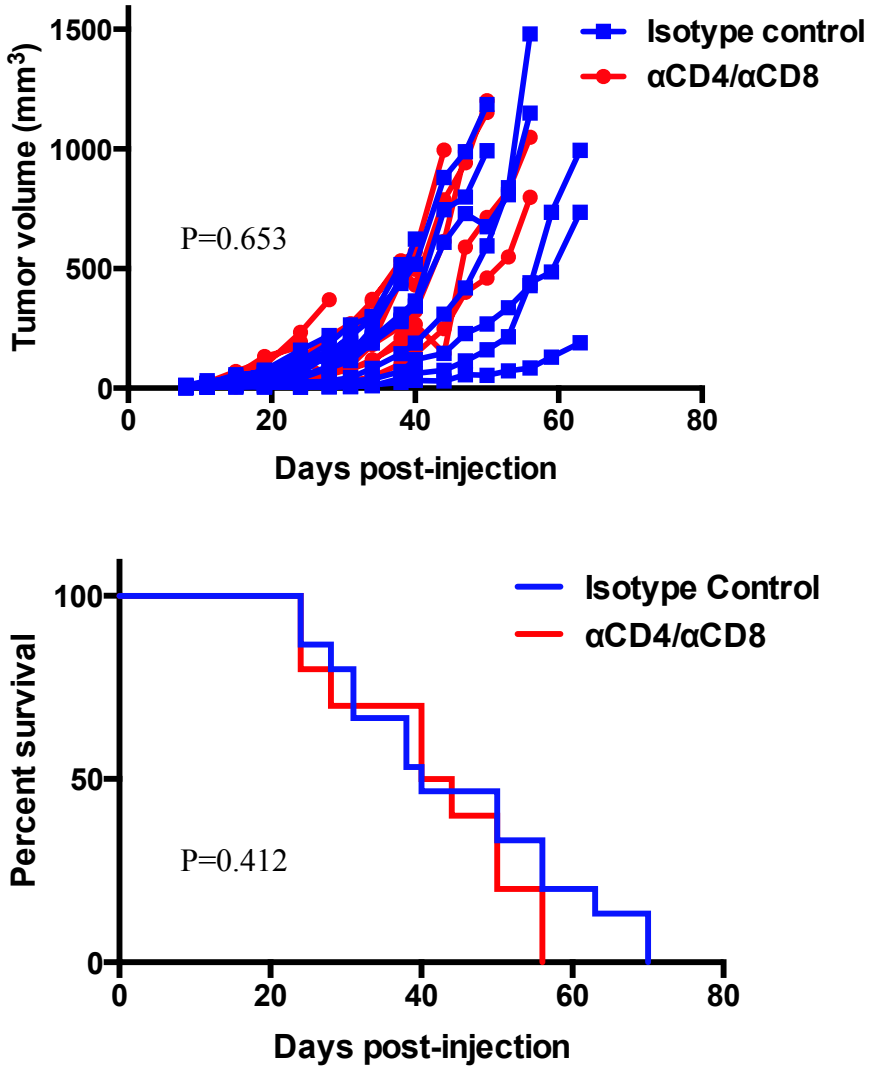


Fig. 11. Low doses of 4662 cells subcutaneously do not elicit T cell-mediated tumor regressions or alter overall survival of recipient mice. Subcutaneous tumor growth of 4662 PDA cells in immune-competent syngeneic hosts (Isotype control) or immune-compromised mice (αCD4/αCD8), shown for low inoculum (10^5 cells). Mice were also monitored for overall survival (bottom panel). Growth data are shown as spaghetti plots of individual mice, and P-value indicates analysis by two-way ANOVA. Survival data were analyzed by Log-rank (Mantel-Cox) test.

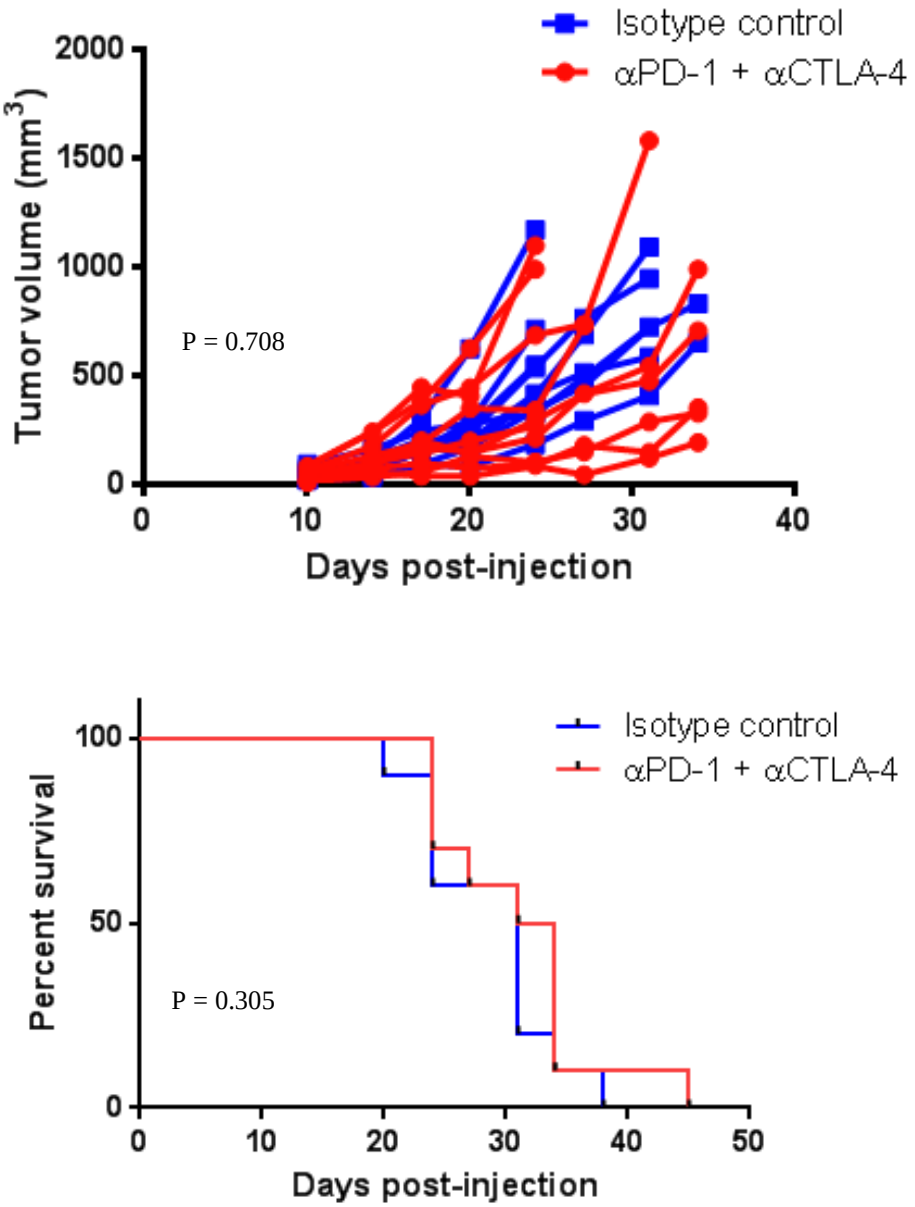


Fig 12. PD-1 and CTLA-4 blockade do not impact 4662 tumor cell growth or overall survival of recipient mice. C57BL/6 female mice were implanted subcutaneously with parental 4662 cells as in Fig. 8 and were treated with a combination of antibodies blocking PD1 and CTLA-4. A second cohort received an isotype control antibody. N=10 mice per cohort. Tumor growth by caliper was analyzed using two-way ANOVA (top), and overall survival was assessed by Log-Rank/Mantel-Cox test (bottom).

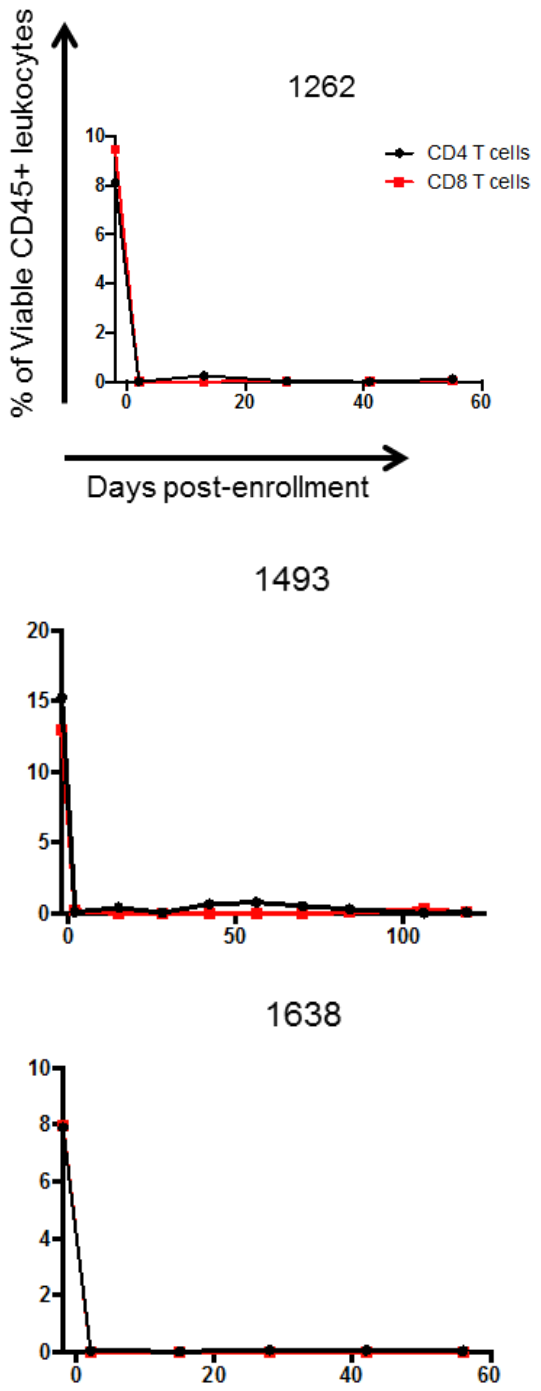


Fig 13. Validation of T cell-depletion in KPC mice used to generate T cell-depleted tumor cell lines. Flow cytometry of peripheral blood from three mice chronically depleted of CD4 and CD8 T cells and used to generate tumor cell lines 1262, 1493, and 1638. T cell levels are shown as a percentage of viable (7AAD-) CD45+ leukocytes at various time points post-enrollment (~4 weeks of age) until tumor-associated morbidity and euthanasia.

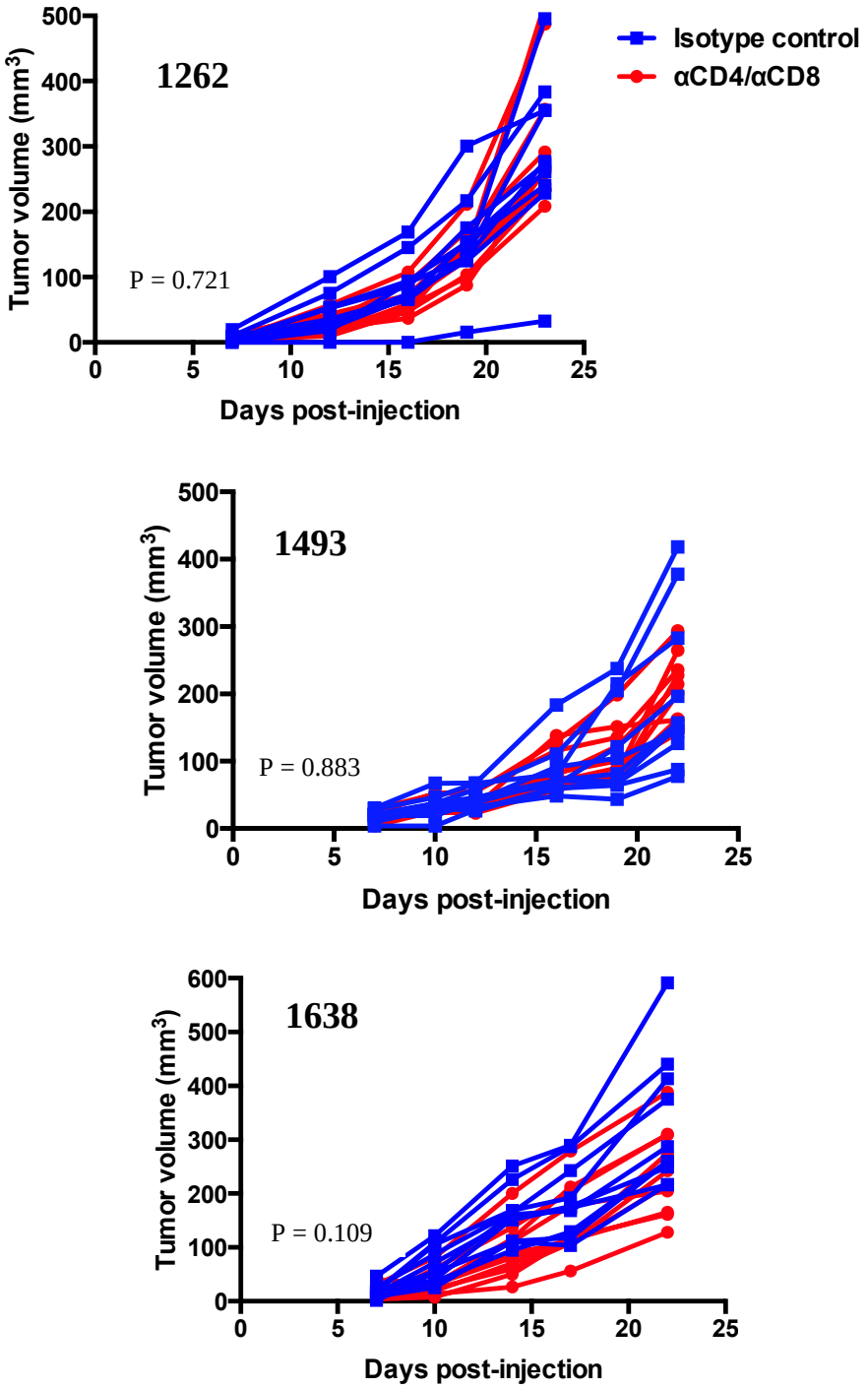


Fig 14. T cell-depleted KPC tumor cell lines grow irrespective of recipient immune status. Subcutaneous growth of CD4/CD8-depleted KPC cell lines (1262, 1493, and 1638; generated as described in Fig. 8) in immune-competent syngeneic isotype control or αCD4/αCD8 mice. N=8-10 mice per cohort. P-values shown were generated by two-way ANOVA.

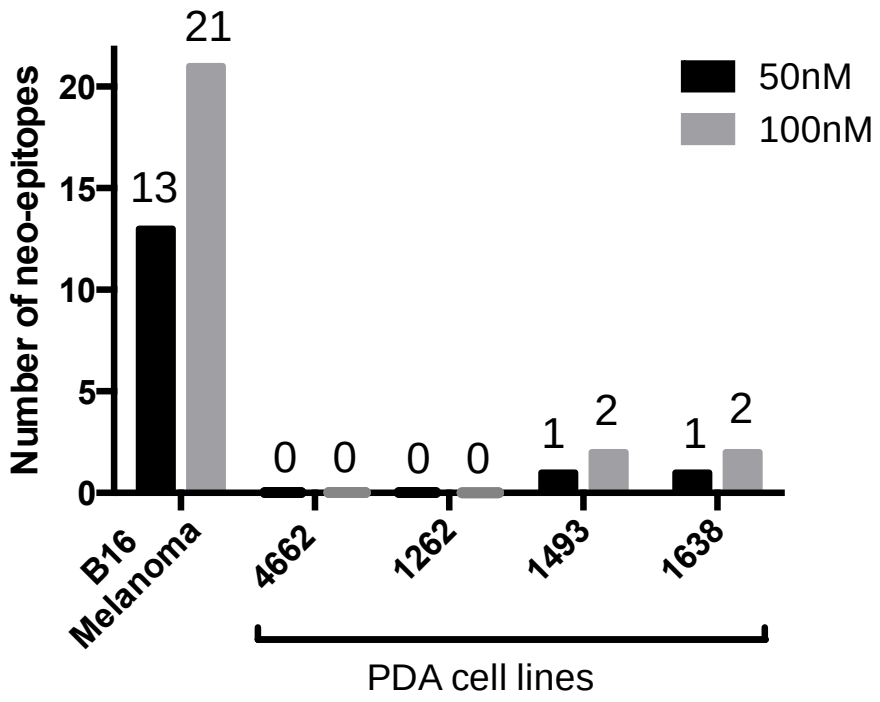


Fig 15. Neo-epitopes in KPC-derived tumors are rare. Numbers of predicted neo-epitopes for the B16 murine melanoma cell line, the 4662 PDA cell line derived from an immune-competent KPC mouse, and three cell lines derived from T cell-depleted KPC mice (1262, 1493, and 1638). Predictions are shown for both the 50nM binding threshold (black bars) and 100nM threshold (grey bars).

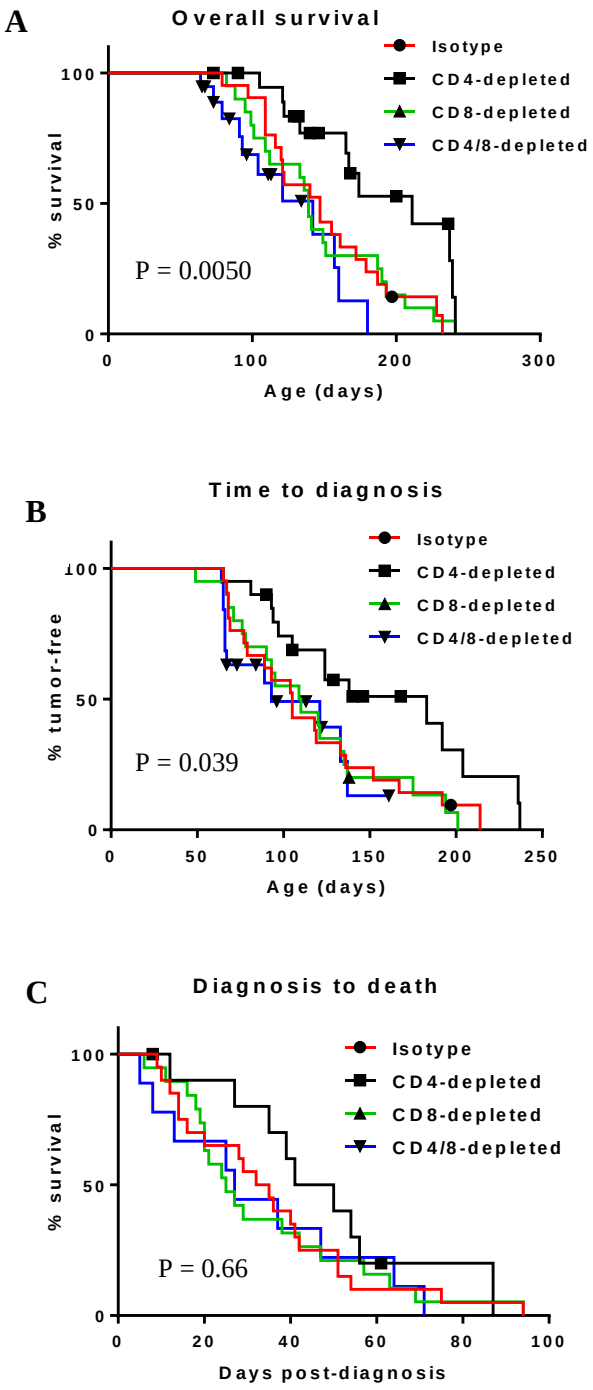
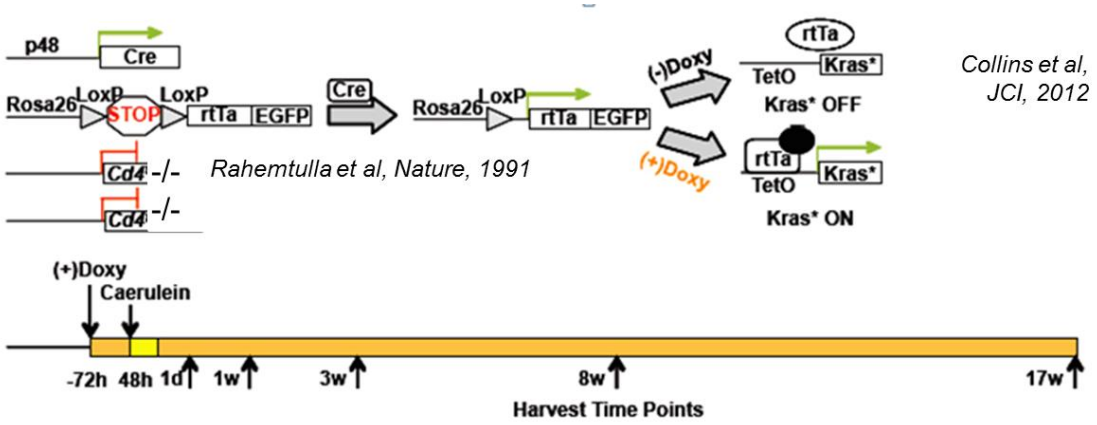


Fig 16. Overall survival and time to diagnosis are increased in CD4-depleted KPC mice. (A) Overall survival according to daily monitoring. (B) Tumor-free survival (i.e. time to diagnosis) according to ultrasound monitoring. (C) Time from diagnosis to death (i.e. tumor progression). Data include CD4-depleted mice as well as the three cohorts described in Fig 5. P-values were determined by Log-rank (Mantel-Cox) analysis.

A



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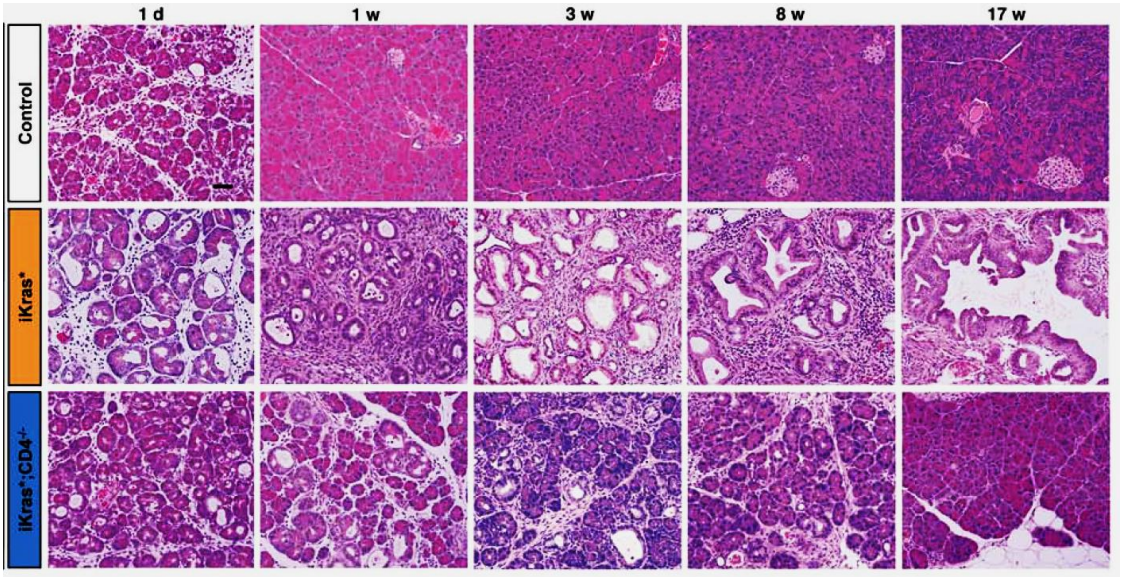


Fig 17. Genetic deletion of CD4 in iKras mice abrogates PanIN formation following cerulein-induced pancreatitis. A) Schematic of CD4-null iKras mouse model and induction of pancreatitis. B) H&E of pancreata of control littermates, iKras mice, and CD4-null iKras mice at various timepoints following administration of cerulein. (Zhang, *et al.*, Cancer Immunology Research, 2014).

CHAPTER 4 – Neo-antigens override the tumor microenvironment and dictate immune surveillance in cancer.

*The majority of these studies have been described in the manuscript submitted for publication

INTRODUCTION

Through the baseline studies of T cell immunosurveillance in the KPC mouse model, I determined that immunoediting does not manifest in the conventional sense, and that the dynamic interaction between the tumor and the immune system is more nuanced and complex in this particular context. The intersection of tumor microenvironmental factors, T cell-intrinsic factors, and sub-threshold immunogenicity results in failed immunosurveillance without driving tumor escape. Which of these elements dominates tumor outgrowth in PDA requires further exploration in order to understand the potential for various immunotherapies.

Clearly the microenvironment and T cells can be inhibited or stimulated, respectively, to drive an immune response; however, these factors may be secondary to the sheer "invisibility" of tumors as predicted by the lack of mutation-derived epitopes. In fact, the tumor microenvironment may very well be negligible in comparison, and T cells may not be truly "dysfunctional" in PDA if provided the appropriate antigen. Recent evidence suggests that neo-antigens are the "holy grail" of cancer immunology; despite the eminent success of chimeric antigen receptors against various blood cancers (Porter et al., 2011; Grupp et al., 2013), these therapies are limited to antigens expressed

on the surface of tumor cells (e.g., CD19 on malignant B cells) and result in toxicity to non-cancerous cells. Given these limitations, neo-antigens represent the next generation of immunotherapy. By definition, neo-antigens are specific to the tumor cells, and they may be intracellular or extracellular; the rate-limiting step is an ability to be processed and presented by MHC class I alleles with significantly higher affinity than the wild-type protein, as recently exemplified in MCA-induced sarcomas (Yewdell and Bennink, 1999; Matsushita et al., 2012). Schreiber and colleagues determined that the loss of mutant β-spectrin protein in escape clones was driven by high-affinity binding to the MHC class I molecule in mice, and importantly resulted from a single amino acid change compared to the wild-type protein. In a subsequent study, Schreiber and colleagues uncovered similar neo-antigens by checkpoint blockade, suggesting that T cell tolerance or exhaustion were co-determinants of immunoediting in this model (Gubin et al., 2014). Our model system again deviated from these findings, such that a dearth of naturally occurring neo-antigens (as suggested by sequencing) required additional T cell "help" in the form of CD40 antibodies and gemcitabine/abraxane to utilize weaker (self) antigens; again, these findings implicate the immune-quiescence of the tumor itself in failed surveillance of PDA, not its microenvironment or T cell-intrinsic dysfunction.

The preeminence of neo-antigens in driving immunosurveillance of human cancers was highlighted by Brown and colleagues in their analysis of deep sequencing data available through The Cancer Genome Atlas (Brown et al., 2014). Using another epitope-prediction tool across human samples from six tumor types, they determined that tumor mutational burden alone did not correlate with survival; rather, it was the presence or absence of a predicted neo-epitope that held prognostic significance. Higher

immunogenicity likewise correlated with *CD8A* expression, suggestive of a T cell response. Moreover, immunogenicity resulted in the increased expression of T cell exhaustion markers like *PDCD1* and *CTLA4*, encoding the same checkpoint proteins targeted in the Schreiber study.

Based on these findings, I hypothesized that the lack of a strong neo-antigen distinguishes PDA from MCA-driven tumors and from tumors responsive to immunotherapy, and may be more critical than an immunosuppressive microenvironment in determining rejection. I therefore predicted that introducing a strong neo-antigen into the same model of PDA would prompt a T cell-dependent response and reproduce key findings from the MCA-driven model.

RESULTS

In lieu of an endogenous neo-antigen, I chose to introduce a known epitope to our non-immunogenic 4662 line that would mimic the activity of a mutation-derived antigen. In particular, ovalbumin (OVA) protein includes a well-established immunodominant peptide (SIINFEKL) that binds with high affinity to H2-K^b and therefore mimics immunological properties of a tumor neo-epitope (Rotzschke et al., 1991). I therefore retrovirally transduced 4662 PDA cells with an OVA-expressing construct (Wang et al., 2008) labeled with a Td-Tomato marker to create PDA cells that express full-length OVA for processing and presentation by H2-K^b MHC class I alleles (Fig 18A).

Cells were sorted post-transduction for expression of Td-Tomato, and a representative clone (V6.Ova) was selected for further studies (Fig. 18B). V6.Ova cells were found to effectively process and present SIINFEKL on H2-K^b, based on reactivity with a fluorochrome-labeled mAb specific for the peptide-MHC complex (Fig. 18B). Baseline expression of MHC class I was similar in V6.Ova and parental 4662 cell lines, and was comparably upregulated following stimulation with IFNγ; in contrast, MHC class II was not found to be expressed on either cell line despite treatment with IFNγ (Fig 19).) Negative sorting of transduced cells was then used to derive a cell line ("OvaNeg") to be used as a control for the three TdT-Ova clones (G10.Ova, G7.Ova, and V6.Ova) (Fig. 20A).

Subcutaneous implantation of V6.Ova cells even at a high dose (10^6 cells) resulted in lymphocytic infiltration at day 8, followed by complete tumor rejection; however, depletion of CD8 T cells prior to implantation permitted rapid outgrowth of V6.Ova tumors (Fig. 21). Neither administration of αCD4- nor αNK1.1-depleting antibodies prevented tumor rejection (Fig. 21), suggesting that CD8-dependent immunity was necessary and sufficient for rejection of V6.Ova cells. I also tested a lower dose of V6.Ova ($7.5x10^5$ cells) and likewise observed poor survival when CD8 cells were depleted, in contrast to 100% cure and long-term survival of isotype-treated mice implanted with this number of V6.Ova cells (Fig. 22). Similar findings were observed for two other 4662-derived Ova-expressing clones (Fig. 20B).

To assess whether this anti-tumor response could be recapitulated in the pancreatic microenvironment, orthotopic implantation and ultrasound monitoring for tumor growth were performed in syngeneic mice. Parental 4662 grew rapidly in all experiments when implanted in the pancreas of untreated wild-type mice (Fig. 23A) and recapitulated the histology of

autochthonous KPC tumors (Fig 23B). In contrast, 14 of 17 immune-competent mice (82%) tested in multiple independent experiments rejected the same dose of orthotopically implanted V6.Ova cells (Fig 24A, isotype cohort; Fig. 25A) and achieved long-term survival compared to the parental 4662 line (Fig. 25B). Antibody-mediated depletion of CD8 T cells abrogated this effect, such that orthotopically implanted V6.Ova cells grew aggressively (Fig. 24A) and required euthanasia of mice in all cases (Fig. 24B). Thus, OVA expression by tumor cells is sufficient to mediate and maintain CD8-dependent tumor rejection in multiple microenvironments, including the pancreas (Fig. 25C).

Moreover, mice cured of V6.Ova subcutaneous implantation rejected or resisted re-challenge with 4662 parental cells; this effect was lost with CD4/CD8 cell depletion immediately prior to re-challenge (Fig. 26). These data are consistent with epitope spreading, whereby T cell immunity can be established against 4662 endogenous antigens in the setting of a strong, acute inflammatory immune response; in this case, the immune response to OVA cultivates the evolution of immunity against covert antigens. These data further underscore that, as previously observed in the setting of chemotherapy and CD40 agonists (Winograd et al., 2015), PDA tumor cells express otherwise immunologically quiescent endogenous (non-OVA) antigens that are nevertheless capable of mediating T cell-dependent tumor rejection when provided with the necessary immunological impetus.

Although these findings with the V6.Ova clone recapitulated critical features of the elimination phase of immunosurveillance, the powerful anti-tumor response may have masked potential immune escape and thereby truncated the immunoediting process. To allow further study of the escape phase, V6.Ova cells were mixed with OvaNeg cells at high ratios (90%

V6.Ova: 10% OvaNeg, and 80% V6.Ova: 20% OvaNeg) for a constant subcutaneous dose of $7.5x10^5$ cells (Fig. 27). Pure populations of both the V6.Ova clone and the OvaNeg cells were included for comparison, and each cohort was treated with αCD8 antibody or an isotype control.

Despite exhibiting slower growth compared to parental 4662 or OvaNeg cells, tumors ultimately emerged from each 90% V6.Ova or 80% V6.Ova isotype implant. In each case, with CD8-depletion, tumor growth accelerated to the same rate as observed for the pure population of Ova-negative cells treated with or without CD8 depletion (Fig. 28). Overall survival was intermediate in the 90% and 80% V6.Ova cohorts compared to either pure population of cells (Fig. 29). The only cohort to achieve cure and long-term survival was the CD8-replete cohort implanted with 100% V6.Ova, as I had anticipated (Fig. 28 and Fig. 29).

To understand antigen-specific responses in these experiments, I established tumors from 80% V6.Ova and control lines for two weeks, at which point complete remission is achieved in mice administered 100% V6.Ova cells. Peptide-MHC tetramer staining for the presence of intratumoral Ova-specific CD8+ T cells revealed a 40-fold enrichment of tetramer-positive cells in the tumors from 80% V6.Ova implants compared to the spleens of the same mice; these intratumoral Ova-specific CD8+ cells were likewise significantly enriched compared to tumors from mice implanted with an equivalent dose of parental 4662 cells, or tumors from mice depleted of CD8 T cells, thereby demonstrating a robust adaptive immune response *in vivo* (Fig. 30). Tumor-infiltrating Ova-specific CD8+ T cells prominently expressed granzyme B and Tbet, consistent with an effector phenotype; moreover, these Ova-specific CD8 T cells were highly functional even after 3 weeks,

expressing Ki67 and IFNγ upon stimulation *ex vivo* (Fig. 31). Overall, this effector phenotype was most prominent in tumor compared to spleen (Fig. 30 and 31). These results suggest a highly specific CD8 T cell response to Ova-expressing cells that bypasses Ova-negative cells. However, the rapid kinetics in this experiment did not provide sufficient time for the development of epitope spreading, which might have otherwise prevented outgrowth of an Ova-negative tumor (as in Fig. 26).

To determine whether the emerging tumor cells themselves had been sculpted by a host T cell response, Td-tomato expression was assessed in tumors allowed to establish for two weeks. Consistent with immunoediting, tumor cells that eventually grew from 80% V6.Ova implants in isotype-treated mice did not express Td-tomato, resulting in expression identical to parental 4662 tumors (Fig. 32). Moreover, the stromal architecture of these tumors resembled that of 4662 parental tumors (Fig. 33). These results are consistent with the escape and subsequent proliferation of Ova-negative tumor cells from the original mixed implant; the data also demonstrate that elimination of antigen-rich cells can occur even in the presence of the immunosuppressive microenvironment orchestrated by escaping tumor cells. In contrast, CD8-depleted cohorts challenged with 100% V6.Ova and 80% V6.Ova significantly and proportionately retained their tumor expression levels of Td-tomato (Fig. 32). Loss of Td-tomato expression in the presence of activated intratumoral CD8+ T cells likely represents selective outgrowth of the Ova-negative tumor cell subset that escapes T-cell mediated elimination. Thus, tumor-sculpting effects were mediated by antigen-specific T cells responding to a neo-antigen, homing to the tumor and executing effector function despite the presence of a desmoplastic microenvironment.

DISCUSSION and CONCLUSIONS

This study demonstrates that ectopic expression of a strong neo-antigen in PDA cells fully rescues the immunoediting and escape phenotype despite an immunosuppressive stroma, without the need for additional therapeutic interventions. I also showed that a well-developed primary response against a neo-epitope can instigate epitope spreading to endogenous antigens and foster a memory response against tumor cells lacking such epitopes. Thus, cancer immunosurveillance manifests according to the antigenic strength of the tumor, with the capacity to override tumor microenvironmental regulation. These findings have clinical implications. First, variable expression of neo-epitopes may explain differences in *de novo* and therapy-induced cancer immunity; human melanoma and murine MCA tumors reside at one end of the spectrum ("hot" tumors) and human and murine pancreatic carcinoma represent the other extreme ("cold" tumors) (Schumacher and Schreiber, 2015; Sharma and Allison, 2015). Second, tumor rejection on the basis of non-mutated endogenous antigens reaffirms the biological importance of such antigens and the therapeutic potential – and perhaps necessity – of vaccines that target them. Overall, this study reconciles seemingly incongruous manifestations of immunosurveillance across the cancer spectrum by highlighting the dominant role of antigen strength.

Schreiber recently reviewed the importance of neo-antigens in tumor immunology, and asserted that these protein-altering mutations (the source of tumor-specific antigens, or TSAs) are the basis of future therapeutics (Gubin et al., 2015). He attributed a lesser role to tumor-associated antigens (TAAs), because these proteins are "self" antigens and therefore rely upon aberrant or excessive expression to overcome

tolerance and induce immune responses. He described a complex pipeline for personalized immunotherapy based on genomic mining for such neo-antigens, various algorithms for predicting MHC Class I binding, and subsequent use of peptide pulsing and tetramer extraction of TILs for adoptive transfer. Although these methods have been given credence by a variety of studies (Snyder et al., 2014; Carreno et al., 2015; Rizvi et al., 2015), PDA has proven highly refractory to various immunotherapies, and moreover lacks a relevant mutational burden that would supply such neo-epitopes. Indeed, simply introducing a "neo-antigen" brought PDA back to the realm of immune-sensitive cancers – so robustly that additional checkpoint inhibition was unnecessary. Clearly, neo-antigens reign supreme in terms of their potential for initiating robust and durable immune responses, but those tumors with a dearth of such antigens at baseline will remain refractory.

I discovered that a "neo-antigen" in the form of ovalbumin can drive a primary immune response and can induce T cell-mediated protection upon subsequent re-challenge with tumor cells *lacking* the neo-antigen, presumably on the basis of epitope spread. This novel finding provides hope for treatment of immunologically "cold" tumors – a subset that Schreiber does not address in his review. Thus, the importance of TAAs should not be underestimated in the context of certain cancers like PDA. These cancers indeed harbor weaker antigens that remain dormant until unveiled in the setting of a strong primary response. Although OVA may be critiqued as an "irrelevant" peptide in terms of tumor immunology, its immunogenicity alone appears to provide the key to unlocking relevant TAAs. This concept might be pertinent in a vaccination setting for cancers that would otherwise remain immunologically quiescent, and would not rely

upon personalized therapy and the extensive pipeline described by Schreiber. Epitope spread and TAAs expand the current armamentarium of immunotherapy against "cold" tumors.

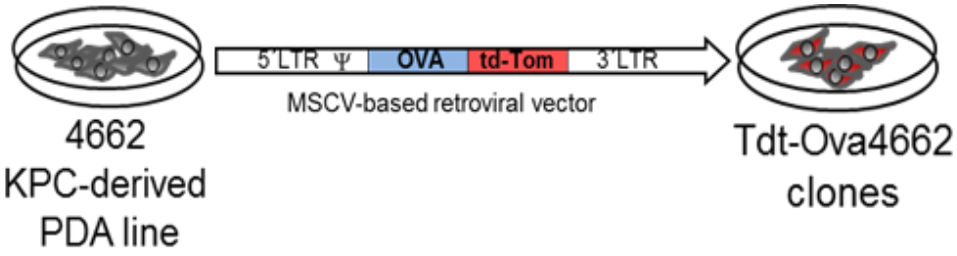
Another advantage of targeting TAAs is the diminished susceptibility to antigen-loss variants, which may ultimately negate the benefit of neo-antigen-based therapies. Additionally, the T cell-dependent protection generated by epitope spread did not require additional T cell stimulation in the form of CD40 agonists or checkpoint inhibitors, and demonstrated efficacy in the presence of an intact CD4+ compartment. The obsolescence of additional therapies would reduce the risk for auto-immunity and related side effects. Most importantly, the potential for universal efficacy of this vaccination approach using *any* strong antigen demands further investigation.

Ovalbumin can induce central tolerance under circumstances of germline expression (Gallegos and Bevan, 2004), but further studies could determine if such tolerance would permit outgrowth of the V6.Ova cells that are invariably rejected when expressed as a "neo-antigen." Using transgenic mice that constitutively express ovalbumin under the actin promoter, outgrowth of a V6.Ova implant would demonstrate that this antigen can, in fact, be subjected to immune tolerance. This study is currently ongoing in the laboratory. Additionally, constructs encoding alterations in the immunodominant SIINFEKL peptide (or class II epitopes) could be introduced to the parental 4662 cell line to induce variable degrees of immunogenicity, thereby elucidating the threshold of antigen strength required for immune control. Other documented tumor antigens – including neo-antigens such as β-spectrin, as well as self-antigens like

Gp100—could be evaluated for an ability to override the immunosuppression of PDA and induce tumor regressions with similar efficacy to ovalbumin (Bakker et al., 1994; Kawakami et al., 1994; Matsushita et al., 2012). Thus, ovalbumin has provided a useful tool for studying the balance between strong antigens and immunosuppression, and will continue to provide a means of studying the "antigenic threshold" required for anti-tumor immunity. It would be additionally informative to re-assess these findings using documented tumor neo-antigens or self-antigens.

FIGURES

A



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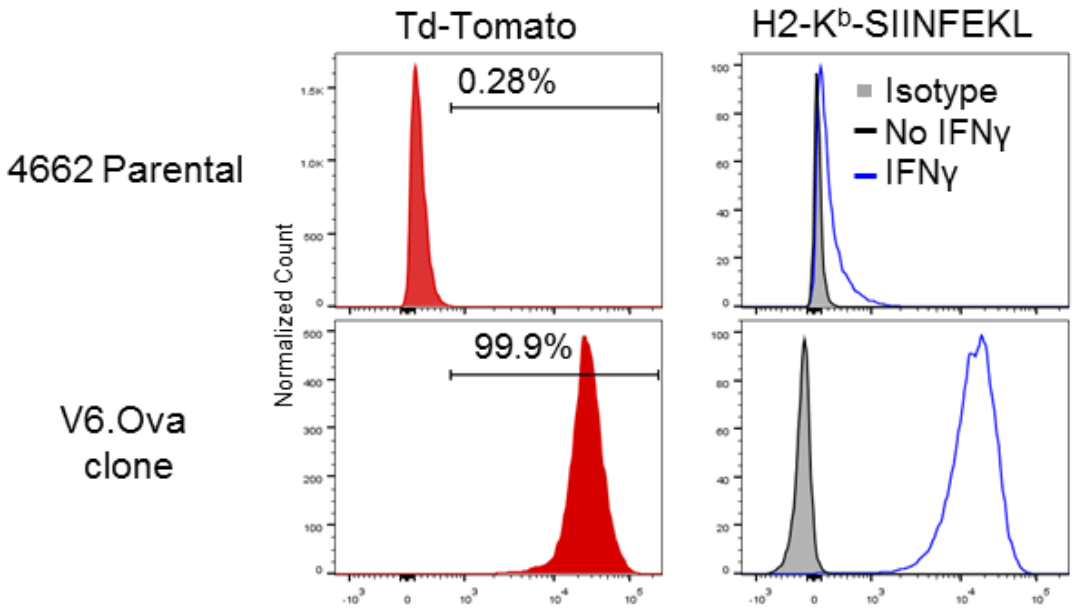


Fig 18. Tdt-Ova 4662 processes and presents SIINFEKL peptide on MHC class I. (A) Parental 4662 cells were retrovirally transduced with a Td-Tomato/OVA-expressing (Tdt-OVA) construct, and sorted to generate single-cell clones. (B) Flow cytometric analysis of the parental 4662 cell line (top panels) compared to the V6.Ova clone (bottom panels). Cells were assessed for expression of Td-tomato (left panels), incubated with or without IFNγ and stained using a mAb against SIINFEKL-bound H2-K^b (MHC class I, right panels), gating on viable (Live/Dead aqua-negative) cells. Data represent three independent experiments.

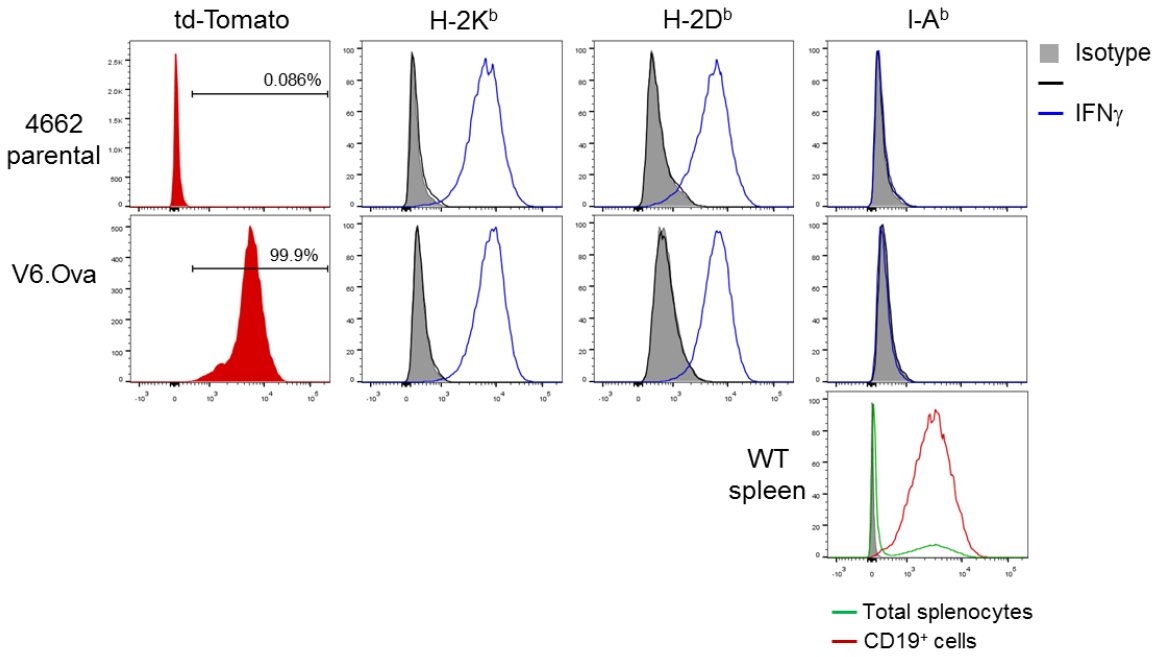
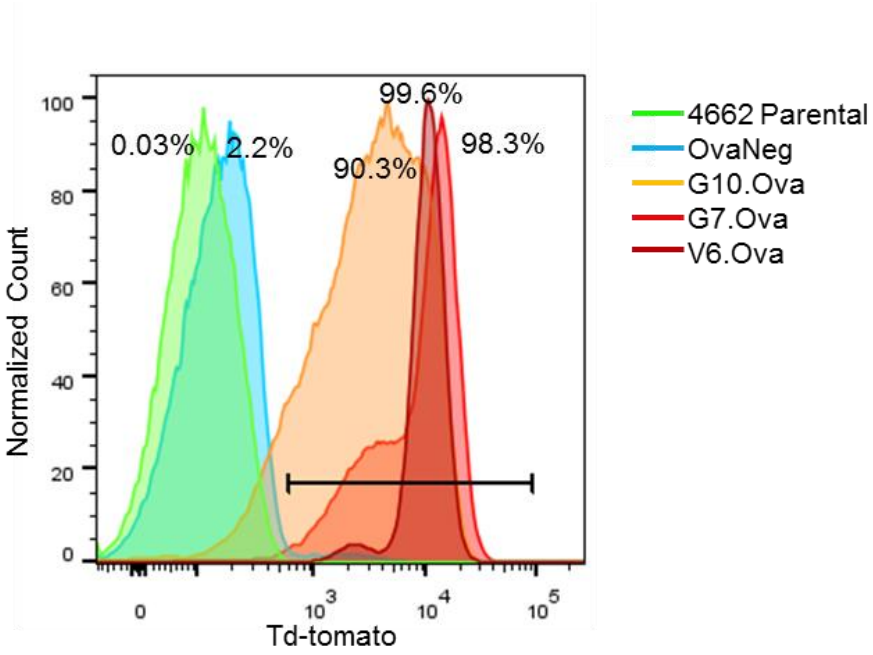


Fig 19. Expression of MHC class I and class II on 4662 parental and V6.Ova cell lines. Parental 4662 and V6.Ova cells were analyzed by flow cytometry for expression of Td-Tomato, MHC class I (H2-K^b and H2-D^b) and MHC class II (I-A^b) with or without IFNγ stimulation *in vitro*. Positive control for MHC class II staining is shown below for total wildtype (WT) splenocytes and CD19+ B cells.

A



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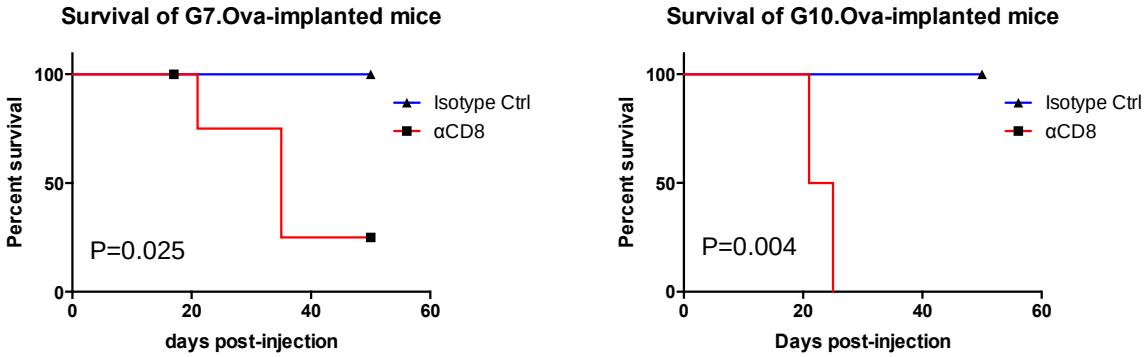


Fig 20. Additional Tdt-Ova expressing 4662 PDA clones. (A) Td-tomato expression levels of three 4662 Tdt-Ova tumor clones (V6.Ova, G7.Ova and G10.Ova) compared to 4662 parental and OvaNeg (negatively sorted) cell lines, gating on viable (7-AAD-negative) cells. (B) Survival data of immune-competent or CD8-depleted C57BL/6 mice implanted subcutaneously with $0.75x10^6$ cells of the additional G7.Ova and G10.Ova clones (N=4-5 mice per cohort). P-values were determined by Log-rank (Mantel-Cox) analysis. One mouse was censored for non-tumor related mortality (CD8-depleted cohort, G7.Ova clone).

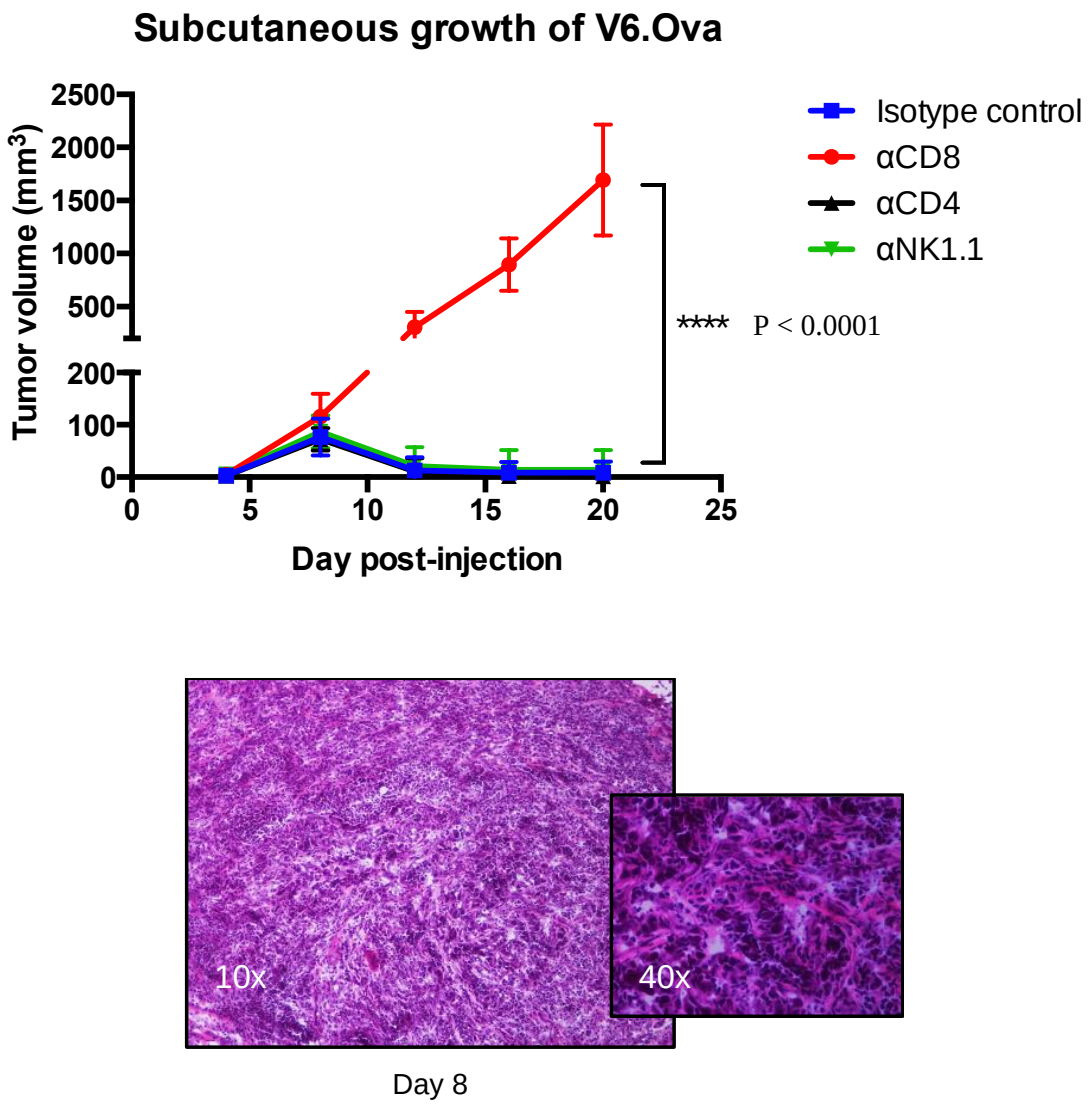
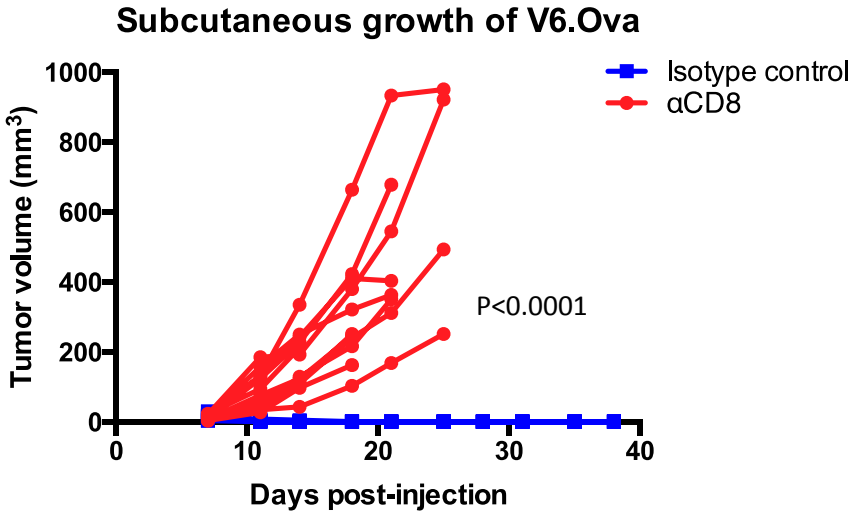


Fig 21. Subcutaneous rejection of V6.Ova clone is CD8 T cell-dependent. A high dose of the V6 clone (10^6 cells) was subcutaneously implanted in syngeneic mice (top) treated with an isotype control antibody, CD8-depleting antibody (αCD8), CD4-depleting antibody (αCD4), or an NK cell-depleting antibody (αNK1.1) and monitored for growth over time by caliper. N=7-8 mice per cohort (P<0.0001 by two-way ANOVA). Below, H&E of an implant at day 8 of an isotype-treated mouse showing presence of tumor cells with marked infiltration of lymphocytes (10x, and inset 40x).

A



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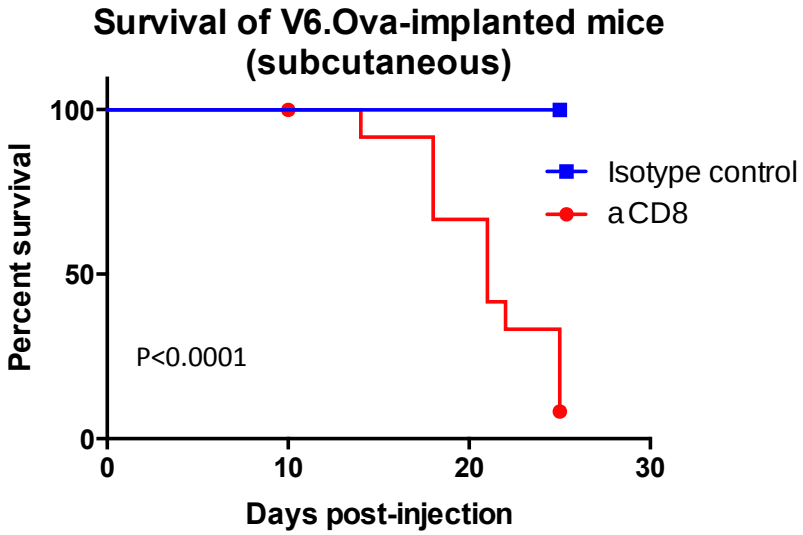
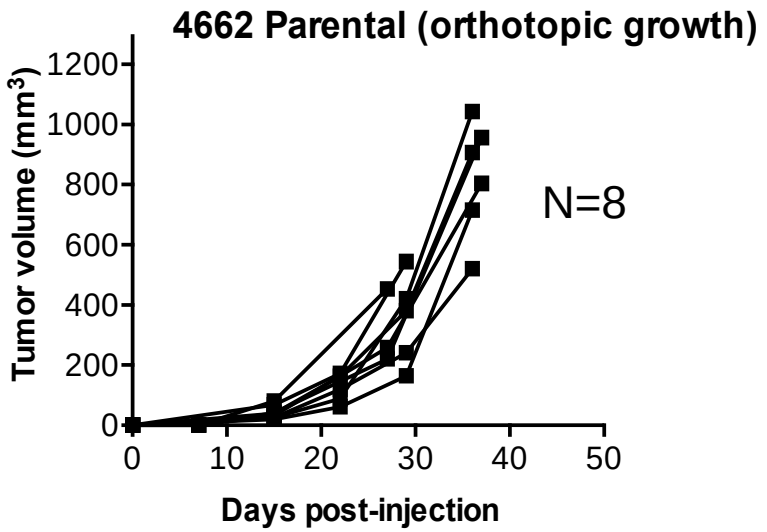


Fig 22. V6.Ova clone is subcutaneously rejected in immune-competent mice, resulting in long-term survival. Tumor growth at a lower inoculum of $0.75x10^6$ V6.Ova cells (A) was assessed in isotype-treated and CD8-depleted cohorts, which were also monitored for overall survival (B). N=12-13 mice per cohort; P-values represents analysis by two-way ANOVA (tumor growth) and Log-rank/Mantel-Cox test (survival).

A



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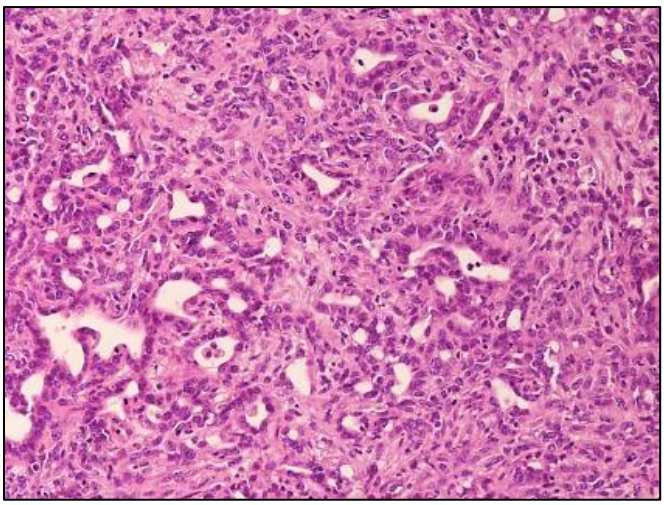
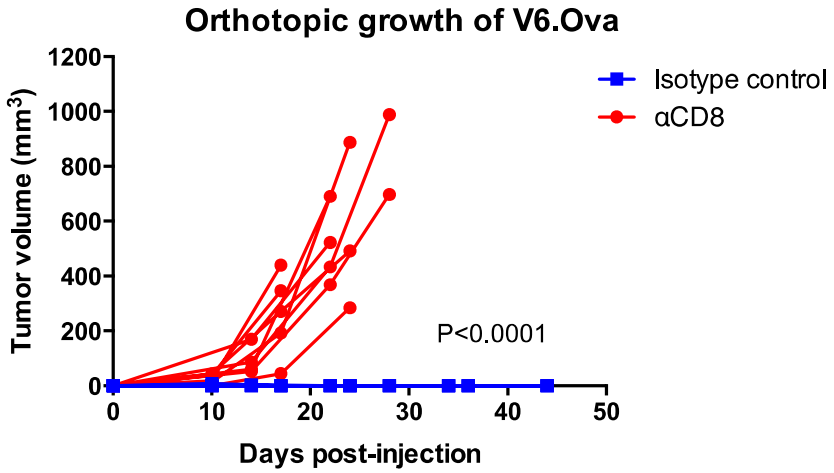


Fig 23. Orthotopic implantation of the 4662 cell line grows out in immune-competent mice and reproduces classic PDA histology. (A) The parental 4662 cell line was implanted orthotopically in immune-competent C57BL/6 host mice at a dose of $0.125x10^6$ cells (N=8). Tumor growth was assessed by serial ultrasound and is shown for each individual mouse post-injection until the time of death. (B) H&E analysis of a representative 4662 tumor 22 days post-injection (10x).

A



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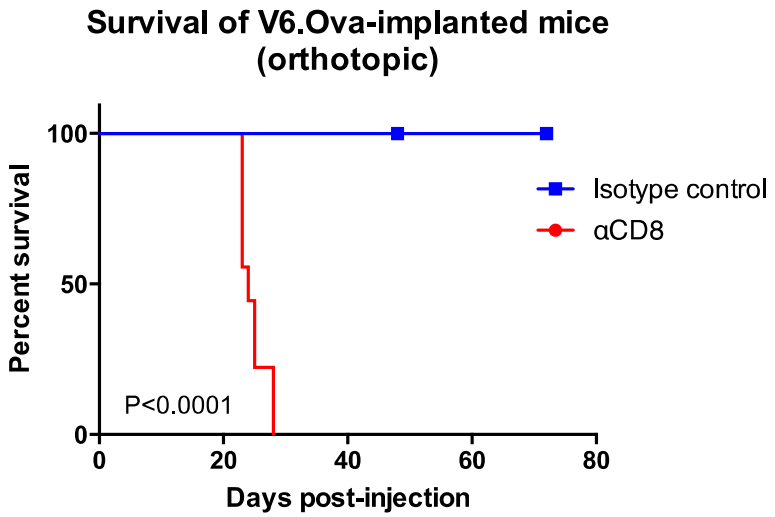


Fig 24. Orthotopic rejection of V6.Ova implants and long-term survival of recipient mice is CD8 T–cell dependent. Growth of V6.Ova tumor cells implanted orthotopically in mice treated with isotype control or αCD8 with an inoculum of $0.125x10^6$ cells. Mice were monitored for tumor growth by ultrasound (A) and assessed for overall survival (B). N=9-10 mice per cohort; data shown are pooled from two independent experiment experiments. Analysis was performed by two-way ANOVA (tumor growth) and Log-rank/Mantel-Cox (overall survival).

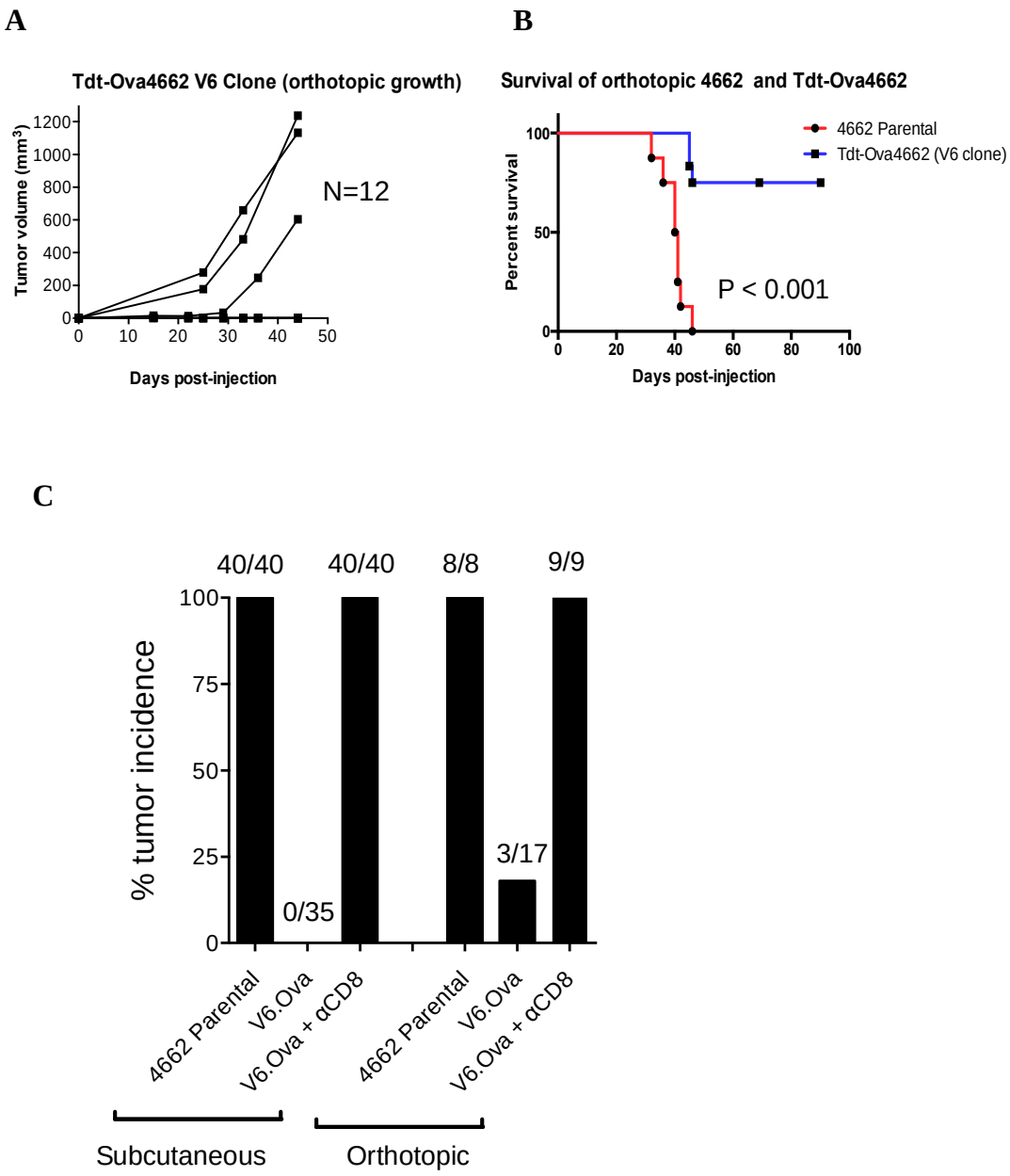


Fig 25. Summary of orthotopic and subcutaneous implant studies. (A) The V6.Ova clone was orthotopically implanted in untreated immune-competent C57BL/6 mice, and tumor growth was monitored by ultrasound. (B) Comparison of overall survival for orthotopic experiments in Fig 23 (4662 parental) and 25A (V6.Ova); P-value calculated by Log-rank/Mantel-Cox test. (C) A summary of subcutaneous and orthotopic growth of parental 4662 cells, and isotype-treated or CD8-depleted V6.Ova-implanted cohorts.

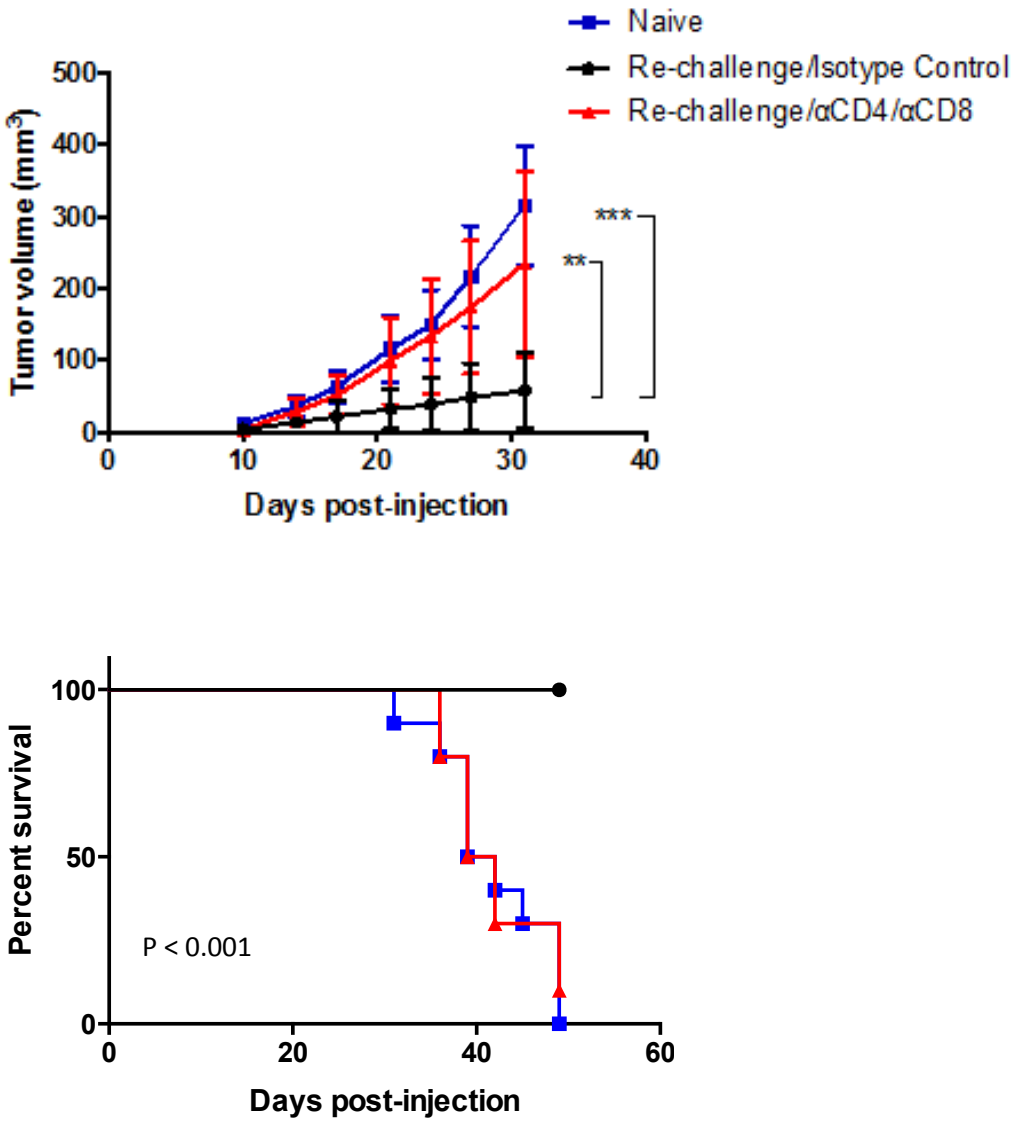
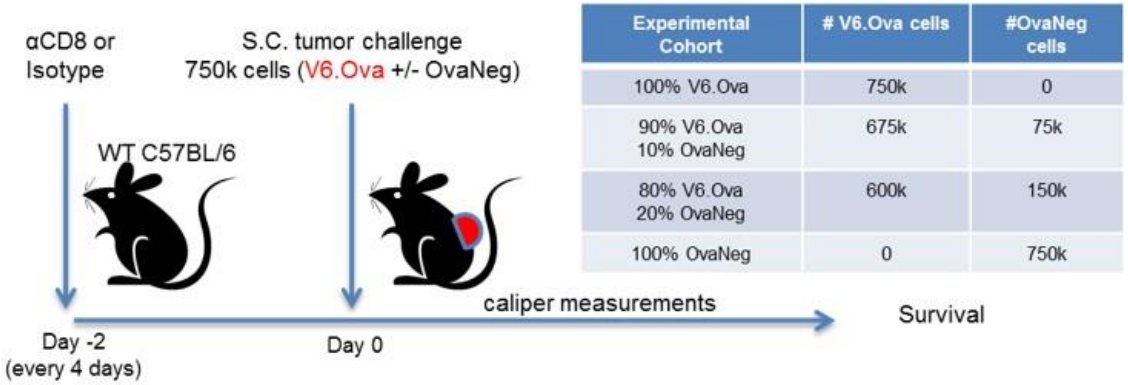


Fig 26. Re-challenge with Parental 4662 results in T cell-dependent tumor regressions and delayed tumor outgrowth. C57BL/6 mice that rejected a subcutaneous V6.Ova implant after six weeks were either CD4/CD8-depleted or administered an isotype control, and then re-challenged with parental 4662 on the opposite flank. A third, naïve cohort was simultaneously challenged with parental 4662 at the same dose of 10^5 cells. N=9-10 mice per cohort. Mice were followed by caliper for tumor growth and monitored for overall survival. P-values represent analysis by two-way ANOVA or Log-rank/Mantel-Cox tests, respectively. ** represents P<0.01 and *** represents P<0.001.



Experimental Cohort	# V6.Ova cells	#OvaNeg cells
100% V6.Ova	750k	0
90% V6.Ova 10% OvaNeg	675k	75k
80% V6.Ova 20% OvaNeg	600k	150k
100% OvaNeg	0	750k

Fig. 27 Experimental design of competition assay between V6.Ova cells and negatively sorted (OvaNeg) cells. Ova+ and Ova– 4662 cells were implanted subcutaneously at various ratios in immune-competent or CD8-depleted cohorts as shown (N=5 mice per cohort) and monitored for tumor growth and overall survival.

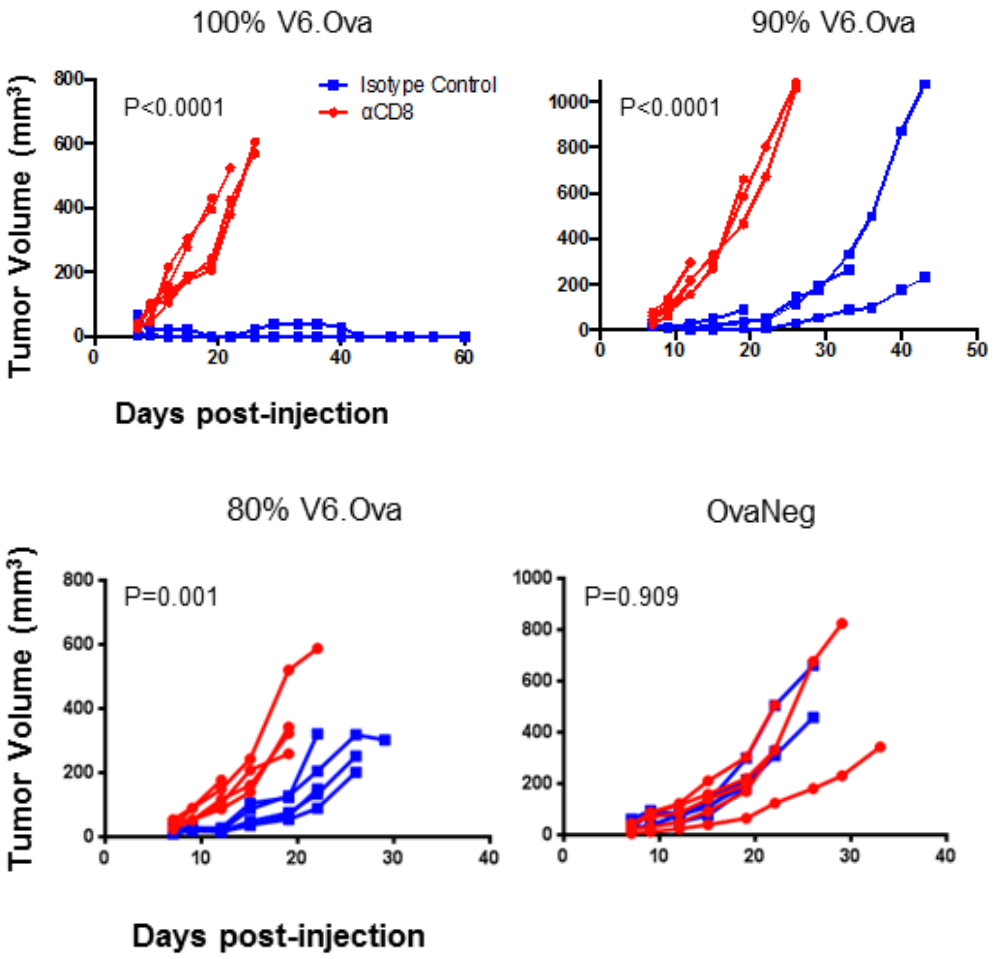


Fig 28. Mixed implants of V6.Ova and OvaNeg cells grow in immune-competent recipients with kinetics proportional to the admixture ratio. Subcutaneous implants containing either a combination of Ova+ and Ova- cells (90% V6.Ova and 80% V6.Ova) or a homogenous population of V6.Ova or OvaNeg cells were assessed for tumor growth. Data are shown as the individual growth curves for each mouse per cohort; P-values represent two-way ANOVA.

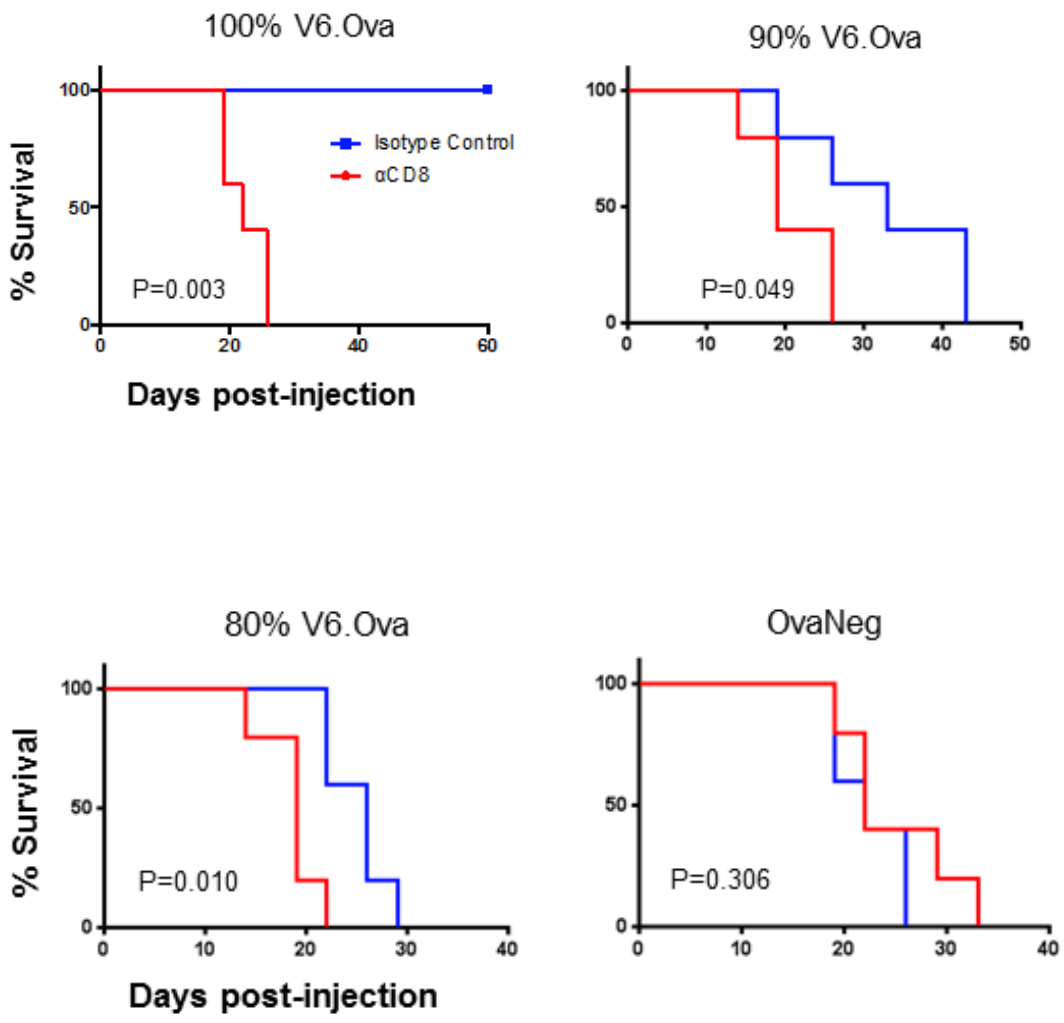


Fig 29. Survival of mice implanted with mixtures of V6.Ova and OvaNeg cells is dependent upon the admixture ratio. Mice implanted with a combination of Ova+ and Ova- cells (90% V6.Ova and 80% V6.Ova) or a homogenous population of V6.Ova or OvaNeg cells were assessed for overall survival; P-values were determined by Log-rank/Mantel-Cox for each cohort.

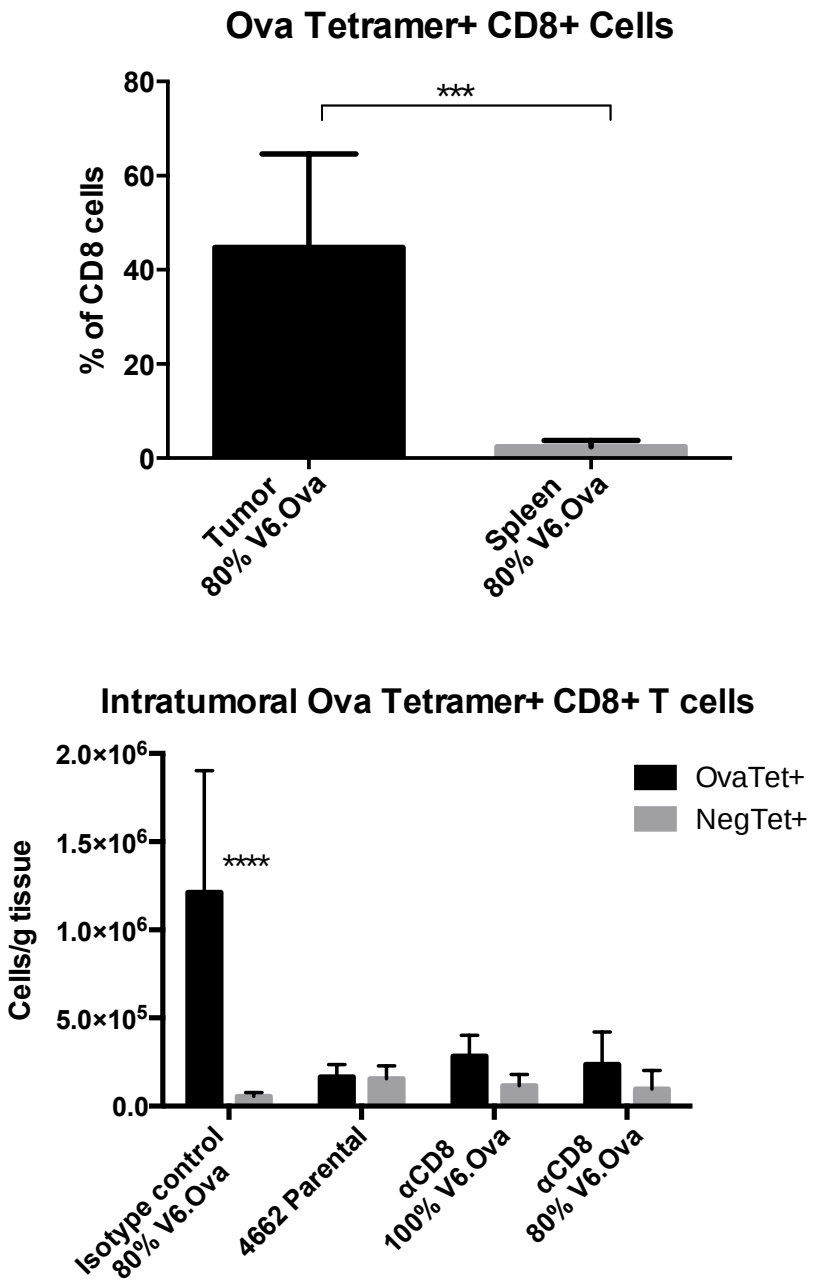


Fig 30. Ova-specific T cells are highly enriched in the tumors of mice implanted with 80% V6.Ova. Tetramer staining of Ova-specific CD8+ T cells in 80% V6.Ova tumors compared to spleens at day 14 (top), and quantified in the tumor across all cohorts (bottom). N=5-7 mice per cohort; data are representative of three independent experiments. *** indicates P<0.001 by unpaired two-tailed Student's *t*-test (top), and **** P<0.0001 by two-way ANOVA (bottom).

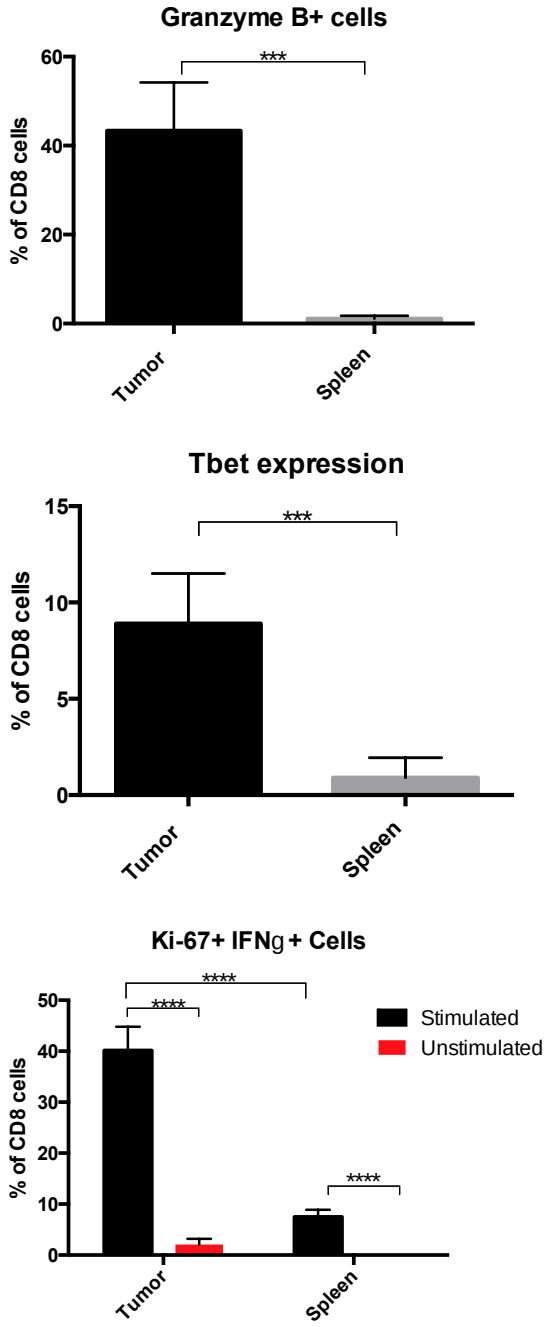


Fig 31. Intratumoral CD8+ T cells in 80% V6.Ova implants are highly functional and proliferative. The tumor-infiltrating CD8+ T cells in mice implanted with 80% V6.Ova cells were assessed by flow cytometry for intracellular levels of Granzyme B, Tbet, and Ki67 at day 21. IFNγ expression was also assessed by stimulation with PMA/ionomycin. N= 6 mice per cohort. *** indicates P<0.001 by unpaired two-tailed Student's *t*-test (upper graphs) and **** represent P<0.0001 by two-way ANOVA (lower graph).

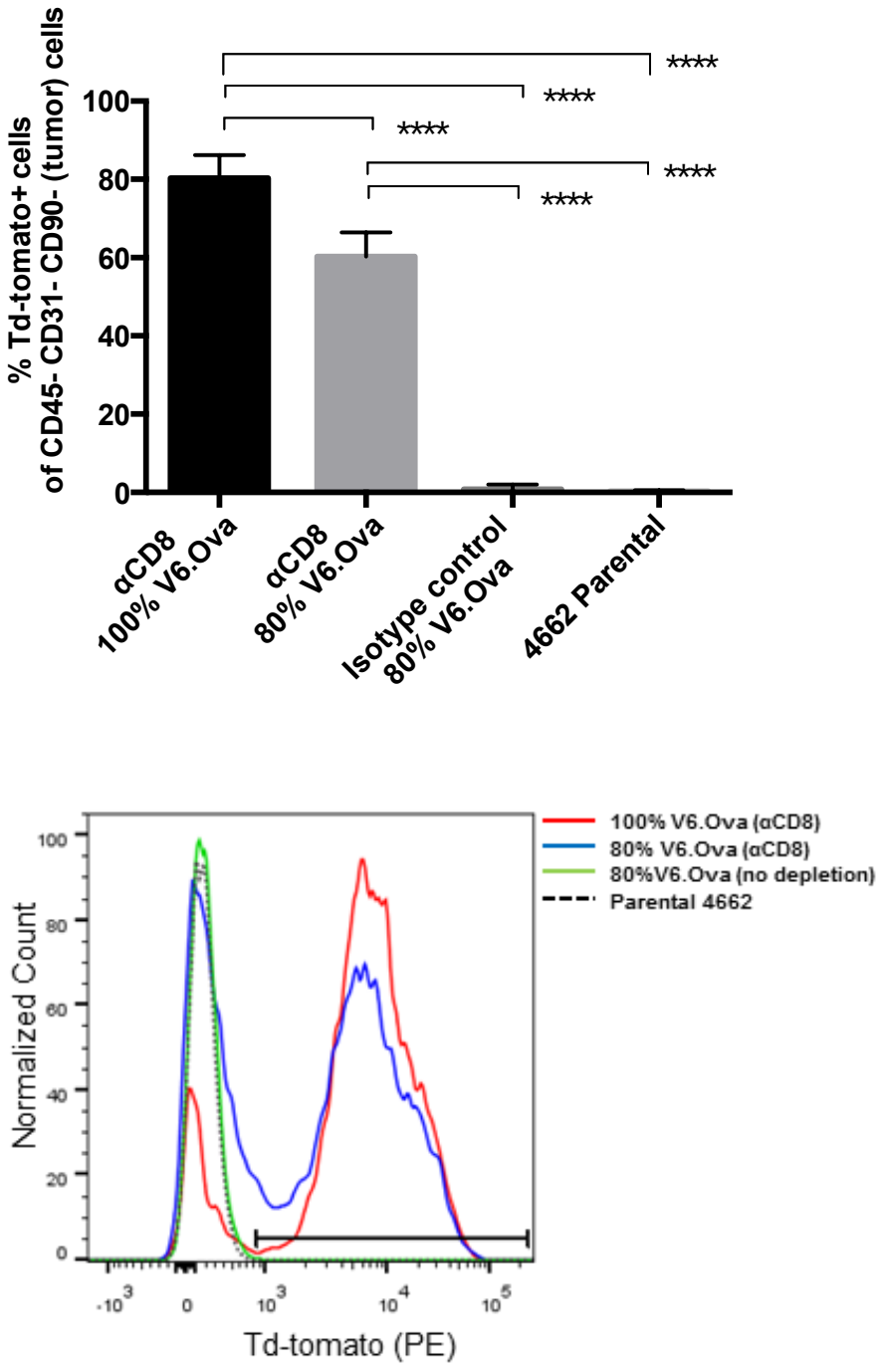


Fig 32. Loss of Td-tomato expression in V6.Ova implants is abrogated by CD8-depletion. Tumor-enriched cells (CD45- CD31- CD90-) were assessed for Td-Tomato expression by flow cytometry; Td-tomato+ cells are shown as a percentage of this tumor-enriched population (top graph). N=5-7 mice per cohort. **** indicates P-value<0.0001 calculated by two-way ANOVA. At bottom, representative data are shown as a histogram of Td-tomato+ cells for each cohort.

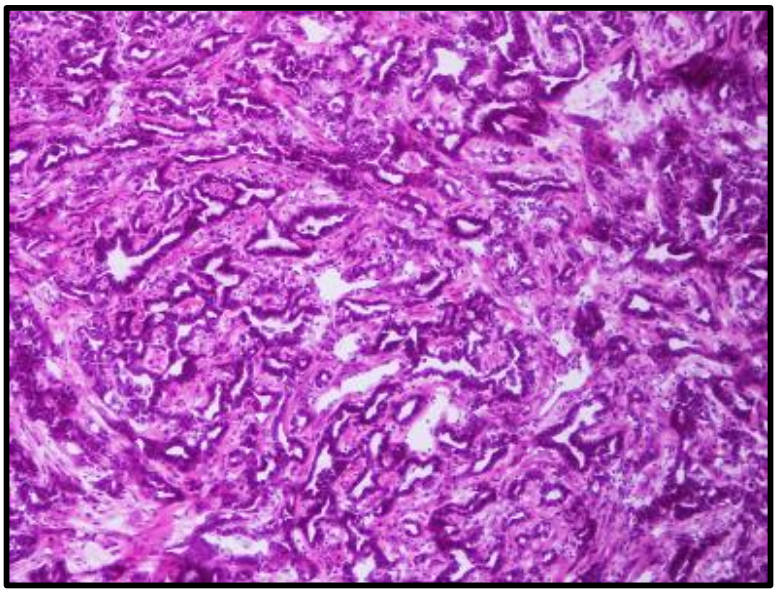


Fig 33. Tumor histology of 80% V6.Ova implants resembles parental 4662 implants. H&E stain of representative isotype-treated 80% V6.Ova tumor (10x).

CHAPTER 5 – Discussion and Future Directions

My thesis work has addressed a critical gap in our understanding of immunoediting and how it manifests in an oncogene-driven cancer model with a known immunosuppressive microenvironment, compounded by refractoriness to current immunotherapies in the clinic. PDA is thus set apart from other tumors such as melanoma and non-small cell lung cancer, in which clinical benefits have been readily demonstrated on the basis of checkpoint therapy or adoptive T cell transfer (Lennerz et al., 2005; Zhou et al., 2005; Rizvi et al., 2015). These successes ultimately rely upon the presence of tumor-specific antigens, especially neo-antigens, which distinguish the tumor from "self" and therefore are not subject to central tolerance in the developing immune system. Despite the dominance of neo-antigens in driving anti-tumor immunity, I have shown that the clinical outcome of surveillance can be manipulated therapeutically regardless of the presence or absence of classically described immunoediting (Fig. 34).

Re-visiting the immunoediting paradigm

I systematically utilized our KPC mouse model of PDA, which is a GEMM faithful to the human disease in both its histology and presentation, to reassess the landmark studies performed by Schreiber and others. I observed that the presence or absence of T cells is irrelevant to the natural history of PDA in this model, eschewing the fundamental tenet of tumor immunology that T cells interact with tumors and invariably sculpt their development. My thorough assessment of the immunoediting hypothesis in the KPC model has highlighted a number of key differences from the current theory:

- T cell-depleted KPC mice do not demonstrate a decreased median overall survival compared to their immune-competent counterparts; RAG2-/- mice subjected to MCA-induced carcinogenesis demonstrate an increase in tumor incidence and a decrease in median overall survival. *Thus, the growth of KPC tumors is independent of T cell surveillance, whereas MCA-induced tumors are subject to T cell surveillance.*
- Tumor cell lines derived from T cell-depleted KPC mice are not susceptible to immune rejection in immune-competent mice; 40% of MCA-derived tumors arising in RAG2-/- mice are rejected in immune competent mice. *Thus, KPC tumors do not undergo immunoediting. MCA-induced tumors undergo editing in immune-competent hosts.*
- Checkpoint blockade does not induce immunosurveillance and rejection of KPC-derived cell lines in immune-competent hosts; the same checkpoint blockade in MCA progressor cell lines renders them susceptible to antigen-specific T cell surveillance. *Thus, even checkpoint blockade does not elicit surveillance in KPC-derived cell lines.*
- Ectopic expression of a strong "neo-antigen" in an immune-refractory KPC cell line fully restores immunosurveillance; MCA-derived tumor cell lines naturally express neo-epitopes such as β-spectrin, Lama4, and Alg3 without a need for ectopic expression to drive surveillance. *Thus, the genomic landscape of KPC tumors and MCA-derived tumors affects the outcome of immunosurveillance.*

T cells therefore appear to be ignorant of the presence of these tumors, suggesting that the microenvironment pre-empts a T cell response, and/or these tumors are inherently non-antigenic. The immunoediting hypothesis does not address either of these possibilities. Thus, the theory that tumor outgrowth in immune-competent individuals is predicated upon tumor escape is *not* upheld in PDA and likely other cancers of the same ilk (Fig 35).

Microenvironment vs. Immunogenicity in the KPC model

I found that two factors (tumor microenvironment, and presence of neo-epitopes) ultimately play a role in failed immunosurveillance of murine or human PDA, but that they are not equally accountable for that failure. Using whole exome sequencing (WES) of my various PDA cell lines to observe mutations and potential neo-antigens on a genomic level, I validated that regardless of the immune status of the donor mouse, these cells lines exhibited few mutations. Moreover, virtually none of these mutations was predicted to bind strongly to MHC class I in a biologically relevant manner. It therefore appears that murine PDA is immunologically "quiescent," a finding substantiated by human data from the TCGA. According to cytolytic and CD8 signatures from the publicly available RNA-seq data, PDA is non-responsive in comparison to melanoma and lung cancers. Thus, both murine and human data indicate that certain tumor subsets are immunologically "cold", while others are "hot," and that the clinical literature mirrors this finding in terms of responsiveness to current immunotherapies.

Despite the lack of overt immunogenicity in KPC-derived tumors, we determined that disruption of the microenvironment can lead to anti-tumor responses based on

weaker, endogenous antigens. Long-term CD4 depletion in the KPC mouse increased survival, but this benefit was lost upon concurrent ablation of CD8 cells, suggesting a CD4-mediated tumor microenvironment that restrains an otherwise relevant cytotoxic T cell response. Although the CD4-depleted mice ultimately succumbed to tumor burden, some degree of CD8-mediated protection was present in these mice and delayed the time to tumor onset. In support of this finding, a cocktail of checkpoint inhibitors (αCTLA-4 and αPD-1) and an immune-stimulatory vaccine (agonistic CD40/gemcitabine/abraxane) resulted in subcutaneous regressions and CD8-dependent memory responses. Both studies indicate that disrupting the tumor microenvironment and/or stimulating T cells can elicit reactions against endogenous antigens.

To determine the *degree* to which each of these factors contributed to failed immunosurveillance, I induced expression of a strong antigen to the otherwise non-immunogenic 4662 cell line. Introduction of Ova in this setting, without TME disruption or further immune modulation, allowed for an endogenous response that resulted in complete subcutaneous regressions and nearly universal responses in the pancreas. Thus, expression of a strong antigen can override the tumor microenvironment without need for further intervention, suggesting that the microenvironment is not as drastically immunosuppressive as previously believed. With this single manipulation of the tumor itself, as proven by competition studies admixing Ova-expressing and parental 4662 cells, immunosurveillance and immunoediting are fully restored. Thus, the ultimate failure of surveillance is tumor-intrinsic, with a supporting role by immunosuppressive factors in the microenvironment.

Clinical implications

Perhaps the most intriguing finding of my study was that a primary response against Ova-4662 cells could allow for subsequent protection against re-challenge with the parental "non-immunogenic" cell line. In this setting, neither immunotherapy *nor* strong antigens were required to elicit durable responses or stable disease in the mice. This finding has the most striking implications for the clinic. Variable expression of neo-epitopes may indeed underscore differences in response to immune checkpoint therapy, for which human melanoma and murine MCA tumors reside at one end of the spectrum ("hot" tumors) and human and murine pancreatic carcinoma represent the other extreme ("cold" tumors) (Schumacher and Schreiber, 2015; Sharma and Allison, 2015). These findings argue a case for sequence-based decisions in predicting efficacy of cancer immunotherapies. However, the need for such immunotherapies and personalized medicine may be rendered obsolete with the simple use of a vaccine, hearkening back to the initial implications of Coley's toxins (Coley, 1891). In Coley's studies, a completely unrelated set of antigens generated by infection with erysipelas allowed for concurrent regressions of various lymphomas.

Although such findings may seem antiquated, contemporary literature validates the pursuit of vaccines against cancers of a non-viral etiology. A recent study used the same KPC model to evaluate the effects of immunization with an attenuated form of *Listeria monocytogenes*, which was engineered to express the Kras mutation (Keenan et al., 2014). Similar to my studies, Keenan *et al* utilized a completely unrelated but highly immunogenic bacteria to induce a response that overcomes central tolerance.

Interestingly, the effect only delayed progression of early stage PanINs, and required concomitant T-reg depletion. However, the study provides proof of principle that a vaccine against a non-viral cancer can be efficacious and allows for specific targeting of any cell expressing mutant Kras, which is present in greater than 95% of PDA patients. The potential for universal application of such an approach is highly appealing, and complements Schreiber's personalized method predicated on neo-antigens.

Another broadly applicable approach re-emerging in the field of tumor immunology is immunization by whole-tumor lysate (Chiang et al., 2015). This method can be used with allogeneic or autologous whole tumor cell lysates; the benefit of the former is the universality, as demonstrated by the use of GVAX against prostate cancer, but the latter offers greater specificity for an individual's unique pool of tumor antigens. Tumor cells can also be genetically modified to secrete GM-CSF and other DC-activating cytokines to enhance their efficacy. In either case, the process of necrosis (for example, by freeze-thaw cycles) induces release of HMGBI, heat shock proteins, and other cellular components from the mitochondria and nucleus that induce an inflammatory response (Sauter et al., 2000). Interaction of dendritic cells with these cellular components via TLR-4 induces their maturation and allows for presentation of local antigens at the site of vaccination. Moreover, dead tumor cells do not secrete the classical immunosuppressive factors previously described (see introduction).

Here, the re-challenge data (Fig. 26) suggest that a vaccine-like effect may have occurred during the primary anti-Ova4662 response. Necrosis induced by cytolytic Ova-specific T cells likely released endogenous antigens in an immunostimulatory setting,

permitting the epitope spread effect observed upon re-challenge. To verify this effect, C57BL/6 mice could be immunized with V6.Ova lysate and re-challenge with parental 4662 to see if the same protective effect is observed.

Future studies might include vaccination of juvenile KPC mice with tumor lysate from the V6.Ova cell line, followed by monitoring of tumor incidence and overall survival compared to a control cohort receiving a sham vaccine. Although I previously attempted to use a freeze-thaw lysate of parental 4662 cells to induce regression in spontaneous KPC tumors, CD8+ T cells were unable to traffic into the tumor even in combination with gem/CD40 therapy (Fig. 36); only CD4+ cells achieved infiltration, and no regressions were observed (Beatty et al., 2015). Vaccination with a lysate of Ova-expressing 4662 might prompt a robust enough primary CD8+ response to permit full T cell trafficking to the autochthonous tumor microenvironment. If effective, this technique would represent an allogeneic whole tumor vaccine, reliant upon the presence of shared antigens between 4662 and other KPC-derived tumors. The vaccine effect could be further boosted with TLR-agonists like LPS or a CD4-depleting therapy to reduce immunosuppression in the developing tumor microenvironment. Various combinations of these approaches may yield a highly effective prophylactic vaccine against pancreatic cancer in the KPC mouse model, which would have significant clinical implications. A tumor vaccine would allow for the benefits of immunosurveillance by undermining tumor development at inception, thereby obviating the escape phase of immunoediting and minimizing treatment-related toxicities – the current limiting factors of immunotherapy as a whole.

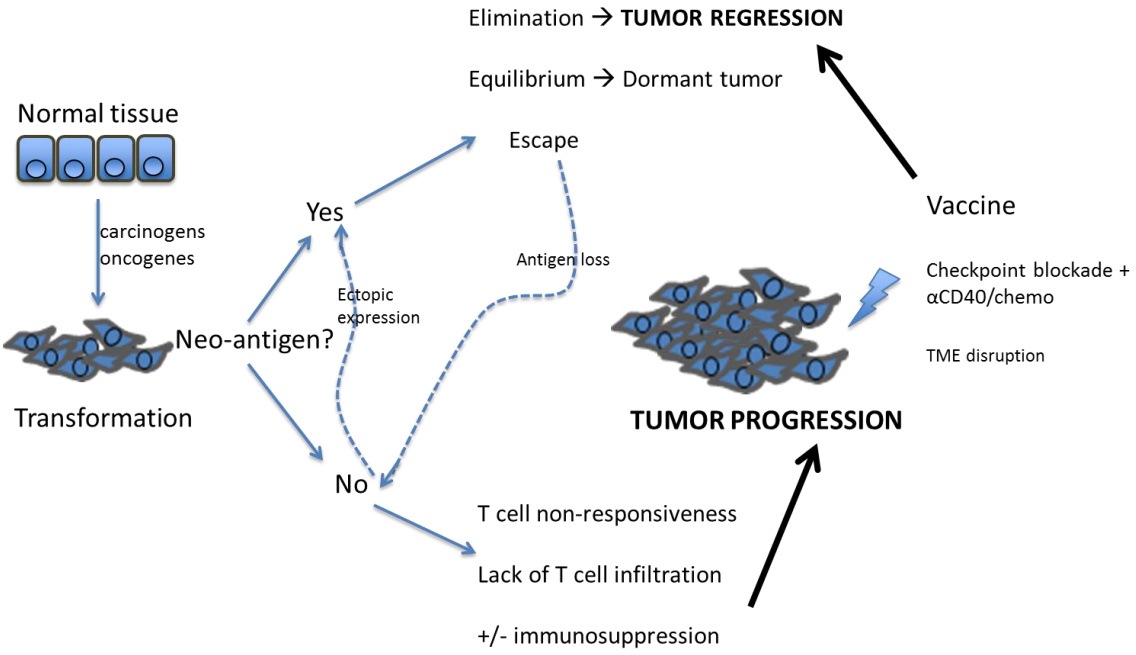


Fig 34. Revised model of tumor immunosurveillance. Updated schematic of tumor surveillance, demonstrating divergent host-immune interactions based on expression of neo-antigens, as well as potential therapeutic interventions for both immunologically "hot" and "cold" tumor subtypes.

A

MCA
KPC

IMMUNE STATUS		Implant Growth
Tumor source ("donor")	Implant host ("recipient")	
WT WT	WT WT	+++ +++
	Rag2null CD4/8dep	+++ +++
Rag2null CD4/8dep	WT WT	+/- +++
	Rag2null CD4/8dep	+++ +++

Tumor cell line	Treatment	Implant growth
MCA (progressor) KPC	Ctrl ab Ctrl ab	+++ +++
	αPD1/CTLA-4 αPD1/CTLA-4	---- +++

B

Tumor Antigen	Recipient immune status	Implant growth
B-spectrin Ova	WT WT	---- ----
	Rag2-/- CD8dep	+++ +++

Fig 35. Comparison of Immunoediting in MCA and KPC models of tumorigenesis. (A) Subcutaneous growth of MCA (black) or KPC (red) cell lines derived from immune-competent or immune-compromised mice upon implantation in immune-competent or immune-compromised recipients. Right panel, growth of MCA or KPC cell lines in the context of checkpoint inhibitors. (B) Subcutaneous growth of antigen-expressing MCA or KPC cell lines in immune-competent or immune-compromised recipients.

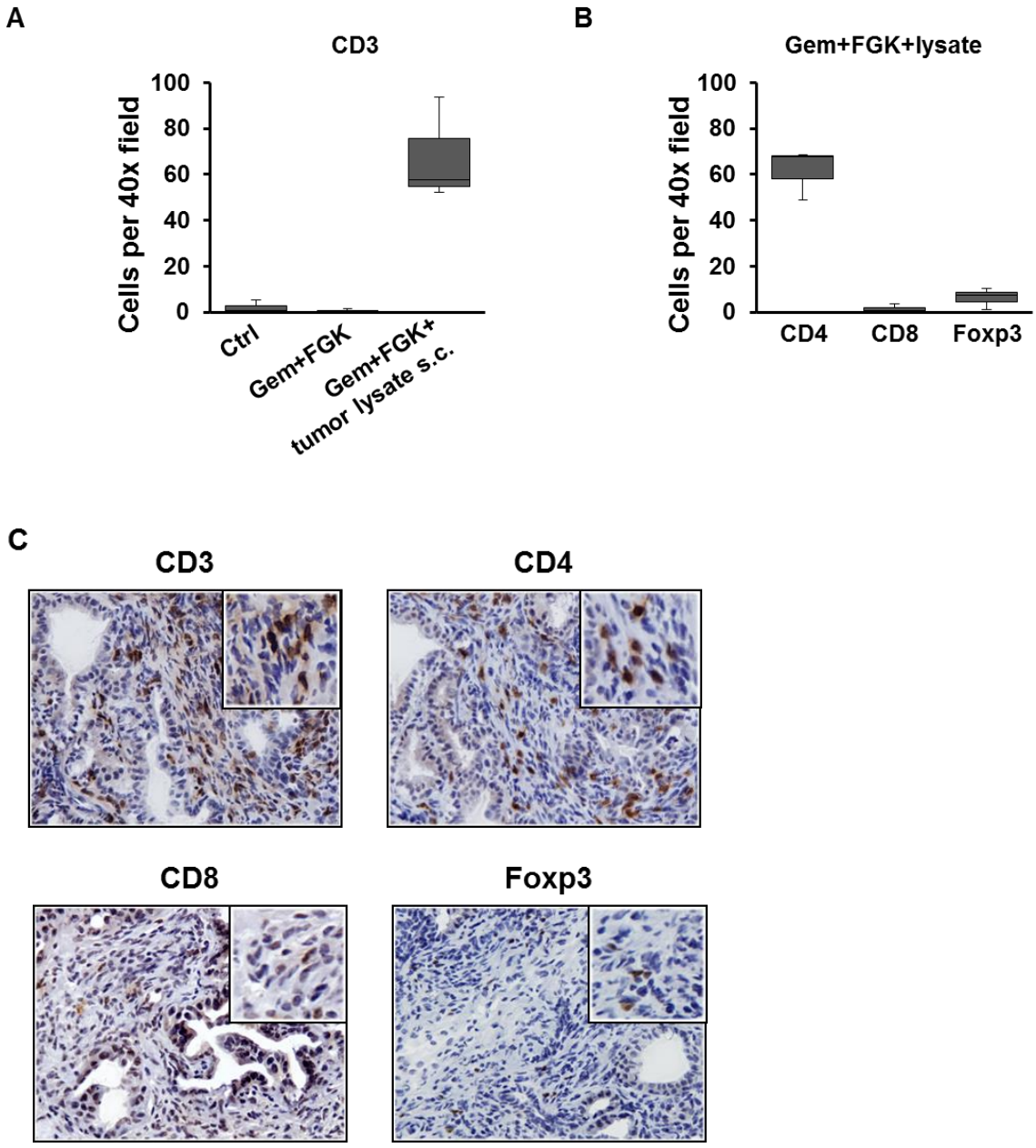


Fig 36. 4662 Tumor lysate fails to induce a vaccine effect in tumor-bearing KPC mice. (A) Quantification of immunohistochemistry for CD3+ cells in KPC pancreatic tumors 14 days post-treatment (n = 4 per group). Data shown as box and whisker plots. (B) Quantification of immunohistochemistry for intratumoral T cell subsets in the triple therapy cohort. (C) Representative staining for each intratumoral T cell subset in pancreatic tumors of KPC mice receiving the triple therapy.

REFERENCES

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*, 1355-1363.

Albina, J.E., and Henry, W.L., Jr. (1991). Suppression of lymphocyte proliferation through the nitric oxide synthesizing pathway. J Surg Res *50*, 403-409.

Bach, E.A., Aguet, M., and Schreiber, R.D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu Rev Immunol *15*, 563-591.

Bakker, A.B., Schreurs, M.W., de Boer, A.J., Kawakami, Y., Rosenberg, S.A., Adema, G.J., and Figdor, C.G. (1994). Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J Exp Med *179*, 1005-1009.

Balner, H., and Dersjant, H. (1966). Neonatal thymectomy and tumor induction with methylcholanthrene in mice. J Natl Cancer Inst *36*, 513-521.

Bayne, L.J., Beatty, G.L., Jhala, N., Clark, C.E., Rhim, A.D., Stanger, B.Z., and Vonderheide, R.H. (2012). Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. Cancer Cell *21*, 822-835.

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*, 1612-1616.

Beatty, G.L., Winograd, R., Evans, R.A., Long, K.B., Luque, S.L., Lee, J.W., Clendenin, C., Gladney, W.L., Knoblock, D.M., Guirnalda, P.D., *et al.* (2015). Exclusion of T Cells From Pancreatic Carcinomas in Mice Is Regulated by Ly6C(low) F4/80(+) Extratumoral Macrophages. Gastroenterology *149*, 201-210.

Billingham, R.E., Brent, L., and Medawar, P.B. (1956). The antigenic stimulus in transplantation immunity. Nature *178*, 514-519.

Braumuller, H., Wieder, T., Brenner, E., Assmann, S., Hahn, M., Alkhaled, M., Schilbach, K., Essmann, F., Kneilling, M., Griessinger, C., *et al.* (2013). T-helper-1-cell cytokines drive cancer into senescence. Nature *494*, 361-365.

Bromberg, J.F., Horvath, C.M., Wen, Z., Schreiber, R.D., and Darnell, J.E., Jr. (1996). Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. Proc Natl Acad Sci U S A *93*, 7673-7678.

Brown, S.D., Warren, R.L., Gibb, E.A., Martin, S.D., Spinelli, J.J., Nelson, B.H., and Holt, R.A. (2014). Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. Genome Res *24*, 743-750.

Buhtoiarov, I.N., Lum, H., Berke, G., Paulnock, D.M., Sondel, P.M., and Rakhmilevich, A.L. (2005). CD40 ligation activates murine macrophages via an IFN-gamma-dependent mechanism resulting in tumor cell destruction in vitro. J Immunol *174*, 6013-6022.

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

Burnet, M. (1957). Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J *1*, 841-847.

Burnet, M. (1964). Immunological Factors in the Process of Carcinogenesis. Br Med Bull *20*, 154-158.

Burstein, N.A., and Law, L.W. (1971). Neonatal thymectomy and non-viral mammary tumours in mice. Nature *231*, 450-452.

Carreno, B.M., Magrini, V., Becker-Hapak, M., Kaabinejadian, S., Hundal, J., Petti, A.A., Ly, A., Lie, W.R., Hildebrand, W.H., Mardis, E.R., *et al.* (2015). Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science *348*, 803-808.

Castle, J.C., Kreiter, S., Diekmann, J., Lower, M., van de Roemer, N., de Graaf, J., Selmi, A., Diken, M., Boegel, S., Paret, C., *et al.* (2012). Exploiting the mutanome for tumor vaccination. Cancer Res *72*, 1081-1091.

Chappell, D.B., Zaks, T.Z., Rosenberg, S.A., and Restifo, N.P. (1999). Human melanoma cells do not express Fas (Apo-1/CD95) ligand. Cancer Res *59*, 59-62.

Chiang, C.L., Coukos, G., and Kandalaft, L.E. (2015). Whole Tumor Antigen Vaccines: Where Are We? Vaccines (Basel) *3*, 344-372.

Chu, G.C., Kimmelman, A.C., Hezel, A.F., and DePinho, R.A. (2007). Stromal biology of pancreatic cancer. J Cell Biochem *101*, 887-907.

Cibulskis, K., Lawrence, M.S., Carter, S.L., Sivachenko, A., Jaffe, D., Sougnez, C., Gabriel, S., Meyerson, M., Lander, E.S., and Getz, G. (2013). Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol *31*, 213-219.

Clark, C.E., Beatty, G.L., and Vonderheide, R.H. (2009). 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*, 9518-9527.

Coley, W.B. (1891). II. Contribution to the Knowledge of Sarcoma. Ann Surg *14*, 199-220.

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., Becouarn, Y., Adenis, A., Raoul, J.L., Gourgou-Bourgade, S., de la Fouchardiere, C., *et al.* (2011). FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med *364*, 1817-1825.

Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J.R., Zhang, L., Burow, M., *et al.* (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med *10*, 942-949.

DeNardo, D.G., Barreto, J.B., Andreu, P., Vasquez, L., Tawfik, D., Kolhatkar, N., and Coussens, L.M. (2009). CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell *16*, 91-102.

Diefenbach, A., Jensen, E.R., Jamieson, A.M., and Raulet, D.H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature *413*, 165-171.

Dighe, A.S., Richards, E., Old, L.J., and Schreiber, R.D. (1994). Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity *1*, 447-456.

Doedens, A.L., Stockmann, C., Rubinstein, M.P., Liao, D., Zhang, N., DeNardo, D.G., Coussens, L.M., Karin, M., Goldrath, A.W., and Johnson, R.S. (2010). Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. Cancer Res *70*, 7465-7475.

Dubey, P., Hendrickson, R.C., Meredith, S.C., Siegel, C.T., Shabanowitz, J., Skipper, J.C., Engelhard, V.H., Hunt, D.F., and Schreiber, H. (1997). The immunodominant antigen of an ultraviolet-induced regressor tumor is generated by a somatic point mutation in the DEAD box helicase p68. J Exp Med *185*, 695-705.

Duluc, D., Corvaisier, M., Blanchard, S., Catala, L., Descamps, P., Gamelin, E., Ponsoda, S., Delneste, Y., Hebbar, M., and Jeannin, P. (2009). Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. Int J Cancer *125*, 367-373.

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*, 991-998.

Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. Immunity *21*, 137-148.

DuPage, M., Cheung, A.F., Mazumdar, C., Winslow, M.M., Bronson, R., Schmidt, L.M., Crowley, D., Chen, J., and Jacks, T. (2011). Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression. Cancer Cell *19*, 72-85.

DuPage, M., Mazumdar, C., Schmidt, L.M., Cheung, A.F., and Jacks, T. (2012). Expression of tumour-specific antigens underlies cancer immunoediting. Nature *482*, 405-409.

Frisch, M., Biggar, R.J., Engels, E.A., and Goedert, J.J. (2001). Association of cancer with AIDS-related immunosuppression in adults. Jama *285*, 1736-1745.

Gabrilovich, D.I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol *12*, 253-268.

Gallegos, A.M., and Bevan, M.J. (2004). Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med *200*, 1039-1049.

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, 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*, 1960-1964.

Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L.H., Craft, J., and Yin, Z. (2003). Gamma delta T cells provide an early source of interferon gamma in tumor immunity. J Exp Med *198*, 433-442.

Girardi, M., Oppenheim, D.E., Steele, C.R., Lewis, J.M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R.E., and Hayday, A.C. (2001). Regulation of cutaneous malignancy by gammadelta T cells. Science *294*, 605-609.

Grant, G.A., and Miller, J.F. (1965). Effect of neonatal thymectomy on the induction of sarcomata in C57 BL mice. Nature *205*, 1124-1125.

Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K.H., and Spies, T. (1999). Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A *96*, 6879-6884.

Grupp, S.A., Kalos, M., Barrett, D., Aplenc, R., Porter, D.L., Rheingold, S.R., Teachey, D.T., Chew, A., Hauck, B., Wright, J.F., *et al.* (2013). Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med *368*, 1509-1518.

Gubin, M.M., Artyomov, M.N., Mardis, E.R., and Schreiber, R.D. (2015). Tumor neoantigens: building a framework for personalized cancer immunotherapy. J Clin Invest *125*, 3413-3421.

Gubin, M.M., Zhang, X., Schuster, H., Caron, E., Ward, J.P., Noguchi, T., Ivanova, Y., Hundal, J., Arthur, C.D., Krebber, W.J., *et al.* (2014). Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature *515*, 577-581.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hidalgo, M. (2010). Pancreatic cancer. N Engl J Med *362*, 1605-1617.

Hingorani, S.R., Petricoin, E.F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M.A., Ross, S., Conrads, T.P., Veenstra, T.D., Hitt, B.A., *et al.* (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell *4*, 437-450.

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*, 469-483.

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*, 5423-5434.

Ikehara, S., Pahwa, R.N., Fernandes, G., Hansen, C.T., and Good, R.A. (1984). Functional T cells in athymic nude mice. Proc Natl Acad Sci U S A *81*, 886-888.

Jager, E., Ringhoffer, M., Altmannsberger, M., Arand, M., Karbach, J., Jager, D., Oesch, F., and Knuth, A. (1997). Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. Int J Cancer *71*, 142-147.

Ji, H., Houghton, A.M., Mariani, T.J., Perera, S., Kim, C.B., Padera, R., Tonon, G., McNamara, K., Marconcini, L.A., Hezel, A., *et al.* (2006). K-ras activation generates an inflammatory response in lung tumors. Oncogene *25*, 2105-2112.

Jones, S., Zhang, X., Parsons, D.W., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A., *et al.* (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science *321*, 1801-1806.

Kang, T.W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., *et al.* (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature *479*, 547-551.

Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J., and Schreiber, R.D. (1998). Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A *95*, 7556-7561.

Kawakami, Y., Eliyahu, S., Delgado, C.H., Robbins, P.F., Sakaguchi, K., Appella, E., Yannelli, J.R., Adema, G.J., Miki, T., and Rosenberg, S.A. (1994). Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci U S A *91*, 6458-6462.

Keenan, B.P., Saenger, Y., Kafrouni, M.I., Leubner, A., Lauer, P., Maitra, A., Rucki, A.A., Gunderson, A.J., Coussens, L.M., Brockstedt, D.G., *et al.* (2014). A Listeria vaccine and depletion of T-regulatory cells activate immunity against early stage pancreatic intraepithelial neoplasms and prolong survival of mice. Gastroenterology *146*, 1784-1794 e1786.

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.

Khong, H.T., Wang, Q.J., and Rosenberg, S.A. (2004). Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother *27*, 184-190.

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*, 903-907.

Korkolopoulou, P., Kaklamanis, L., Pezzella, F., Harris, A.L., and Gatter, K.C. (1996). Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. Br J Cancer *73*, 148-153.

Kumar, A., Commane, M., Flickinger, T.W., Horvath, C.M., and Stark, G.R. (1997). Defective TNF-alpha-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science *278*, 1630-1632.

Lennerz, V., Fatho, M., Gentilini, C., Frye, R.A., Lifke, A., Ferel, D., Wolfel, C., Huber, C., and Wolfel, T. (2005). The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A *102*, 16013-16018.

Lo, A., Wang, L.C., Scholler, J., Monslow, J., Avery, D., Newick, K., O'Brien, S., Evans, R.A., Bajor, D.J., Clendenin, C., *et al.* (2015). Tumor-Promoting Desmoplasia Is Disrupted by Depleting FAP-Expressing Stromal Cells. Cancer Res *75*, 2800-2810.

MacKie, R.M., Reid, R., and Junor, B. (2003). Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. N Engl J Med *348*, 567-568.

Maleckar, J.R., and Sherman, L.A. (1987). The composition of the T cell receptor repertoire in nude mice. J Immunol *138*, 3873-3876.

Matsushita, H., Vesely, M.D., Koboldt, D.C., Rickert, C.G., Uppaluri, R., Magrini, V.J., Arthur, C.D., White, J.M., Chen, Y.S., Shea, L.K., *et al.* (2012). Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature *482*, 400-404.

McAllister, F., Bailey, J.M., Alsina, J., Nirschl, C.J., Sharma, R., Fan, H., Rattigan, Y., Roeser, J.C., Lankapalli, R.H., Zhang, H., *et al.* (2014). Oncogenic Kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia. Cancer Cell *25*, 621-637.

Medema, J.P., de Jong, J., Peltenburg, L.T., Verdegaal, E.M., Gorter, A., Bres, S.A., Franken, K.L., Hahne, M., Albar, J.P., Melief, C.J., *et al.* (2001). Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci U S A *98*, 11515-11520.

Mills, C.D., Shearer, J., Evans, R., and Caldwell, M.D. (1992). Macrophage arginine metabolism and the inhibition or stimulation of cancer. J Immunol *149*, 2709-2714.

Muller-Hermelink, N., Braumuller, H., Pichler, B., Wieder, T., Mailhammer, R., Schaak, K., Ghoreschi, K., Yazdi, A., Haubner, R., Sander, C.A., *et al.* (2008). TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. Cancer Cell *13*, 507-518.

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*, 1457-1461.

Ozdemir, B.C., Pentcheva-Hoang, T., Carstens, J.L., Zheng, X., Wu, C.C., Simpson, T.R., Laklai, H., Sugimoto, H., Kahlert, C., Novitskiy, S.V., *et al.* (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell *25*, 719-734.

Pardoll, D. (2003). Does the immune system see tumors as foreign or self? Annu Rev Immunol *21*, 807-839.

Porter, D.L., Levine, B.L., Kalos, M., Bagg, A., and June, C.H. (2011). Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med *365*, 725-733.

Pradeu, T., and Carosella, E.D. (2006). On the definition of a criterion of immunogenicity. Proc Natl Acad Sci U S A *103*, 17858-17861.

Provenzano, P.P., Cuevas, C., Chang, A.E., Goel, V.K., Von Hoff, D.D., and Hingorani, S.R. (2012). Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. Cancer Cell *21*, 418-429.

Pylayeva-Gupta, Y., Lee, K.E., Hajdu, C.H., Miller, G., and Bar-Sagi, D. (2012). Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. Cancer Cell *21*, 836-847.

Rabinovich, G.A., Gabrilovich, D., and Sotomayor, E.M. (2007). Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol *25*, 267-296.

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*, 2913-2921.

Restifo, N.P., Esquivel, F., Kawakami, Y., Yewdell, J.W., Mule, J.J., Rosenberg, S.A., and Bennink, J.R. (1993). Identification of human cancers deficient in antigen processing. J Exp Med *177*, 265-272.

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*, 349-361.

Rhim, A.D., Oberstein, P.E., Thomas, D.H., Mirek, E.T., Palermo, C.F., Sastra, S.A., Dekleva, E.N., Saunders, T., Becerra, C.P., Tattersall, I.W., *et al.* (2014). Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer Cell *25*, 735-747.

Rizvi, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J.J., Lee, W., Yuan, J., Wong, P., Ho, T.S., *et al.* (2015). Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science *348*, 124-128.

Robbins, P.F., El-Gamil, M., Li, Y.F., Kawakami, Y., Loftus, D., Appella, E., and Rosenberg, S.A. (1996). A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J Exp Med *183*, 1185-1192.

Rodriguez, P.C., Quiceno, D.G., Zabaleta, J., Ortiz, B., Zea, A.H., Piazuelo, M.B., Delgado, A., Correa, P., Brayer, J., Sotomayor, E.M., *et al.* (2004). Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res *64*, 5839-5849.

Rooney, M.S., Shukla, S.A., Wu, C.J., Getz, G., and Hacohen, N. (2015). Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell *160*, 48-61.

Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., and Rammensee, H.G. (1991). Exact prediction of a natural T cell epitope. Eur J Immunol *21*, 2891-2894.

Rustgi, A.K. (2006). The molecular pathogenesis of pancreatic cancer: clarifying a complex circuitry. Genes Dev *20*, 3049-3053.

Sanda, M.G., Restifo, N.P., Walsh, J.C., Kawakami, Y., Nelson, W.G., Pardoll, D.M., and Simons, J.W. (1995). Molecular characterization of defective antigen processing in human prostate cancer. J Natl Cancer Inst *87*, 280-285.

Sausen, M., Phallen, J., Adleff, V., Jones, S., Leary, R.J., Barrett, M.T., Anagnostou, V., Parpart-Li, S., Murphy, D., Kay Li, Q., *et al.* (2015). Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. Nat Commun *6*, 7686.

Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med *191*, 423-434.

Scarlett, U.K., Rutkowski, M.R., Rauwerdink, A.M., Fields, J., Escovar-Fadul, X., Baird, J., Cubillos-Ruiz, J.R., Jacobs, A.C., Gonzalez, J.L., Weaver, J., *et al.* (2012). Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. J Exp Med *209*, 495-506.

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.

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

Sette, A., Vitiello, A., Reherman, B., Fowler, P., Nayersina, R., Kast, W.M., Melief, C.J., Oseroff, C., Yuan, L., Ruppert, J., *et al.* (1994). The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol *153*, 5586-5592.

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*, 1107-1111.

Sharma, P., and Allison, J.P. (2015). The future of immune checkpoint therapy. Science *348*, 56-61.

Shi, Y., Evans, J.E., and Rock, K.L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. Nature *425*, 516-521.

Siegel, R., Naishadham, D., and Jemal, A. (2012). Cancer statistics, 2012. CA Cancer J Clin *62*, 10-29.

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*, 1695-1710.

Skoulidis, F., Byers, L.A., Diao, L., Papadimitrakopoulou, V.A., Tong, P., Izzo, J., Behrens, C., Kadara, H., Parra, E.R., Canales, J.R., *et al.* (2015). Co-occurring Genomic Alterations Define Major Subsets of KRAS-Mutant Lung Adenocarcinoma with Distinct Biology, Immune Profiles, and Therapeutic Vulnerabilities. Cancer Discov *5*, 860-877.

Smyth, M.J., Thia, K.Y., Street, S.E., MacGregor, D., Godfrey, D.I., and Trapani, J.A. (2000). Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med *192*, 755-760.

Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., *et al.* (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med *371*, 2189-2199.

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*, 192-197.

Street, S.E., Trapani, J.A., MacGregor, D., and Smyth, M.J. (2002). Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med *196*, 129-134.

Stutman, O. (1974). Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science *183*, 534-536.

Stutman, O. (1979a). Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose. J Natl Cancer Inst *62*, 353-358.

Stutman, O. (1979b). Spontaneous tumors in nude mice: effect of the viable yellow gene. Exp Cell Biol *47*, 129-135.

Tempero, M., Plunkett, W., Ruiz Van Haperen, V., Hainsworth, J., Hochster, H., Lenzi, R., and Abbruzzese, J. (2003). Randomized phase II comparison of dose-intense gemcitabine: thirty-minute infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. J Clin Oncol *21*, 3402-3408.

Teng, M.W., Vesely, M.D., Duret, H., McLaughlin, N., Towne, J.E., Schreiber, R.D., and Smyth, M.J. (2012). Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state. Cancer Res *72*, 3987-3996.

Tiemessen, M.M., Jagger, A.L., Evans, H.G., van Herwijnen, M.J., John, S., and Taams, L.S. (2007). CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci U S A *104*, 19446-19451.

Trutin-Ostovic, C., Golubic, M., Matovic, M., and Marusic, M. (1986). Incidence and growth of methylcholanthrene-induced tumors in mice with altered immunological status. Cancer Immunol Immunother *23*, 130-136.

Tumeh, P.C., Harview, C.L., Yearley, J.H., Shintaku, I.P., Taylor, E.J., 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*, 568-571.

van den Broek, M.E., Kagi, D., Ossendorp, F., Toes, R., Vamvakas, S., Lutz, W.K., Melief, C.J., Zinkernagel, R.M., and Hengartner, H. (1996). Decreased tumor surveillance in perforin-deficient mice. J Exp Med *184*, 1781-1790.

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*, 1691-1703.

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*, 949-954.

Waddell, N., Pajic, M., Patch, A.M., Chang, D.K., Kassahn, K.S., Bailey, P., Johns, A.L., Miller, D., Nones, K., Quek, K., *et al.* (2015). Whole genomes redefine the mutational landscape of pancreatic cancer. Nature *518*, 495-501.

Wang, L., Pino-Lagos, K., de Vries, V.C., Guleria, I., Sayegh, M.H., and Noelle, R.J. (2008). Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. Proc Natl Acad Sci U S A *105*, 9331-9336.

Westcott, P.M., Halliwill, K.D., To, M.D., Rashid, M., Rust, A.G., Keane, T.M., Delrosario, R., Jen, K.Y., Gurley, K.E., Kemp, C.J., *et al.* (2015). The mutational landscapes of genetic and chemical models of Kras-driven lung cancer. Nature *517*, 489-492.

Willimsky, G., and Blankenstein, T. (2005). Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. Nature *437*, 141-146.

Willimsky, G., Czeh, M., Loddenkemper, C., Gellermann, J., Schmidt, K., Wust, P., Stein, H., and Blankenstein, T. (2008). Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness. J Exp Med *205*, 1687-1700.

Winograd, R., Byrne, K.T., Evans, R.A., Odorizzi, P.M., Meyer, A.R., Bajor, D.L., Clendenin, C., Stanger, B.Z., Furth, E.E., Wherry, E.J., *et al.* (2015). Induction of T-cell Immunity Overcomes Complete Resistance to PD-1 and CTLA-4 Blockade and Improves Survival in Pancreatic Carcinoma. Cancer Immunol Res *3*, 399-411.

Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K.H., and Beach, D. (1995). A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science *269*, 1281-1284.

Yewdell, J.W., and Bennink, J.R. (1999). Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu Rev Immunol *17*, 51-88.

Ying, H., Kimmelman, A.C., Lyssiotis, C.A., Hua, S., Chu, G.C., Fletcher-Sananikone, E., Locasale, J.W., Son, J., Zhang, H., Coloff, J.L., *et al.* (2012). Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. Cell *149*, 656-670.

Zaks, T.Z., Chappell, D.B., Rosenberg, S.A., and Restifo, N.P. (1999). Fas-mediated suicide of tumor-reactive T cells following activation by specific tumor: selective rescue by caspase inhibition. J Immunol *162*, 3273-3279.

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*, 203-213.

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*, 423-435.

Zhou, J., Dudley, M.E., Rosenberg, S.A., and Robbins, P.F. (2005). Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. J Immunother *28*, 53-62.

Zitvogel, L., Kepp, O., and Kroemer, G. (2010). Decoding cell death signals in inflammation and immunity. Cell *140*, 798-804.