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Determinants Of Adaptive Immunity In Cancer

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Determinants Of Adaptive Immunity In Cancer

Abstract
Immune checkpoint blockade results in T cell antitumor responses but most patients fail to respond. This raises fundamental questions about mechanisms of tumor immune recognition and resistance. Here, I first report tumor regressions in a subset of patients with metastatic melanoma treated with anti-CTLA4 antibody and radiation (RT) and reproduced this effect in mouse models. Although combined treatment improved responses in irradiated and unirradiated tumors, resistance was common due to upregulation of PD-L1 on tumor cells and corresponding T cell exhaustion. Accordingly, optimal response in melanoma and other cancer types required RT, anti-CTLA4, and anti-PD-L1/PD1. When I investigated determinants of improved responses to combination therapy, I found that RT enhanced the antigenic diversity of intratumoral T cells, anti-CTLA4 predominantly inhibited regulatory T cells, and anti-PD-L1 reversed T cell exhaustion. I next extended my investigation of this combination therapy to pancreatic ductal adenocarcinoma (PDA), finding that optimal responses required addition of agonist CD40 monoclonal antibody.

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DETERMINANTS OF ADAPTIVE IMMUNITY IN CANCER

Andrew J. Rech

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Cell and Molecular Biology

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ABSTRACT

Immune checkpoint blockade results in T cell antitumor responses but most patients fail to respond. This raises fundamental questions about mechanisms of tumor immune recognition and resistance. Here, I first report tumor regressions in a subset of patients with metastatic melanoma treated with anti-CTLA4 antibody and radiation (RT) and reproduced this effect in mouse models. Although combined treatment improved responses in irradiated and unirradiated tumors, resistance was common due to upregulation of PD-L1 on tumor cells and corresponding T cell exhaustion. Accordingly, optimal response in melanoma and other cancer types required RT, anti-CTLA4, and anti-PD-L1/PD1. When I investigated determinants of improved responses to combination therapy, I found that RT enhanced the antigenic diversity of intratumoral T cells, anti-CTLA4 predominantly inhibited regulatory T cells, and anti-PD-L1 reversed T cell exhaustion. I next extended my investigation of this combination therapy to pancreatic ductal adenocarcinoma (PDA), finding that optimal responses required addition of agonist CD40 monoclonal antibody. To further understand determinants of response and resistance in PDA, I next examined the immune landscape of PDA in humans. I report that human PDA displays a range of intratumoral cytolytic T cell activity: PDA tumors with low cytolytic activity exhibited significantly increased copy number alterations, high cytolytic activity in PDA did not correlate with increased
neoepitope load, and PDA tumors exhibited a unique pattern of immune suppressive molecule expression. To place PDA in a wider context of human adaptive antitumor immunity, I then extended this analysis to 35 solid tumor types, finding abundant neoepitopes across human cancer at the global level and identifying immune gene sets predictive of neoepitope load. Overall, distinct rates of neoepitope generation, cytolytic activity, and immune suppressive molecule expression define disease types across human cancer. In summary, these findings characterize mechanisms of response and resistance to immune checkpoint blockade in distinct tumor types and investigate determinants of adaptive immunity in human cancer.
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CHAPTER 1 - Introduction

Immunosurveillance and the cancer immunoediting hypothesis

Leukocytes in the tumor microenvironment exert critical influence on the process of oncogenesis, playing a dual role of both promoting and suppressing tumor growth through complex, dynamic interactions (Hanahan & Weinberg 2011). Paul Ehrlich first suggested in 1909 that immune function could, under normal homeostasis, exert control over clinically significant disease in a manner similar to the highly efficient and sensitive immune control of infections (Ehrlich 1909). This hypothesis was refined by Burnet and Thomas half a century later when they proposed that ‘immune surveillance’ could restrain early malignant lesions by recognizing and destroying nascent tumor cells, and then subsequently proposed that such a mechanism may be required for survival of long-lived organisms (Burnet 1957, Cellular and Humoral Aspects of the Hypersensitive States: A Symposium at the New York Academy of Medicine 1959). Interest in these early theories was renewed by Schreiber et al. in 2001 when he showed that newly-available immunodeficient lacking key adaptive immune proteins (interferon-γ and perforin) had increased incidence of tumors (Shankaran et al. 2001). This discovery led to a refinement by Schreiber et al. of the immune surveillance concept into the current paradigm of
immune-tumor interaction, the cancer immunoediting hypothesis (Dunn et al. 2004). This hypothesis recognizes both tumor-promoting and host-protective immune effects and is codified into three temporally distinct phases: immune elimination, immune equilibrium, and immune escape.

The elimination phase is Burnet and Thomas’ immune surveillance hypothesis revisited, and is the first and most prevalent interaction between the host immune system and tumor. The mechanisms of initial immune activation against tumors are diverse and incompletely understood, but this process likely requires the coordination of innate and adaptive cell types, beginning with the release of ‘danger signals’ in a process analogous to that during infection (Gallucci & Matzinger 2001). For instance, early tumor development results in release of type I interferons, (Dunn et al. 2005) and various damage-associated molecular patterns or stress ligands can be released directly from dying tumor cells or due to disruption of surrounding stroma (Sims et al. 2010). This initial activating signal is subsequently propagated by local release of proinflammatory cytokines such as interferon-\(\gamma\), leading to increased recruitment of innate immune populations that result in a local environment conducive to immune priming (Guerra et al. 2008). Following activation of naïve CD4 and CD8 T cells, CD8 effector T cells infiltrate the tumor site and directly engage tumor cells in a MHC class I-dependent manner. This destruction mechanism is reliant on continued expression of tumor-specific antigen and MHC; thus, the determinants of whether immune elimination is
successful depends on the profile of available tumor antigens as well as the sustained existence of a proinflammatory milieu that supports T cell effector function.

Nascent malignant cells occasionally avoid this early immune-mediated destruction, leading to a relatively rare state of equilibrium between host and tumor. In this phase, a dynamic balance exists in which overall tumor outgrowth is restrained by the immune system but complete eradication never occurs. Entrance into the equilibrium phase is likely marked by selection of relatively low immunogenicity tumor clones; however, enough immune pressure exists to sustain the equilibrium state for extended periods of time. In humans, transfer of undetected cancer from an organ donor into immunosuppressed recipients can occur (MacKie et al. 2003) and dormant tumor cells can exist in patients for several decades before resuming growth (Aguirre-Ghiso 2007). That this period of time can stretch into years implies that the most prevalent state of the tumor microenvironment from an immune standpoint is one in which tumor cells are unable to accumulate sufficient adaptation to become clinically detectable. In immunocompetent mice exposed to carcinogen, tumor cells can apparently exist for extended periods of time, only developing into clinically detectable masses upon depletion of T cells and blockade of interferon-γ (Koebel et al. 2007). Additional evidence of an equilibrium phase comes from the finding that antigen-specific T cells can suppress pancreatic tumors in a Tag-induced carcinogenesis model, dependent on tumor necrosis factor and interferon-γ (Müller-Hermelink et al. 2008). In this study, in the absence of
these proinflammatory factors, T cells promoted angiogenesis and multistage carcinogenesis, denoting the primary important of maintaining a local proinflammatory milieu. Continued expression of tumor antigen is not sufficient for arresting tumor growth, a principle that has profoundly impacted therapeutic attempts to augment endogenous immunity.

The escape phase occurs when growth of tumor cells progress into a clinically significant mass, unrestrained by host immune function. This state arises due to a combination of acquired tumor immune privilege, immune-intrinsic limitations in response, active tumor-induced immunosuppression, and direct resistance to destruction by T cells, that together result in progression out of the equilibrium phase. First, acquired tumor immune privilege is a state of inadequate recognition by the immune system of tumor cells, (Mellor & Munn 2008) and has been demonstrated to occur after loss of antigen expression against which CD8 effector T cells are directed. This can be due to selection of tumor cells that no longer produce highly immunogenic proteins, have inadequately functioning antigen-processing machinery, or have downregulated MHC class I. Intrinsic tumor cell genetic instability provides a fertile ground for selection of these T cell-resistant clones.

Second, upregulation of immune suppression, both through pathways intrinsic to the immune system designed to prevent unrestrained activation and through active co-option of these same pathways by tumor cells, are key contributors to immune escape.
Immune suppressive cytokines such as vascular endothelial growth factor, transforming growth factor-beta or indoleamine 2,3-dioxygenase, and a plethora of newly identified or recently established molecules, can potently suppress adaptive immunity by directly limiting effector cell trafficking and function or by promoting suppressive populations such as regulatory T cells and myeloid-derived suppressor cells (Mittal et al. 2014). A major additional axis of immune suppression is through the increased expression of immune checkpoint pathways such as CTLA4 and PD1/PD-L1. In the case of immune checkpoints, increased signaling occurs both in direct response to a T cell-inflamed microenvironment and due to increased oncogene-driven ligand expression by tumor cells (Pardoll 2012).

Third, direct resistance to T cell cytolytic function can occur through upregulation of pro-survival molecules that prevent apoptotic cell death. Tumor cells under selective immune pressure are shown to upregulate molecules such as STAT3 or the anti-apoptotic molecule Bcl2 (Gajewski et al. 2011). Apoptotic cell death is regulated in a complex manner by interactions between pro-survival and pro-apoptotic molecules. This provides ample evolutionary surface for tumor cell adaptation. Furthermore, aberrations in these same pathways are often cancer-initiating events, increasing the likelihood that cancer cells are intrinsically resistant to T cell effector function (Fernald & Kurokawa 2013). Taken together, these three categories of tumor escape mechanisms
ensure that clinically significant tumors have, in most cases, a long history of selection for immune-evasive properties at the time of clinical presentation.

The central tenet of the cancer immunoediting hypothesis is that T cell recognition of specific antigens drives immune elimination. This is supported by the finding that RAG2-deficient mice lacking T cells harbor more immunogenic tumors (Schreiber et al. 2011). In theory, non-synonymous somatic mutations, whether drivers of malignancy or passengers accrued during oncogenesis, are a source of mutated peptides distinct from self tissue. No tolerance against such peptides, termed neoepitopes, is likely to exist because they are not expressed in the thymus during T cell development and are not a substrate of peripheral tolerance mechanisms in normal tissue. In two landmark 2012 studies investigating the antigenicity of nascent tumor cells, neoepitopes were sufficient to induce antitumor immunity and selected against during tumor growth (DuPage et al. 2012, Matsushita et al. 2012). Matsushita et al. demonstrated that carcinogen-induced sarcomas in RAG2-deficient mice harbored a point mutation in Spectrin-beta2 capable of subsequently driving tumor rejection in immune competent mice, demonstrating immune selection against a neoepitope. DuPage et al. demonstrated, using a autochthonous murine sarcoma model, that primary tumors from similarly immunodeficient mice were edited to become less immunogenic in immunocompetent mice (DuPage et al. 2012). Additionally, using the neoepitope pipeline described in this thesis, colleagues and I reported that a lack of immune editing could be reversed in a
murine model of PDA with the addition of a single highly immunogenic neoantigen (Evans et al. 2016). Taken together, these results established the primacy of neoepitopes in immune surveillance, implying these may also be critical in augmenting antitumor immunity therapeutically.

Several lines of evidence support the immunoediting hypothesis’ relevance to human disease. First, patients who are immunosuppressed after organ transplant or due to AIDS exhibit higher incidence of tumors than the general population, suggesting that adaptive immune function exerts a large effect on background rate of tumorigenesis (Vesely et al. 2011). In particular, the dramatically increased frequency of virus-associated malignancies in AIDS patients may reflect the relatively efficient elimination of these immunogenic cells in normal individuals (Boshoff & Weiss 2002). Second, correlative clinical evidence from diverse disease types shows that the presence of CD8 tumor-infiltrating lymphocytes (TILs) is associated with improved clinical outcome (Clemente et al. 1996, Naito et al. 1998, Zhang et al. 2003). Third, in rare but well-documented cases, patients with cancer can develop profound, spontaneous adaptive immune responses to non-viral antigens expressed by tumors (Boon & van der Bruggen 1996). Of course, recent clinical breakthroughs using combinatorial blockade of specific negative adaptive immune checkpoints provides significant evidence of baseline immune activity in tumors, consistent with the immunoediting hypothesis (Brahmer et al. 2012, Hamid et al. 2013, Hodi et al. 2010, Postow et al. 2015b, Schadendorf et al. 2015, Topalian et al. 2015).

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Recent investigations have also provided mechanistic insight into cancer immunoediting in humans. First, whole exome sequencing of non–small cell lung cancers treated with antibodies that block negative immune checkpoints demonstrated that therapeutic efficacy correlated with higher nonsynonymous mutation and neoepitope burden (Rizvi et al. 2015). Rizvi et al. additionally found that total exonic mutation burden was a poorer correlation of response, implying that neoantigens derived from somatic missense mutations – not overall genomic errors or instability, which may correlate strongly with tumor aggressivity – is an important factor in predicting response to therapy. Similarly, responses to anti-CTLA4 in patients with metastatic melanoma similarly correlated with overall mutation and neoepitope load (Van Allen et al. 2015). Here, durable response, despite initial fluctuations in disease, correlated with these measures as well as tumor cytolytic activity as assessed by expression of granzyme A and perforin, a pattern consistent with partial reversion to a state of immune equilibrium. These studies specifically support the hypotheses that neoepitope-directed T cells are of primary importance to therapeutic induction of antitumor immunity in humans, and when taken together with studies of immune interactions with nascent tumors, support the hypothesis that the immune system exerts continuous impact on tumor biology.

In summary, the cancer immune editing hypothesis is the underlying framework for much of the current field of cancer immunology. This includes work discussed in chapters 3 (page 63), which investigates mechanisms of resistance to immunity acquired
by tumors in response to immune pressure, and Chapter 4 (page 103), which investigates immune determinants and recognition of tumors by T cells across human cancer (Figure 1).

Figure 1. The cancer immune editing hypothesis.

This hypothesis states that early during tumorigenesis, nascent tumor cells result in an upregulation of inflammatory signals, leading to an effective T cell response and tumor elimination (top). However, this process can go awry; tumors escape this immune-mediated destruction, leading to clinically significant disease (bottom). For the purpose of this dissertation, tumor escape can occur either through the active upregulation in the tumor microenvironment of immune suppressive pathways or a state of acquired immune privilege (red). These processes can be reversed therapeutically with immune checkpoint blockade or immune agonist strategies (blue). This immune biology is investigated in Chapters 3 and 4.
Negative immune checkpoints and cancer

Cytotoxic T-lymphocyte-associated protein 4

It is now well understood that immunotherapy capable of stimulating CD8 effector T cells can eradicate established tumors in mice and humans. Yet even in the setting of immune-stimulating agents such as vaccines, tumors often escape destruction. This likely occurs through further selection, now in the therapeutic setting, for suppressive mechanisms similar to those that result in initial escape from endogenous immunity. Understanding these suppressive mechanisms is of great importance for improving immune therapy. The most notable therapeutically-leveraged immune suppressive pathway is CTLA4, a critical regulator of T cell activation. Early work first demonstrated that CTLA4 knockout mice rapidly succumb to lymphoproliferative disease with concomitant T cell infiltration in multiple organ systems (Pardoll 2012, Tivol et al. 1995, Waterhouse et al. 1995). CTLA4 primarily counteracts the T cell co-stimulatory receptor CD28, which during T cell activation provides a required amplifying signal to TCR stimulation (Linsley et al. 1990, 1991). CD28 and CTLA4 have identical ligands, CD80 and CD86, which bind CTLA4 with greater affinity; thus, interaction with CTLA4 outcompetes that with CD28 when CTLA4 expression is induced (Schneider et al. 2006). Despite this role of CTLA4 expression on effector CD8 T cells, the predominant mechanism by which CTLA4 dampens adaptive immunity is by augmenting regulatory T
cell suppressive function and limiting CD4 T cell activity (Lenschow & Walunas 1996, Peggs et al. 2009, Wing et al. 2008). CTLA4 is a target of FOXP3, the regulatory T cell master transcription factor, regulatory T cells constitutively express CTLA4, (Gavin et al. 2007, Hill et al. 2007) and CTLA4 signaling in this cell population promotes suppressive functions such as production of transforming growth factor beta (Chen et al. 1998).

Consistent with these mechanisms, blockade of CTLA4 was demonstrated in a landmark study by Allison and colleagues to significantly improve antitumor T cell responses in mice bearing moderately immunogenic tumors in which some baseline, endogenous immunity is present (Leach et al. 1996).

Based on this work and other preclinical models, two humanized anti-CTLA4 antibodies began clinical testing in 2000, (Hodi et al. 2003) producing objective responses in approximately 10% of patients despite severe immune related adverse events (Phan et al. 2003). A subsequent randomized, three-arm clinical trial showed a 3.5-month overall survival benefit in patients with metastatic melanoma treated with anti-CTLA4 (Hodi et al. 2010). Interestingly, an accompanying vaccine against the universal melanoma antigen GP100 did not further improve responses, suggesting that re-direction of immunity against known antigens was not essential for response. This objective response rate corresponded to an improvement in long-term survival of 18% at two years, a highly impressive durability of response that serves as a foundation for the promise of immune checkpoint blockade to revert some advanced tumors to a state of immune equilibrium.
**Programmed cell death protein 1**

Leading the class of emerging, second-generation immune therapies is blockade of programmed cell death protein 1 (PD1). Therapeutic antibodies against PD1 or its ligand PD-L1 have produced objective responses in patients with melanoma, non-small cell lung cancer, and other malignancies (Brahmer et al. 2012, Topalian et al. 2012). Like CTLA4, PD1 is expressed principally on T cells. Unlike CTLA4, which reduces the amplitude of T cell activation, PD1 primarily appears to limit the function of already activated T cells (Pardoll 2012), and the PD1 ligands PD-L1 and PD-L2 are upregulated on a plethora of cells during inflammation, including tumor cells. PD-1 ligation can result directly in apoptosis of activated T cells, (Dong et al. 2002) yet other effects are more complex, involving at least 5 interacting molecules that induce transition to an anergic state in activated T cells (Barber et al. 2006, Goldberg et al. 2007). The PD1/PD-L1 pathway also augments suppressive regulatory T cells, a second mechanism by which this pathway influences immune responses (Dong et al. 1999). These insights led to the hypothesis that PD-1 pathway blockade might extend antitumor T cell function, was which proven to be the case in several preclinical murine models (Curiel et al. 2003). The FDA recently approved 2 anti-PD1 monoclonal antibodies and multiple additional antibodies are being actively investigated in hundreds of clinical trials. PD1 blockade has now been shown to generate clinically meaningful tumor regressions in a wide diversity of cancer types,
including advanced and metastatic disease resistant to RT and chemotherapy, with durable effects and tolerable toxicity (Chen et al. 2015).

Combinatorial immune checkpoint blockade

Despite broad classification as similar agents, therapeutic blockade of CTLA4 and PD1/PD-L1 are based on distinct scientific rationale. Summarizing mechanistic work in murine models, the major physiologic function of CTLA4 appears to be exerting control over T cell responses to self-antigens. Clinical evidence supports this conclusion: infiltration of activated T cells occurs both in tumor and normal tissue, often leading to broad off-target autoimmunity in multiple major organs such as the skin, gastrointestinal track and liver (Weber et al. 2012). Additionally, it remains to be determined how important regulatory T cell suppression of endogenous antitumor immune activity is in human melanoma or more generally, therefore it appears that anti-CTLA4 primarily works by directly removing restraints from largely nonspecific effector T cell reactivity (Lühder et al. 1998, Maker et al. 2005, Perrin et al. 1996). In contrast to this, consistent with the lack of spontaneous autoimmunity in PD1-deficient mice, (Dong et al. 2004) clinical evidence thus far shows a relatively low burden of autoimmunity following treatment with anti-PD1/L1 (Arance et al. 2015). This may also be due to the limited expression of PD1/L1 during normal homeostasis; in contrast to CTLA4, the PD1 pathway’s main function appears to be as an inducible regulator at sites of tissue...
inflammation. Indeed, a recent clinical trial in patients with metastatic melanoma comparing anti-CTLA4 and anti-PD1 was stopped ahead of schedule due to superiority of the PD1 group with adverse events, as well as superior overall survival (Arance et al. 2015). Thus, though anti-CTLA4 was initially viewed as the backbone of combinatorial immune therapy, this role now belongs to anti-PD1 due in part to its more subtle basic immune function and corresponding favorable clinical profile (Mahoney et al. 2015).

Despite these apparent clinical shortcomings of anti-CTLA4, combinatorial immune checkpoint blockade is a critical therapeutic strategy that has resulted in improved responses compared to either single modality and is now FDA approved for advanced melanoma (Postow et al. 2015a). Only a small subset of patients respond to single agent therapy; while improvements in biomarkers may increase response rates due to refinements in patient selection, monotherapy is fundamentally limited by the fact that mechanisms of immune evasion during the natural history of cancer progression are heterogeneous. Many tumor types appear to harbor an indolent endogenous immune response, yet different suppressive mechanisms are operative or predominate across clinically homogenous patient populations. Resistance pathways to immune therapy are similarly diverse. Thus, combinatorial immune therapy in some form is likely to be required for durable responses in most patients. Chapter 3 (page 63) reports results of a phase I trial evaluating anti-CTLA4 and investigates mechanism of action of dual
immune checkpoint blockade with anti-CTLA4 and anti-PD1 in murine tumor models.
Agonists of adaptive immunity in cancer

Effective activation of naïve T cells against tumor cells requires several barriers to be overcome that are distinct from those for immune responses against foreign targets. First, a high-affinity peptide-MHC interaction must be present, requiring expression by tumor cells of genomic regions that are sufficiently non-self that reactive T cells bypass mechanisms of central and peripheral tolerance without deletion or anergy. Second, the engagement of costimulatory molecules expressed by antigen presenting cells is required. Without costimulatory signals, activated naïve T cells undergo apoptosis or transition to an anergic state, (Jenkins & Schwartz 1987) an intrinsic immune suppressive mechanism that prevents rampant autoimmunity. The canonical costimulatory molecule expressed on naïve T cells is CD28, which is required for T cell expansion and effector function. However, many other costimulatory molecules exist and contribute to T cell activation following antigen encounter, and these molecules have been targeted with monoclonal antibodies in the context of checkpoint blockade to augment antitumor responses. This strategy has particular relevance in diseases such as pancreatic ductal adenocarcinoma where evidence suggests there may be little underlying endogenous antitumor immunity in most patients.
Several therapeutically-leveraged costimulatory molecules are members of the tumor necrosis factor superfamily, which were first described as mediators of immune response expressed by antigen presenting cells, activated T cells, and activated B cells (Moran et al. 2013). CD40 is one such a tumor necrosis factor receptor superfamily member and is most notably expressed on dendritic cells (Grewal & Flavell 1998). The CD40 ligand CD154 is expressed on activated T cells; ligation of CD40 on dendritic cells augments antigen presentation ability and has been shown to be a critical component of T cell help (Diehl et al. 1999, French et al. 1999, Sotomayor et al. 1999). Anti-CD40 agonist monoclonal antibodies can reverse T cell tolerance in tumor-bearing mice via this mechanism, circumventing a potential barrier of CD8 T cell priming by stimulating tumor antigen presentation (Bennett et al. 1998, Ridge et al. 1998, Schoenberger et al. 1998, Wing et al. 2008). CD40 is also expressed by tumor cells in several disease types; thus, targeting CD40 may additionally augment antitumor responses by activating antibody-dependent cellular cytotoxicity (Li & Ravetch 2011). A further mechanism is activation of antitumor macrophages that were capable of mediating T cell-independent responses in a murine model of PDA (Beatty et al. 2011).

Literature shows that anti-CD40 and anti-CTLA4, combined with an ovalbumin liposomal peptide vaccine, induce CD8 effector T cells that control ovalbumin-expressing leukemia (Ito et al. 2000). Similarly, this combination leads to CD8-dependent complete
regression of murine melanoma expressing a highly antigenic, virus-derived protein when combined with an adenoviral vaccine directed against this protein (Sorensen et al. 2010). In phase I clinical trials, anti-CD40 monoclonal antibody was associated with immune activity and resulted in partial responses in patients with metastatic melanoma, (Bajor et al. 2014, Vonderheide et al. 2001, 2007) and clinical trials investigating the combination of anti-CD40 and immune checkpoint blockade are currently underway.

**Radiotherapy**

Increasing evidence suggests that RT can similarly function as an immune agonist, augmenting responses to checkpoint blockade or other strategies in tumors with little baseline immunity (Demaria et al. 2016). A role for immune cells in RT-induced tumor regression was first demonstrated in 1979 (Stone et al. 1979) and later definitively confirmed using T cell receptor-transgenic mice (Lee et al. 2009, Lugade et al. 2005). RT induces immunogenic cell death, (Apetoh et al. 2007, Golden & Apetoh 2015) responses are mediated in part by intratumoral CD8⁺CD103⁺ dendritic cells in mice, and responses are dependent on the innate DNA damage-sensing cGAS–Stimulator of Interferon Genes pathway (Deng et al. 2014b). Crucially, RT has been shown to induce an abscopal effect – tumor regression at unirradiated sites such as distant metastases – that depends on CD8 T cells (Seung et al. 2012) This is likely due to release of tumor antigen and secretion of proinflammatory cytokines from irradiated tumor, as shown in the murine B16-F10
melanoma model, (Lugade et al. 2005) though the precise mechanisms underlying this effect are poorly understood. Local RT in B16-F10 melanoma also promotes regulatory T cells, (Lugade et al. 2005) suggesting that anti-CTLA4, which inhibits regulatory T cell function, may synergize with RT. Indeed, anti-CTLA4 augments local RT-induced abscopal effects on a second, non-irradiated tumor and this effect correlates with CD8 T cell tumor infiltration and proinflammatory cytokine production (Dewan et al. 2009). Four early case reports showed complete responses to RT + anti-CTLA4 in patients with metastatic melanoma, further contributing to excitement (Golden et al. 2013, Hiniker et al. 2012, Postow et al. 2012, Stamell et al. 2013). Results from preclinical models also support the investigation of combinatorial therapy with RT and anti-PD1 – synergistic responses in multiple disease models are mediated by CD8 T cells and correlate with decreased markers of immune suppression in the tumor microenvironment (Deng et al. 2014a, Dovedi et al. 2014, Zeng et al. 2013). Chapter 3 explores this biology, investigating the combination of RT and dual immune checkpoint blockade with anti-CTLA4 and anti-PD1 in melanoma and pancreatic ductal adenocarcinoma.

In summary, a broad rationale exists, based on animal models and preliminary human data, for combining immune checkpoint blockade with immune agonist strategies such as anti-CD40 or RT. This rationale is particularly strong in the case of ‘cold’ tumor types with less endogenous immune activity because immune checkpoint blockade primarily functions by augmenting pre-existing adaptive immunity. The current
challenge is rational selection of combinatorial therapy, especially given that, based on the complexity of these immune pathways, mechanisms of action of individual agents may differ in combinatorial use. Chapter 3 (page 63) investigates mechanisms and determinants RT and CD40 combinatorial therapy with immune checkpoint blockade in melanoma and pancreatic ductal adenocarcinoma.

Melanoma in humans and the B16-F10 murine model

Melanoma incidence is increasing worldwide and now accounts for 80% of all skin cancer deaths, despite representing less than 5% of skin cancer incidence (World Health Organization). Accordingly, the prognosis of late stage melanoma remains grim: median survival is 6-9 months with chemotherapy despite an excellent survival for local disease of greater than 95% (Garbe et al. 2011). Prognosis of advanced disease varies based on extent of dissemination, such as the presence of visceral disease or brain metastases (Bedikian et al. 2009). These statistics reflect the underling reality that melanoma is highly chemotherapy resistant; the wide range of treatments to which melanoma is refractory further suggests that underlying mechanisms of resistance are complex, seemingly linked as much to the intrinsic biology of melanocytes as to acquired therapeutic resistance (Soengas & Lowe 2003).
Prior to immune checkpoint blockade, the only FDA-approved therapy for metastatic melanoma was dacarbazine, hydroxyurea and interleukin-2, none of which were tested in large, randomized clinical trials, and standard of care was enrollment in experimental therapy. In 2006, a fully-human anti-CTLA4 monoclonal antibody was shown to have mild toxicity, a 7% complete response rate, and long-term durable responses in an initial phase I dose escalation study (Gomez-Navarro et al. 2006). This result was subsequently confirmed in a landmark 2010 phase III study, (Hodi et al. 2010) resulting in anti-CTLA4 becoming the first drug to receive FDA approval for the treatment of metastatic cancer. Anti-PD1, the most promising second generation immune checkpoint antibody, has also shown promising results in patients with metastatic melanoma based on initial studies demonstrating objective response rates in a quarter of patients (Hamid et al. 2013). Furthermore, an expansion cohort trial of 173 patients with anti-CTLA-refractory disease demonstrated improvements in overall response with anti-PD1 treatment, and subsequent randomized phase II and III studies has established anti-PD1 as effective in this patient subpopulation (Ribas & Flaherty 2015). Thus, it has been difficult in recent years to overstate the perceived promise of immune checkpoint blockade to change clinical management of advanced melanoma.

Melanoma is an ideal model to study CD8 effector T cell resistance mechanisms because endogenous antitumor immunity in melanoma is clearly mediated by this cell population (Erdag et al. 2012, van Houdt et al. 2008). Infiltrating immune cells were first
described in cutaneous melanoma, in one of the first such discoveries in any tumor type, by Clark and Mihn in 1969, (Clark et al. 1969), and many years passed before these cells were subsequently shown to correlate with overall survival (Day et al. 1981, Tuthill et al. 2002). Syngeneic murine melanoma models have been a cornerstone for mechanistic investigations of tumor-immune interactions. The most widely used murine melanoma model, and likely the most widely used murine cancer model, is the B16 cell line (Kuzu et al. 2015). B16-F10 is an established sub-clone with high metastatic potential, rapid dissemination of metastatic disease, (Herlyn & Fukunaga-Kalabis 2010) and relatively poor endogenous immunogenicity due in part to low levels of MHC class I expression (Becker et al. 2010). B16-F10 shares characteristics with human melanoma, including inactivating mutations in Cdkn2a. Indeed, compared with initial murine studies showing efficacy of single agent anti-CTLA4, (Leach et al. 1996) B16-F10 does not respond to single agent CTLA4; instead, anti-CTLA4 must be combined with other agents thought to stimulate endogenous antitumor T cell response such as granulocyte-macrophage colony-stimulating factor (van Elsas et al. 1999). Similarly, anti-PD1 combined with RT, but not either modality alone, induces antitumor T cell responses in this model against tumor-specific antigens (Sharabi et al. 2015). In summary, B16-F10 is a useful murine model for combinatorial immune therapy due to its verisimilitude to human melanoma resistance to adaptive immunity. An additional advantage is rapid cell proliferation that permits multiple in vivo passages, further facilitating this study of resistance mechanisms.
New clinical trials in patients with metastatic melanoma focus on investigating potential advantages of combinatorial immune therapy with radiation or chemotherapy. The critical outstanding scientific objectives, with multiple immunotherapeutic agents approved or emerging, is to develop biomarkers and rational strategies for combination therapy.

Pancreatic adenocarcinoma in humans and the Kras^{G12D}, p53^{R172H}; Pdx1-Cre murine model

Pancreatic ductal adenocarcinoma (PDA) is a nearly universally lethal form of cancer, with an 5-year survival rate that remains below 5% despite decades of attempts at improvement (Hidalgo 2010). It is the third most common cause of cancer death and PDA incidence appears to be increasing – PDA will overtake colorectal adenocarcinoma to become the second most common cause of cancer death by 2030 (Rahib et al. 2014). PDA lethality is due in part to the anatomical location of the pancreas, which allows for long periods with few specific clinical manifestations, such that more than 80% of patients have advanced disease at the time of diagnosis (Hidalgo 2010). New combinatorial chemotherapy strategies in PDA have resulted in minimal impact on patient outcomes; though these recent strategies have modest response rates of 20-30%, there has been little change in rates of durable response (Conroy et al. 2011, Hoff et al. 2013). Immune therapy, having demonstrated profound potential for impact on response rates in other disease types, has thus been a strategy of great promise in PDA (Foley et al.
Furthermore, exciting preliminary studies have suggested that a small subset of PDA tumors have a relatively immunogenic profile, characterized by greater infiltration of T cells and prominent proinflammatory signal, (Bailey et al. 2016a, Fukunaga et al. 2004) and survival of PDA patients whose tumors are resectable appears to correlate positively with T cell infiltration (Fukunaga et al. 2004, Hiraoka et al. 2015, Ino et al. 2013). Despite this rationale and myriad successes in other disease types, immune-modulating strategies such as anti-PD1/PD-L1 and anti-CTLA4 have thus far uniformly failed to show efficacy in more than a very small proportion of patients with PDA (Brahmer et al. 2012, Royal et al. 2010). This is perhaps due to the fact that hallmarks of PDA in the majority of patients are a predominant desmoplastic stroma, (Vonderheide & Bayne 2013, Wörmann et al. 2013) essentially no T cell infiltration in most cases, and prominent upregulation of immune suppressive pathways (Bailey et al. 2016b, Beatty et al. 2011). This clinical reality demands deeper mechanistic understanding of determinants of immune activity in PDA, which is explored in both chapters 3 (page 63) and 4 (page 103).

PDA develops from pancreatic intraepithelial neoplasia in linear fashion, with each progressive stage typified by a greater load of genetic abnormalities in common oncogenic driver and tumor suppressor genes (Rahib et al. 2014). Aspects of PDA oncogenesis are incompletely understood – other pancreatic lesions such as intraductal papillary mucinous neoplasia can also generate PDA, (Kopp et al. 2012) and recent
evidence indicates that PDA oncogenesis may sometimes be an abbreviated, catastrophic process (Notta et al. 2016). Regardless of etiology, the genetic hallmark of PDA is a dominate mutation in the KRAS2 oncogene leading to constitutive activation of the canonical downstream MAPK/ERK pathway. Progression from pancreatic intraepithelial neoplasia is also associated with increasingly frequent mutations in p16INK4a, p53, DPC4/SMAD4, and BRCA2 (Hruban et al. 2000).

The high prevalence and recognized importance of mutations in KRAS2 led to development of a murine PDA model using targeted endogenous expression of Kras\textsuperscript{G12D}, the most common KRAS2 activating mutation. Expression is restricted to presumed progenitor cells using Cre-lox recombination tied to the pancreatic-specific promoters p48 or Pdx1 (Hingorani et al. 2003). These mice recapitulate with high fidelity the progression from pancreatic intraepithelial neoplasia to PDA; spontaneous evolution occurs as mice age with eventual invasive primary lesions and metastatic disease. The addition of a second hallmark PDA genetic lesion with restricted pancreatic expression, TP53\textsuperscript{R172H} (a dominant-negative TP53 isoform similar to that found in Li-Fraumeni syndrome), resulted in a murine PDA model (KPC, Kras\textsuperscript{LSL-G12D/+};p53\textsuperscript{LSL-R172H/+};Pdx-1-Cre) with 100% penetrance within 6 months of birth (Clark et al. 2009, Hingorani et al. 2005). Furthermore, the KPC model recapitulates the dense desmoplastic stroma, prominent immune suppressive pathways, and absence of CD8 T cell infiltrate that characterizes the majority of human PDA tumors (Beatty et al. 2011, Clark et al. 2007). Myeloid-derived
suppressor cells, which limit CD8 effector T cell function through secretion of arginase and inducible nitric oxide synthase, and other tumor-associated macrophages characterized by secretion of suppressive cytokines IL-10 and IL-6, are hallmarks of the KPC immune microenvironment (Bayne et al. 2012, Beatty et al. 2011, Clark et al. 2007, Gabrilovich et al. 2012).

This presence of immune suppressive pathways and lack of endogenous CD8 T cell infiltration suggest that the PDA tumor microenvironment may be a site of acquired immune privilege, in contrast to other tumor types such as melanoma in which endogenous T cell activity may itself drive immune suppression (Spranger et al. 2013). In this context, acquired immune privilege is defined as a state of suppressed lymphocyte responses to antigens that is actively and locally enforced, and is distinct from central tolerance (Mellor & Munn 2008). The canonical example of this biology is the immune homeostasis present at large mucosal surfaces where the risk of immune activation against benign foreign antigens is omnipresent. In the context of cancer, this hypothesis suggests that PDA tumors may be therapeutically susceptible to T cell-mediated destruction provided T cell infiltration and activation can be sufficiently augmented. In other words, paradoxically, the lack of baseline T cell infiltration in PDA may result in a tumor microenvironment that does not strictly adhere to the immune editing hypothesis. In PDA, no or minimal selective immune pressure may result in clinically-detectable tumors of high potential, if not apparent, antigenicity. Indeed, increasing data from
mouse models suggest that T cell responses against KPC tumors can be strikingly robust, and even curative, when immune suppressive pathways are inhibited or antigenicity is sufficiently strong (Byrne & Vonderheide 2016, Winograd et al. 2015). In particular, recent data demonstrate that combinatorial treatment with anti-CD40 and chemotherapy can overcome baseline refractoriness to immune checkpoint blockade (Winograd et al. 2015).
Targeting of tumors cells by CD8 T cells

The ability of checkpoint blockade to induce durable responses in an increasing range of malignancies creates an urgent need to understand why the majority of tumors across disease types are either not responsive or develop resistance to T cell-mediated immunity. (Sharma & Allison 2015) Many efforts to identify predictive biomarkers of checkpoint blockade have identified immune and tumor pathways of importance; (Herbst et al. 2014, Taube et al. 2014, Topalian et al. 2015, Tumeh et al. 2014) however, there remains a need for immune dynamics across human tumor types to be extensively characterized.

Tumor antigens targeted by T cells

The etiology of antigens targeted by antitumor T cell responses is one such inadequately explored area. In vitro expression library screening of tumor-infiltrating lymphocytes was the initial method of identifying tumor antigens. In these experiments, PBMCs were sensitized against autologous tumor or normal cells pulsed or transfected with potential tumor epitopes. Five classes of antigens emerged from these studies: viral antigens, over-expressed gene projects, tissue-specific differentiation antigens, cancer germline antigens, and mutated antigens, also called neoepitopes (Lu & Robbins 2015). Availability of viral antigens as targets for T cells, such as HPV E6 and HPV E7, is limited.
to specific tumor types such as cervical and head and neck cancers. Furthermore, immune responses can be limited by viral immune evasion mechanisms, perhaps contributing to the fact that targeting these antigens is effective at preventing disease but does not produce clinically meaningful antitumor responses in patients whose disease has progressed (van Poelgeest et al. 2013). Over-expressed gene products, such as PRAME, (Epping & Bernards 2006) are enticing targets due to their wide expression across tumor types. However, this class of antigens is also expressed at nominal levels in normal tissue, and responses are likely to generate either peripheral tolerance or, if this tolerance is broken, autoimmunity. Tissue-specific differentiation antigens and cancer germline antigens are expressed less widely, restricted to a single tissue of origin or germ cells, respectively, but nevertheless may have the same fundamental limitation. Germ cells lack expression of MHC, reducing risk of autoimmunity, and clinical trials evaluating adoptive transfer of the cancer germline antigen NY-ESO-1 have achieved objective responses in more than 50% of selected patients with myeloma and melanoma (Rapoport et al. 2015, Robbins et al. 2015). Yet the relatively small number of these antigens that have been identified – and a lack of truly robust endogenous responses in most cases – has motivated efforts to identify tumor-specific peptides capable of generating immunity.
**Neoepitopes in cancer immunotherapy**

Increasing evidence indicates that neoepitopes, antigens derived from somatic mutations and seen as foreign by the immune system, are critical mediators of tumor immunity (Schumacher & Hacohen 2016). Targeting neoepitopes has, by definition, the theoretical advantage of bypassing both immune-intrinsic suppressive mechanisms as well as the threat of autoimmunity. Furthermore, though precise antigens likely differ across patients, this class of antigens is a tractable target in virtually all forms of cancer with more than a handful of genetic abnormalities that generate non-self-peptides that can be presented on MHC. For these reasons, investigating the determinants of immune responses against neoepitopes has generated tremendous interest. Higher predicted neoepitope load is associated with baseline lymphocyte infiltration and survival in colorectal tumors in the absence of immunotherapy. (Giannakis et al. 2016)

Therapeutically, degree of DNA damage is associated with improved response to anti-CTLA4, (Snyder et al. 2014) and responses to anti-PD1 in patients with NSCLC also correlate with neoepitope load (Rizvi et al. 2015). Similarly, a strong correlation exists between mismatch repair deficiency and response to anti-PD1 (Le et al. 2015) and between mutation or MHC class I neoepitope load and response in melanoma or urothelial carcinoma, respectively (Hugo et al. 2016, Rosenberg et al. 2016). A recent analysis of whole exome sequencing from patients with metastatic melanoma identified neoepitopes recognized by adoptively-transferred tumor-infiltrating lymphocytes, and
neoepitope load was associated with objective tumor regressions (Robbins et al. 2013).

Taken together, this preponderance of recent findings suggests neoepitopes can drive productive antitumor responses in humans and may be predominant targets of these responses.

Studies in mice and humans have suggested that neoepitopes derived from mutant Kras can serve as tumor rejection antigens, (Fossum et al. 1995, van Elsas et al. 1995) and this was recently demonstrated to be the case in a patient treated with adoptively-transferred autologous T cells specific for mutant Kras$^{G12D}$ (Tran et al. 2016). Kras$^{G12D}$-specific T cells made up 75% of adoptive-transferred cells and mediated objective regression of 100% of lung metastases. A single lesion that progressed after 9 months was found to have lost the crucial HLA allele required for KRAS activity. This unusual case report is direct, albeit anecdotal, evidence of neoepitope-directed antitumor responses in a therapeutic setting.

Murine models have provided further mechanistic support for the hypothesis that neoepitopes are predominant tumor rejection antigens, including the hallmark study from Robert Schreiber et al. supporting the cancer immunoediting hypothesis, which demonstrated that a point mutation in Spectrin-beta2 could drive tumor rejection (Matsushita et al. 2012). In B16-F10 melanoma, 11 of 50 mutated peptides identified on the basis of predicted affinity for MHC class I from whole exome sequencing preferentially induced immune responses (Castle et al. 2012). Therapeutically,
immunization of mice against MHC class I or II neoepitopes resulted in slowed tumor growth and enhanced survival (Kreiter et al. 2015, Yadav et al. 2014). In summary, correlative studies suggest that neoepitopes may be primary targets in the context of immune checkpoint blockade, and that these immune targets can mediate tumor regression in mice and humans.

_Cancer immunotherapy neoepitope prediction_

The emerging importance of neoepitopes in antitumor responses necessitates the ability to understand determinants of T cell immunity to this class of antigens. Tumor DNA and RNA sequencing provide a vast landscape of tumor-specific gene expression and mutations that can be used to identify MHC class I and II neoepitopes. Computational neoepitope prediction using these genomic data is a complex problem, in large part due to the MHC restriction of antigen presentation. Murine and human cells express 6 MHC class I molecules and antigen presenting cells express 8 or more MHC class II molecules. Determinants of preferential binding differ substantially across MHC type, allele, and peptide length, and more than 3000 HLA alleles exist (Leone et al. 2013). Furthermore, expression of mutant proteins – as well as processing and presentation of peptides – differs substantially within and across tumors. This vast diversity requires that the ability of a mutated peptide to function as a neoepitope is assessed at the level of individual tumor samples.
High throughout computational pipelines to mine tumor mutations, expression levels, and MHC types have arisen to meet this need (Gfeller et al. 2016). These analyses generally begin with whole exome sequencing of tumor and normal tissue to identify somatic variants present only in tumor tissue, which can be readily identified with high accuracy if coverage depth is sufficient (Sims et al. 2014). Ideally, matched RNA sequencing is then used to identify genes at least minimally expressed in tumors and therefore capable of being processed and presented in the context of MHC. RNA sequencing additionally allows for identification of cancer-specific splice variants and gene fusions that can also result in novel peptides.

Predicting neoepitopes that drive T cell responses with high specificity and sensitivity, particularly those that are good vaccine candidates, has been enormously challenging (Gfeller et al. 2016). Several high throughout pipeline components for increasingly accurate identification of MHC alleles, DNA variant candidates, expressed genes, and high-affinity peptides have been developed to meet this demand. To generate effector T cells, a genetic variant must result in peptide fragments of 8-14 amino acids that bind specific MHC with high enough affinity to persist stably in the MHC binding groove for presentation. Peptide N- and C-terminal residues are generally involved in binding MHC and are therefore critical for this stability, and evidence suggests that higher affinity interactions between peptide and MHC may preferentially result in epitopes capable of stimulating T cells (Leone et al. 2013). Based on these rules, predictive
computational methods have been developed to align peptides and, using large sets of training data, infer novel peptide affinity for MHC according to the presence or absence of conserved motifs (Falk et al. 1991). The most accurate of these methods use machine learning approaches such as neural networks, hidden Markov models or support vector machines capable of considering complex sequence patterns as high-dimensional problems (Dönnes & Elofsson 2002, Nielsen & Lund 2009). Across most HLA types, as shown to be the case for diverse algorithms designed to replicate biological processes, consensus methods combining methodologically orthogonal approaches have yielded the best results (Vita et al. 2015). Various downstream steps have been investigated to improve peptide predictions, such as consideration of peptide cleavage site preference, complex peptide-MHC structures, or peptide transport predictions, (Antes et al. 2006, Larsen et al. 2005, Nielsen et al. 2005) but it is not yet clear if these computationally-intensive added steps lead to improved predictions.

CD4 T cells are a major component of tumor-infiltrating lymphocytes and have been shown to mediate tumor regression independent of CD8 T cells in murine models of adenocarcinoma (Kreiter et al. 2015, Tran et al. 2014). Consequently, computational methods for predicting MHC class II affinity may also be useful for modeling antitumor responses. The relatively laxity of peptide interactions with MHC class II make these predictions more challenging; experimental evidence indicates that a greater diversity of peptide lengths are able to stably interact with MHC class II and, in the case of HLA-DR,
polymorphic alpha and beta chains reduce the ability of algorithms to extrapolate from conserved binding rules based on peptide motifs. For both MHC class I and class II predictions, a minority of predicted high-affinity peptides generate T cell reactivity in animal models or patients, (McGranahan et al. 2016) yet computational neoepitope prediction does substantially enrich for reactive peptides (Castle et al. 2012).

The multiple order of magnitude decrease in the cost of genomic sequencing over the past 20 years has enabled cancer sequencing efforts that can now be used to investigate tumor antigens across human cancer (Hayden 2014). The Cancer Genome Atlas (TCGA), now Genome Data Commons, provides a large, multidimensional set of genome-wide sequencing ideally suited for investigating pan-cancer and disease-level interactions between genetic and immune factors. TCGA includes paired normal and tumor exome sequencing required for somatic variant identification and HLA-typing, as well as RNA sequencing required for determining variant expression and inferring tumor microenvironment immune activity. Previous work using the first subset of TCGA disease types available showed that predicted MHC class I neoepitopes correlate with patient survival across colorectal adenocarcinoma, ovarian serous cystadenocarcinoma, breast carcinoma, glioblastoma, kidney renal clear cell carcinoma, and lung adenocarcinoma, (Brown et al. 2014) and that MHC class I neoepitopes correlate with cytolytic immune activity in lung and stomach adenocarcinoma, head and neck squamous cell carcinoma, uterine carcinosarcoma and thyroid carcinoma (Rooney et al.
Beyond these initial compelling studies, the broad landscape of interactions between neoepitope load and immune dynamics in the tumor microenvironment remains inadequately explored.
CHAPTER 2 – Materials and methods

Clinical trial patients and study design

A clinical protocol of ipilimumab (anti-CTLA4) and RT for patients with metastatic melanoma was registered on clinicaltrials.gov (NCT01497808). Eligible patients were at least 18 years of age with previously treated or untreated stage IV melanoma with multiple metastasis. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 or 1, adequate renal, hepatic, and hematological function, no current or history of CNS metastasis, no prior radiation that precludes use of stereotactic body radiation (SBRT), and at least one tumor between 1-5 cm that could be treated with SBRT. The primary objectives of this phase I study were to determine feasibility, dose-limiting toxicities (DLT) and maximum tolerated SBRT fraction when given in conjunction with ipilimumab. The secondary objectives were to determine late toxicity, immune-related clinical responses and changes. The study treated successive cohorts of patients with escalating doses of SBRT to a single tumor (index lesion), followed 3-5 days later by ipilimumab every three weeks for four doses. Moderate RT doses were used since higher RT dose has not been clearly correlated with better immune response but would likely increase toxicity. Patients were stratified into two stratum based on treatment site (lung or bone vs. liver or subcutaneous) and dose escalation of SBRT
were determined as follows: For lung/bone lesion, dose level 1 (DL1) was 8 Gy x 2; dose level 2 (DL2) was 8 Gy x 3; and for liver/subcutaneous lesion, DL1 was 6 Gy x 2; DL2 was 6 Gy x 3. The study followed a “treat six” design with the goal of accruing 6 patients to each dose level, or 24 patients total. Enrollment to a dose level would stop if 2 or more patients had a DLT. If 0-1 patients out of the 6 had a DLT at DL1, escalation to DL2 would proceed. No DLTs were observed, defined by the protocol as any treatment-related grade 4 or higher immune-related toxicity (NCI CTC Version 4.0) or grade 3 or higher non-immune related toxicity experienced during study treatment or within 30 days after the last injection of ipilimumab. Pre- and post-treatment blood, CT, and PET/CT were obtained to follow tumor response and assess immune responses. Response evaluation by imaging was performed within 60 days of the last ipilimumab treatment using either RECIST v1.1 (Eisenhauer et al. 2009) or PERCIST. The study protocol was approved by the University of Pennsylvania institutional review board. All participating patients provided written informed consent.
Murine studies

Cell lines and tissue culture

B16-F10 was purchased from ATCC. TSA was a gift from Sandra Demaria. PDA.4662 cell line was derived from single cell suspensions of PDA tissue from LSL-Kras$^{LSL-G12D/+}, LSL-p53^{LSL-R172H/+}, Pdx1-Cre$ mice as previously described (Bayne et al. 2012). B16-F10 and PDA.4662 cell lines were cultured at 37º C in DMEM and TSA cells were cultured at 37º C in RPMI. Media was supplemented with 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin, 2 mM L-glutamine. All cell lines were determined to be free of Mycoplasma (Lonza) and common mouse pathogens (IDEXX).

In vivo tumor growth experiments

Five- to seven-week-old female C57BL/6 and BALB/c mice were obtained from NCI Production (Frederick, MD) and Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen free conditions. All animal experiments were performed according to protocols approved by the Institute of Animal Care and Use Committee of the University of Pennsylvania. For B16-F10 melanoma, $5\times10^4$ B16-F10 cells were mixed with an equal volume of Matrigel (BD Biosciences) and subcutaneously injected on the right flank of C57BL/6 mice on day 0 and the left flank on day 2. The right flank tumor
site was irradiated with 20 Gy on day 8. Blocking antibodies were given on days 5, 8 and 11. For the concurrent vs. sequential RT experiment, the right flank was irradiated on either day 8 (sequential) or 12 (concurrent), while blocking antibodies were given on days 9, 12, and 15. For TSA breast cancer, 1x10^5 TSA cells were mixed with an equal volume of Matrigel (BD Biosciences) and subcutaneously injected on the right flank of BALB/c on day 0 and the left flank on day 2. The right flank of the mice was irradiated with 8 Gy on three consecutive days starting on day 10 or 11 post tumor implantation. Blocking antibodies were started 3 days prior to RT and given every 3 days for a total of 3 doses. For the pancreatic cancer model, 4x10^5 PDA.4662 cells were subcutaneously injected on the right flank. The right flank was irradiated with 20 Gy on day 8. Blocking antibodies were given on days 5, 8, and 11. For melanoma and breast cancer models, I used the optimal dose and fraction of radiation as previously reported (Dewan et al. 2009, Lee et al. 2009). All irradiation was performed using the Small Animal Radiation Research Platform (SARRP). Antibodies used for in vivo immune checkpoint blockade experiments were given intraperitoneally at a dose of 200 µg/mouse and include: CTLA4 (9H10), PD1 (RMP1-14), PDL-1 (10F.9G2), CD8 (2.43), and rat IgG2B isotype (LTF-2) (BioXCell). CD40 (BioXCell) was given at a dose of 100 µg/mouse on day 11 only based on prior studies showing this dose to have equivalent pharmacodynamic effect to higher doses in mice and humans (Beatty et al. 2011, Kedl et al. 2001, Vonderheide et al. 2007). Anti-CD8 was given 2 days prior to tumor implantations (day -2), day 0, then every 4 days for the duration of the experiment. Perpendicular tumor diameters were measured using
calipers. Volume was calculated using the formula $L \times W^2 \times 0.52$, where $L$ is the longest dimension and $W$ is the perpendicular dimension.

**Survival and tumor response analysis**

Differences in survival were determined for each group by the Kaplan-Meier method and the overall p-value was calculated by the log-rank test using the “survival” R package version 2.37+. For mouse studies, an event was defined as death or when tumor burden reached a protocol-specified size of 1.5 cm in maximum dimension to minimize morbidity. To help control for differences in treatment response due to experimental variation or intrinsic growth differences with sublines, tumor volume measurements were also analyzed after normalizing to the average volumes of untreated control mice. These average untreated tumor volumes were determined at day 11-12, a time when tumor dimensions could be accurately measured, and was considered a baseline tumor volume (Vcont). Normalized tumor response to treatment is the measured volume ($V$) relative to Vcont, or $V/V_{\text{cont}}$, a dimensionless value that is relative to a baseline volume. Measurements from different experiments separated by 1-2 days were binned. Differences in growth curves were determined by a mixed effect linear model with normalized data using the “lmerTest” R package version 2.0. Sample size estimations were based on preliminary pilot experiments. For control mice, I expected an average tumor volume of 0.4 cm$^3$ at day 17-21. For most experiments, I assumed the treatment group
would have an effect size resulting in a 50% reduction in average tumor volume. Sigma was estimated to be 1.5. For a 0.80 power at the 0.05 alpha level, this gave us a sample size of 5 mice. Mice were randomly assigned a treatment group. For experiments whereby the effect size was expected to be small and/or non-robust, two independent researchers with at least one researcher blinded to the treatment group assignments performed caliper measurements.

Flow cytometry

For flow cytometric analysis of in vivo experiments, blood, spleen, and tumor were harvested at either day 16 or 18 post tumor implantation. Single cell suspensions were prepared and red blood cells were lysed using ACK Lysis Buffer (Life Technologies). Live/dead cell discrimination was performed using Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) or Sytox Red Dead Cell Stain (Life Technologies). Cell surface staining was done for 20-30 minutes. Intracellular staining was done using a fixation/permeabilization kit (00-5521-00, eBioscience.) T effector cells were phenotyped as CD8\(^+\)CD44\(^+\), myeloid derived suppressor cells (MDSC) as CD11b\(^-\)Gr-1\(^+\), and regulatory T cells as CD4\(^+\)FOXP3\(^+\). All flow cytometric analysis was done using an LSR II (BD) or FACSCalibur (BD) and analyzed using FlowJo software (TreeStar) or the FlowCore package in the R language and environment for statistical computing.
**CRISPR gene targeting**

Gene targeting by CRISPR/Cas9 was accomplished by co-transfection of a Cas9 plasmid (Addgene, 56503), the guide sequence (selected using ZiFit Targeter) cloned into the gBlock plasmid, and a plasmid with the puromycin selection marker. Successful targeting of PD-L1 was determined by flow cytometry screening of clones treated with and without 100 ng/mL of interferon-γ (PeproTech). Confirmed clones were pooled. Clones without knockout were also pooled and used as controls.

**Immunohistochemistry for PD-L1**

Formalin-fixed, paraffin-embedded tumors were collected at the time of surgical resection or from biopsy. All patients with available recent biopsy, which was optional for trial enrollment, were used for analysis. After heat-induced antigen retrieval (Bond ER2, 20 min.), the tumor slides were stained with an anti-PD-L1 antibody (E1L3N, Cell Signaling) at 1:50 dilution. Intensity of staining on a 0-3+ scale, the percent tumor cells or macrophages with positive staining, and the cellular pattern (membrane vs. cytoplasm) were analyzed by two pathologists. Samples with membrane PD-L1 staining intensity score of 0-1 were classified as PD-L1lo, and samples with an intensity score of 2+ in at least 1% of the cells were classified as PD-L1hi. To confirm specificity, the anti-PD-L1
antibody was validated by staining Hodgkin’s lymphoma cells (Green et al. 2010) and placenta (Holets 2006).

**Microarray data processing and normalization**

Total RNA was isolated and purified from cells using Isol-RNA Lysis Reagent (Fisher.) Total RNA from tumors was isolated and purified from frozen specimens using Isol-RNA Lysis Reagent and Qiagen RNAeasy extraction kit with DNAase I on column treatment. Labeled RNA was hybridized to the Affymetrix GeneChip Mouse Gene 1.0 and 2.0 ST Array. Affymetrix CEL files for all samples were processed using the RMA method as implemented in the “oligo” R package version 1.26.6. Probe annotations were provided by the “mogene10sttranscriptcluster.db” and “mogene20sttranscriptcluster.db” R package version 8.0.1 and 2.13.0, respectively. Since different array types and different batches were used, each expression set was z-score transformed (Cheadle et al. 2003) and median centered. Multiple probes for the same gene were averaged and only genes common to the 1.0 and 2.0 ST arrays were kept. Batch effects were adjusted using the ComBat method as implemented in the “sva” R package version 3.8.0. The microarray data has been deposited at the GEO (GSE65503). Gene expression data for primary melanoma samples were downloaded from the GEO (GSE22155). For this data set, the post-processed data and provided annotations were used.
Determining differentially expressed genes and enriched gene sets

Non-specific filtering was used to remove genes with an interquartile range less than 0.05. To find differentially expressed genes (DEGs) between parental sensitive and resistant tumors, Significance Analysis of Microarray (Tusher et al. 2001) (“samr” R package version 2.0) was applied using a two class unpaired comparison, minimal fold change of 1.2, and median false discovery rate (FDR) of 0.05. Unannotated transcripts were not considered. To test whether gene sets were enriched in response to different conditions, we utilized Gene Set Analysis as implemented in the “GSA” R package version 1.03 (Efron & Tibshirani 2007). The “maxmean” test statistic was used to test enrichment using a two-class comparison. All p-values and false discovery rates were based on 500-1000 permutations. For restandardization, a method that combines randomization and permutation to correct permutation values of the test statistic and to take into account the overall distribution of individual test statistics, the entire data set was used rather than only the genes in the gene sets tested.

Flow cytometry data processing

Gating was performed using either FlowJo version 9.7.5 or the FlowCore R package version 1.28.24. For computational modeling, values were normalized by subtracting the average values of untreated controls. For the CD8/Treg ratio, the percent
CD8^{+}CD44^{+} cells were divided by the percent CD4^{+}FOXP3^{+} cells. Because these data could be skewed with varying and wide distributions, these data were log transformed for downstream analysis.

**TCR deep sequencing and clonotype diversity analysis**

DNA from pre-treatment blood, post-treatment blood, and tumor was extracted on day 16 using the Qiagen DNA extraction protocol. Samples were sequenced by Adaptive Biotechnologies using “survey” sequencing depth for tumor and “deep” sequencing depth for blood samples. Processed data were downloaded and frequencies/counts for TCR clonotypes were examined by nucleotide sequences after non-productive reads were filtered out. The top 100 most frequent TCR clonotypes in the tumor were used to examine their frequencies in the pre- and post-treatment blood. The Shannon’s diversity index (Rempala & Seweryn 2012) (DI) normalized to the number of reads ($DI = -\sum (pi \ln pi) / \ln n$, where $n$ is the number of clones, $pi$ is the clonal frequency of the $i$th clone, and sigma is summed from $i=1$ to $i=n$) was calculated for each sample. This gives a value between 0 and 1, where 0 is monoclonal and 1 is an even distribution of different clones.
Unsupervised and supervised analysis of CDR3 amino acid properties

Based on previously described methods (Atchley et al. 2005, Thomas et al. 2014), Atchley factors were used to reduce a linear sequence of amino acids into analyzable numeric features of distinct amino acid properties. The five Atchley factors and the attributes they measure are: 1) PAH: accessibility, polarity, and hydrophobicity, 2) PSS: propensity for secondary structure, 3) MS: molecular size, 4) CC: codon composition, 5) EC: electrostatic charge. Each CDR3 was represented as a set of all possible contiguous amino acids of length $p$ ($p$-tuple). $p=3$ was chosen based on previous published reports but examined a range of $p$ values, which gave comparable results (see below). For each $p$-tuple, the Atchley factors for the amino acids were then calculated to give a vector of length $5p$, or 15 (3 amino acids x 5 Atchley factors). Thus, each CDR3 was represented by a set of these vectors. The average values for these vectors were calculated for the top $B$ most frequent clones from the post-treatment blood. A cut-off of $B=5$ was chosen based on examination of the frequency distribution of the TCR clonotypes and an estimate of the number of clones with extreme values compared to the rest of the distribution. These averaged values were then clustered into two groups by k-means clustering with $k=2$. The association between cluster membership and treatment with or without RT was calculated by Fisher’s exact test. This entire process was repeated for the five clones in the pre-treatment blood, for randomly drawn clones from the post-treatment blood, for $p$-
tuple lengths from $p=2$ to 10, and for cut-off values from $B=3$ to 50. In all cases, the 
distribution of p-values was compared to the p-value from the observed data.

Although averaging the Atchley factor values is a simple method to agglomerate 
CDR3 features for unsupervised classification, it does not provide insight into how 
treatment groups influence the amino acids that comprise the CDR3. To understand 
which sets of $p$-tuples were most strongly influenced by treatment groups with RT, 
without RT, and pre-treatment blood, this work used previously described methods 
(Thomas et al. 2014) to assign $p$-tuples into $n$ clusters based on their Atchley factor 
vector. Model based clustering with cluster number determination using the “mclust” R 
package was applied to all $p$-tuples from the top five clones in all treatment groups from 
pre- and post-treatment blood. This gave rise to 17 clusters, or subsets, of $p$-tuples. The 
proportion of $p$-tuples belonging to each of these 17 subsets, denoted $P_i$, was then 
calculated for each clonotype and used as features. The subsets that were most influenced 
by treatment group (treatment group with RT, without RT, or pre-treatment) were then 
analyzed by multivariable RF regression using a design matrix for treatment groups as the 
x-variable and $P_i$ as the response variable. The variables $P_i$ most affected by each 
treatment group were selected by comparing the observed importance scores to the 
importance scores generated by permutation. To determine the location and frequencies 
of amino acids belonging to the selected $p$-tuple subsets across the variable length CDR3 
region, the CDR3 of each clone was divided into 10 bins of equal size. Then, the
proportion of $p$-tuples in each of these 10 bins belonging to the selected subset was calculated and compared between treatment groups.

Guide RNA sequences

Gene block contains 20 bp target size (N), U6 promoter, gRNA scaffold, and termination signal. The sequence and sequences for each guide used are as follows:

```
TGTACAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCC
GGTACCAAGGTGCGGCGAGGAGGGGGCCTATTTCCATGATTTCTTCTATATT
GCATATACGATACAAAGGCTGTAGAGAGGATAATTGAAATTTATGACTGTA
ACACAAAAGATATATTGACAAAAATACGTGACGTAAGAAAATATAATTCTTGG
TAGTTGCAAGTAAAAATATGTAAAAATTTAAATGACTATCATATGCTTACCCG
ACTTGAAGATATTTTCATTGCCTTTATATATCTTGATGGAAAGGACGAAAC
ACCGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAA
ATAAGGCTAGTCGTTATCAACTTGAAGGAAGTGTCGAGTTCCCTTTCTCTAGAC
NNNNNNNNNNNNNNNNNNNNN
```

**G1:** GGCTCCAAAGGACTTGACG

**G2:** GACTTGACGTGGTGAGTA

**G3:** GTATTGCCAAGCTACGTCACGA
Neoeptipe analysis

Tumor and normal sample datasets for neoeptipe analysis

DNA variant, gene expression and clinical data from The Cancer Genome Atlas (TCGA) were obtained in June 2016 from the Genome Data Analysis Center Firehose (Spring 2016 run) following dbGAP approval. DNA variants were obtained in manually-curated mutation annotation format (MAF). Gene expression data used were normalized counts from the TCGA RNASeq Version 2 pipeline and included all available ‘Level_3’ samples. Raw tumor and normal paired whole exome sequencing reads were obtained through dbGaP (accession phs000178.v9,p8 ) (Tryka et al. 2014) and the NCI Cancer Genomics Hub. Samples selected for analysis were those with DNA variant, gene expression and raw DNA exome reads that could be successfully downloaded. Disease types included in neoeptipe analyses were those with at least 15 samples for predicted MHC class I or MHC class II neoeptopes. A list of samples analyzed for neoeptopes is available for all disease types (Table 9); samples with no missense mutations were excluded from predicted neoeptipe analyses. Tumor cellularity, purity, and ploidy estimates were determined using Sequenza and ABSOLUTE (Carter et al. 2012, Favero et al. 2014). Variant, expression, clinical, and raw data from TCGA are now available through Genomic Data Commons.
**HLA class I and II predictions**

Normal tissue whole exome sequencing was used for 4-digit HLA class I and II typing. In cases with multiple normal tissue samples, the sample with the greatest read depth was used. HLA class I typing was performed on reads re-mapped with RazerS3 (version 3.5 [2437c13]) using OptiType (version 1.3.1)(Szolek et al. 2014). This method significantly improves on the accuracy of first-generation HLA typing tools and has been independently validated on TCGA whole exome sequencing. RazerS3 was used with the following settings: percent-identity = 90; max-hits = 1; distance-range = 0. OptiType was used with default settings. HLA class II typing was performed on reads re-mapped with BWA (version 0.7.12) using HLAreporter (version 1.0.3)(Huang et al. 2015), which was updated to allow efficient parallel execution. BWA was used with default settings and the modified HLAreporter version used is available on Github (https://github.com/andrewrech/pHLAreporterII). HLA class I and II tying was validated to have >90% 4-digit accuracy compared to standard clinical typing using sequence-specific oligonucleotide probe and sequence specific primer techniques. For class I and II typing, the highest-scoring HLA type was used for analysis; samples with inadequate read-depth or low-certainty typing results were excluded (< 5%).
**Neoepitope class I and II predictions**

Variants predicted to yield missense mutations present in tumor but not normal samples were selected for neoepitope prediction. Human Genome Organization, RefSeq and Entrez identifiers were converted to Enembl transcript IDs using the R/Bioconductor package biomatR (version 2.30.0) (Durinck et al. 2009) and amino acid sequence variation nomenclature was converted to a standard input form. DNA variants were filtered for those in genes with RSEM normalized count expression of greater than 1. After filtering, a sliding window of 8-15-mer peptides centered on each variant site were generated. Peptides were truncated if the window length extended beyond the predicted translated protein sequence. Estimated binding affinity for each peptide was then calculated using the Python script version of the Immune Epitope Database and Analysis Resource (IEDB) MHC class I and II prediction tools (version 2.15) (Kim et al. 2012). The ‘IEDB recommended’ method was chosen for predictions because it considers algorithm benchmarks in large scale evaluation and availability across HLA alleles (http://www.iedb.org/). Median values were taken when multiple prediction algorithms were used. For MHC class I predictions, peptides with a median half-maximum inhibitory concentration of less than 5000nM or less than 50nM for a sample’s corresponding HLA types were classified as potential MHC binders and potential neoepitopes, respectively. For MHC class II predictions, peptides with a percentile rank of less than 4 or less than 1 were classified as potential MHC binders and potential
neoepitopes, respectively. Generator mutations were defined as missense sites generating one or more neoepitopes. The above pipeline for generating peptides, predicting MHC class I/II binding affinity and interpreting predictions for normal and mutant peptides is freely available as an R package and can be obtained from GitHub (https://github.com/andrewrech/Neoepitopes). See also Chapter 7: Software.
Survival analysis

Survival used in the Kaplan-Meier estimates was determined as the number of days from diagnosis until death or last contact. Analyses were conducted using all samples or by disease type; age, gender and tumor stage were assessed as potential confounders. Disease types with fewer than 15 total samples across subgroups analyzed were excluded. The R package 'survival' (version 2.39-5) was used to construct Kaplan-Meier curves.
Random forest analysis

Random forest analysis was conducted using the randomforestSRC R package (version 2.3.0) as previously described (Ishwaran et al. 2014, Twyman-Saint Victor et al. 2015). Random forest analysis is a multivariable non-parametric ensemble partitioning tree method for modeling classification, regression, or survival problems (Breiman 2001, Chen & Ishwaran 2012). This approach can be used to model the effect of multiple input variables and their interactions on a response variable of interest. Two-thirds of available samples are randomly chosen for model building and the result is then cross-validated using the remaining (out-of-bag, OOB) samples. Missing values were imputed and classification models were built using the following parameters: ntree = 500; nodesize = 2; nsplit = 10; mtry = (number of model variables)3/4. A Gini index splitting rule was used for classification and downsampling was used when the number of samples in each class differed by > 5-fold.

Variables were then ranked by minimal depth (MD), a dimensionless statistic that measures variable predictiveness in tree-based models. MD is defined as the shortest distance between the root node of a tree and the parent node of a maximal subtree, which is the largest subtree whose root node splits on the variable. Smaller MD values indicate greater predictiveness and a tree-averaged threshold MD was used to classify variables as predictive using the model-building two-thirds of samples. Once predictive variables were identified, the model was then re-fit using these variables and tested against the
remaining out-of-bag samples. If the number of input variables exceeded the number of samples by a factor of > 10, variable hunting was employed to calculate minimal depth prior to variable selection and forest re-fitting. Variable hunting is a regularized algorithm that exploits maximal subtrees for more effective variable selection under conditions where many noisy variables exist (Ishwaran et al. 2010). With variable hunting, the high dimensional feature space is divided into multiple smaller subspaces to better estimate minimal depth, which is returned as the average for each variable across subspaces.

I determined model performance using geometric mean accuracy (1-mean OOB error rate across replications) and F-score because I am primarily interested in the predictiveness of models for all response variable classes. Relative stability was determined using the normalized Brier score for each model, a proper score function that measures the mean squared difference between the predicted outcome probabilities from a random forest model and the actual outcome tested using OOB samples. A normalized Brier score of 100 indicates random guessing; a value of 0 indicates perfect prediction. Each analysis was bootstrapped over n > 10 iterations with replacement and model performance was determined by averaging OOB error rate/F-score, normalized Brier score and minimal depth across bootstrap iterations. A randomly selected set of 30 non-predictive genes were used as to generate negative control models. Models were considered significant if they met the following criteria: normalized Brier score < 90,
geometric mean accuracy > 0.6, normalized Brier score relative to negative control < 0.8, and geometric mean accuracy relative to control > 1.2. Negative control models were non-predictive.
Other computational analyses

Gene expression analysis tumor types and datasets

Data for tumor types available from TCGA were accessed in December 2015 following dbGAP approval and represent only untreated primary tumors (defined by the TCGA pathologist as “pancreatic ductal adenocarcinoma” (Table 6). Each tumor sample is paired with a normal tissue sample providing a germline reference. The following tumor types (project code and n = sample size) were selected: kidney renal clear cell carcinoma (KIRC, n = 606), lung adenocarcinoma (LUAD, n = 116), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n = 309), lung squamous cell carcinoma (LUSC, n = 553), pancreatic adenocarcinoma (PAAD, n = 134), stomach adenocarcinoma (STAD, n = 418), head and neck squamous cell carcinoma (HNSC, n = 566), colon adenocarcinoma (COAD, n = 328), skin cutaneous melanoma (SKCM, n = 105 primary tumors), bladder urothelial carcinoma (BLCA, n = 427), esophageal carcinoma (ESCA, n = 196), liver hepatocellular carcinoma (LIHC, n = 371), thyroid carcinoma (THCA, n = 572), ovarian serous cystadenocarcinoma (OV, n = 309), glioblastoma multiforme (GBM, n = 169), and prostate adenocarcinoma (PRAD, n = 555). Manually curated DNA variant mutational annotation format and tumor mRNA expression were obtained for each disease type from Broad Firehose (http://gdac.broadinstitute.org). Raw DNA reads (.bam format) used for BMR calculation
coverage estimates and HLA typing were accessed via the NCI Cancer Genomics Hub (https://cghub.ucsc.edu/). GISTIC2.0 (Mermel et al. 2011) individual copy number data for the PDA dataset was obtained from the TCGA Data Portal (https://tcga-data.nci.nih.gov).

**RNA sequencing-based gene expression data and analysis**

Data were normalized following the method of (Rooney et al. 2015). Briefly, total raw read counts per gene were divided by the gene’s maximum transcript length to represent a coverage depth estimate. Coverage estimates were then scaled to sum to a total depth of 1e6 per sample and can be interpreted as Transcripts Per Million (TPM) (Rooney et al. 2015). Gene set variation analysis (GSVA) was performed using the R/Bioconductor package “GSVA” because it implements a non-parametric unsupervised method to measure gene set enrichment across a dataset. The sample-wise enrichment score for a given gene set is calculated using a Kolmogorov-Smirnov (KS)-like random walk statistic. Statistical ranking for GSVA scores for the cytolytic index by the top decile and bottom quartile were defined as cytolytic-high and cytolytic-low, respectively. Unsupervised hierarchical clustering, using complete linkage with the distance metric equal to 1 minus the Pearson correlation coefficient, was also performed using the GSVA scores for each dataset. Principal components analysis between cytolytic-high and low PDA tumors was calculated using the 5,000 most variable genes. Differential gene
expression analysis between cytolytic-high (top 10\textsuperscript{th} decile cytolytic index) and cytolytic-low (bottom 25\textsuperscript{th} quartile cytolytic index) across TCGA datasets was calculated using gene-level raw counts in the R/Bioconductor package “limma” with voom transformation with quantile normalization (Law et al. 2014). Lowly expressed genes with less than 1 count per million in fewer than half of the samples in a dataset were excluded for differential gene expression analysis. Genes with BH-adjusted p values ≤ 0.1 were considered differentially expressed. All plots and graphs were generated using the R package “ggplot2” (Wilkinson 2011).

\textit{Mutation and copy number analysis}

Significantly mutated genes (SMGs) in cytolytic subtypes of PDA were calculated using the Mutational Significance in Cancer (MuSiC Genome Suite) (Dees et al. 2012). MuSiC identifies SMGs with a significantly higher mutation rate than the background mutation frequency (BMR) for a given gene calculated across the entire sample population. The threshold for significance was a false discovery rate of 0.1. Mutational spectra across cytolytic subtypes of PDA were determined as previously described (Rooney et al. 2015, Witkiewicz et al. 2015). Somatic copy number alterations in each TCGA PDA sample were counted by taking the sum of segment mean changes ≥ 0.6 and ≤ -0.4 between somatic and normal samples. Tumor cellularity and purity estimates were determined using Sequenza and ABSOLUTE (Carter et al. 2012, Favero et al. 2014).
Other computational and statistical software

The following R (https://www.r-project.org/, version 3.3.1) packages were used: data.table (http://r-datatable.com, version 1.9.6) (general data analysis), stats (version 3.3.2, analysis of variance, spearman’s rank correlation coefficients, and Student’s T test, Benjamini & Hochberg false discovery rate adjustment), pheatmap (https://CRAN.R-project.org/package=pheatmap, version 1.0.8) (heatmap generation), ggRandomForests (version 2.0.1)(Ehrlinger 2015) (random forest graphics), HiveR (https://CRAN.R-project.org/package=HiveR, version 0.2.55) (hive plots), ggplot2 (version 2.1.0) (other graphics), cowplot (https://CRAN.R-project.org/package=cowplot, version 0.6.3) (figure layouts). Data ranked for classification into high vs. low groups was scaled from 0 to 100 using an empirical cumulative distribution function and conducted within each disease type to avoid confounding analyses by comparing highly imbalanced sample subsets. Hierachal clustering with complete linkage was used to order heatmap axes.
### Mouse Experiment

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### Human Experiments

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**Table 1:** List of flow cytometry antibodies
CHAPTER 3 – Radiation and Dual Checkpoint Blockade Activates Non-Redundant Immune Mechanisms in Cancer

Much of Chapter 3 has been published, see Manuscripts (page xi).

Introduction and results

Anecdotal clinical reports suggest that RT may cooperate with anti-CTLA4 to systemically enhance melanoma response; (Postow et al. 2012) however, this combination has not been reported in a clinical trial. To examine the feasibility and efficacy of RT combined with immune checkpoint blockade, a phase I clinical trial of 22 patients with multiple melanoma metastases was initiated (Table 2). A single index lesion was irradiated with hypofractionated RT, delivered over two or three fractions, followed by four cycles of the anti-CTLA4 antibody ipilimumab (Figure 6a). Accrual was completed in three out of four RT dose levels, and treatment was well tolerated (Table 3). Evaluation of the unirradiated lesions by CT imaging using Response Evaluation Criteria in Solid Tumors (RECIST) demonstrated that 18% of patients had a partial response (PR) as best response, 18% had stable disease (SD), and 64% had progressive disease (PD) (Figure 2a). For example, patient PT-402 showed a large reduction in sizes of unirradiated tumors and
a partial metabolic response by positron emission tomography (PET) (Figure 2b). None of the 12 patients evaluated by PET had progressive metabolic disease in the irradiated lesion (Figure 6b, Table 4). The median progression-free survival (PFS) and overall survival (OS) was 3.8 and 10.7 months with median follow-up of 18.4 and 21.3 months (18.0 and 21.3 for patients without event), respectively (Figure 2c).

Although responses were observed, the majority of patients in this trial did not respond. To understand the contribution of RT to immune checkpoint blockade and to discover mechanisms of resistance, I utilized the B16-F10 melanoma mouse model in collaboration with Dr. Twyman Saint Victor and others. Mice with bilateral flank tumors received anti-CTLA4, irradiation of one tumor (index) using a micro-irradiator, or both treatments delivered concurrently (Figure 2d). The best responses in both tumors occurred with RT + anti-CTLA4. RT given before or concurrently with CTLA4 blockade yielded similar results (Figure 6c). Complete responses (CRs) were CD8 T cell-dependent, and mice with CRs also exhibited CD8 T cell-dependent immunity to tumor re-challenge (Figure 6d-e). However, similar to the clinical trial, only approximately 17% of mice responded. To better understand determinants of response, cell lines were derived from unirradiated tumors that relapsed after RT + anti-CTLA4 (Res 499 and Res 177). Resistance was confirmed in vivo and was not due to intrinsic RT resistance (Figure 7a-c). Random forest (RF) machine learning analysis (Ishwaran et al. 2010, 2011) of tumor infiltrating lymphocytes (TILs) demonstrated that the top predictor of resistance, as
measured by variable importance scores and selection, was the CD8+CD44+ to Treg (CD8/Treg) ratio (Figure 2e, Figure 7d). In resistant tumors, the CD8/Treg ratio failed to increase after RT + anti-CTLA4 as it did in sensitive tumors because CD8+CD44+ T cells did not significantly expand despite reduction in regulatory T cells (Figure 2f). Other immune variables associated with resistance were also related to the failure to accumulate CD8 effector T cells.

The prevalence of CD8 effector T cells can be blunted by mechanisms that interfere with T cell function. Transcriptomic profiling of Res 499/177 tumors revealed that PD-L1 was among the top 0.2% of upregulated genes that make up a RT + anti-CTLA4 “resistance gene signature” (Figure 7e). Other genes include interferon stimulated genes, which may promote immune suppression through PD-L1 (Teijaro et al. 2013, Wilson et al. 2013). Similarly, PD-L1 was co-expressed with the resistance signature in tumors from a previously reported (Jonsson et al. 2010) cohort of metastatic melanoma patients (Figure 2g). This increase in PD-L1 was observed on melanoma cells devoid of contaminating stromal cells, and a comparable increase was similarly seen in the Res 237 murine breast cancer cells (Figure 2h), which was selected from the TSA line for resistance to RT + anti-CTLA4 (Figure 7f-g). In contrast, expression of other inhibitory receptors and their ligands nominated by gene profiling did not suggest an obvious role in resistance (Figure 7h-i). Indeed, genetic elimination of PD-L1 on Res 499 cells by CRISPR (Figure 7j) restored response to RT + anti-CTLA4 by increasing survival from
0% to 60% (Figure 2i). Thus, an increase in PD-L1 on tumor cells observed in multiple cancer types can be a dominant resistance mechanism to RT + anti-CTLA4.

Elevated levels of PD-L1 can promote T cell exhaustion, a state characterized by dysfunction in T cell proliferation and effector function (Wherry 2011). Exhausted T cells co-express the PD-L1 receptor PD1 and the transcription factor Eomes (Paley et al. 2012). Reversal of exhaustion, known as reinvigoration, is marked by an increase in the proliferation marker Ki67 and the cytotoxic protein GzmB within the exhausted T cell pool. In both untreated parental and resistant tumors, approximately 20% of CD8 effector T cells co-expressed PD1 and Eomes, and only a minority of these cells were Ki67\(^+\)GzmB\(^+\), indicating that a significant fraction was exhausted (Figure 3a-b). In B16-F10 tumors, RT + anti-CTLA4 markedly increased both the proportion of PD1\(^+\)Eomes\(^+\) CD8 T cells and the proportion that were Ki67\(^+\)GzmB\(^+\) within this subset. In contrast, in resistant tumors the average proportion of PD1\(^+\)Eomes\(^+\) T cells that were Ki67\(^+\)GzmB\(^+\) only marginally increased after RT + anti-CTLA4; however, addition of anti-PD-L1 increased this to levels observed in parental tumors treated with only RT + anti-CTLA4. The frequency of CD8\(^-\)CD44\(^+\) TILs and the CD8/Treg ratio also increased (Figure 3c), and these were strongly correlated with the proportion of PD1\(^+\)Eomes\(^+\) CD8 effector T cells that were Ki67\(^+\)GzmB\(^+\) (Figure 8a). Importantly, addition of anti-PD-L1 improved responses of resistant Res 499 tumors after RT + anti-CTLA4 (Figure 8b-c). For treatment naïve tumors, responses were even more dramatic as the addition of either anti-PD-L1 or
anti-PD1 to RT + anti-CTLA4 markedly improved survival and increased CRs to 80% (Figure 3d, Figure 8d-f). On average, 58% of mice with CRs after adding anti-PD-L1 or anti-PD1 were alive 90+ days after tumor rechallenge, and similar improvements were observed with Res 237 breast cancer tumors after addition of PD-L1 blockade (Figure 8g-i). Thus, elevated PD-L1 on tumor cells results in persistent T cell exhaustion that impairs the CD8/Treg ratio. Addition of PD-L1 blockade inhibits resistance and results in long-term immunity.

Notably, RT is needed to achieve high CR rates as dual checkpoint blockade proved inferior to dual checkpoint blockade plus RT (Figure 3d), a requirement additionally seen in a pancreatic cancer model (Figure 8j). The superiority of triple therapy in multiple cancer types suggests non-redundant mechanisms for each treatment. To examine this notion, I assessed treatment-related changes in TILs from unirradiated tumors. RF modeling of immune cell profiles confirmed that anti-CTLA4 predominantly caused a decrease in regulatory T cells, anti-PD-L1 strongly increased CD8 TIL frequency, and the blockade of both increased the CD8/Treg ratio (Figure 4a-b, Figure 12a). In contrast, RT caused only a modest increase in CD8 effector T cells; however, TCR sequencing revealed that this was accompanied by increased diversity of TCR clonotypes, which could be observed even in the presence of CTLA4 blockade (Figure 4c-d). Thus, within the tumor microenvironment, CTLA4 blockade primarily decreases regulatory T
cells, PD-L1 blockade predominantly reinvigorates exhausted CD8 effector T cells, and RT diversifies the TCR repertoire of TILs from unirradiated tumors.

When I extended these initial findings in the pancreatic cancer model to include both an index and unirradiated tumor, I found that unirradiated pancreatic tumors were resistant to RT + dual checkpoint blockade (Figure 9a-b). I hypothesized that this failure may be explained by a relative lack of antitumor immunity and corresponding immune suppressive microenvironment at baseline in the KrasLSL-G12D/+,LSL-p53LSL-R172H/+,Pdx1-Cre (KPC) pancreatic model, (Bayne et al. 2012, Beatty et al. 2011, Clark et al. 2009, Hingorani et al. 2005) mirroring human disease (Clark et al. 2009). Indeed, despite high intratumoral expression of the PD1–PD-L1 axis, PDA tumors are resistant to dual checkpoint blockade alone (Winograd et al. 2015). To evaluate this hypothesis, I induced T cell immunity with anti-CD40, which facilitates vaccination against tumor cells and can potentially synergize with agents that induce immunogenic cell death such as RT (Winograd et al. 2015). Addition of anti-CD40 to RT + dual checkpoint blockade resulted in sustained complete responses in 50% of mice (Figure 9c), restoring the two tumor KPC pancreatic model response rate to that observed in B16-F10 without anti-CD40. However, addition of anti-CD40 did not further improve responses in B16-F10 when mice were challenged with ten times the standard number of tumor cells (Figure 9d), suggesting that the KPC immune microenvironment creates a distinct additional requirement for immune activation.
Further investigation revealed that the early antitumor immune response to RT + dual checkpoint blockade + anti-CD40, but not overall survival, was independent of CD4 and CD8 T cells (Figure 10), consistent with the hypothesis that CD40 exerts an antitumor effect through the activation of innate lymphocytes (Beatty et al. 2011). I found that changes in both adaptive and innate immune populations during the peak immune response scored as top variables in a highly accurate random forest model predicting treatment group, for both RT and RT + dual checkpoint blockade + CD40 (Figure 11).

To investigate if treatment effects on TILs were propagated to the peripheral T cell pool, I examined spleen and blood in the B16-F10 model. As observed in TILs, RT + anti-CTLA4 reinvigorated exhausted PD1'Eomes' splenic CD8 T cells, and this reinvigoration was further enhanced by addition of anti-PD-L1 (Figure 4e-f). Reinvigoration after addition of anti-PD-L1 was also accompanied by a large expansion of a small subset of the top 100 most frequent TCR clonotypes found in TILs (Figure 4g). Remarkably, some clones reached a frequency in the post-treatment blood of over 20% after RT and dual checkpoint blockade (Figure 4h). With anti-CTLA4 +/- RT, peripheral T cell clonal expansion was modest, which parallels the low CR rates following this treatment. RT alone was insufficient to drive peripheral T cell expansion, despite increasing TCR repertoire diversity of TILs, but did promote qualitative alterations in the TCR repertoire of the most expanded clonotypes. Unsupervised analysis using the average CDR3 amino acid features (Atchley et al. 2005, Thomas et al. 2014) demonstrated that the TCRs of the
most frequent clonotypes in the post-treatment blood formed two readily apparent clusters based on RT treatment (Figure 4i). In contrast, the most frequent clonotypes from pre-treatment blood and randomly sampled clonotypes from post-treatment blood did not separate into clusters, consistent with differences in CDR3 amino acid properties being an effect of RT only observed in the most expanded clones (Figure 12b-c). The separation into two clusters was driven by differences in the CDR3 occupancy profile of short amino acid sequences belonging to distinct subsets differing in size, polarity, and electrostatic charge (Figure 12d-e).

To determine if treatment and resistance-related changes in peripheral T cells can constitute a biomarker for tumor response, I modeled the effects of reinvigoration, exhaustion, and the CD8/Treg ratio. Specifically, I used 1) the percent PD1+ splenic CD8 T cells that are Eomes+ to integrate the burden that exhausted T cells might exert, 2) the percent PD1+ CD8 T cells that are Ki67+GzmB+ as a measure of potential reinvigoration, and 3) the CD8/Treg ratio as a barometer for the suppressive potential of regulatory T cells. The overall prediction accuracy of the model was 84%, and variables for T cell reinvigoration and exhaustion were the most predictive, followed by the CD8/Treg ratio (Figure 13a-b). Moreover, the percentage of PD1+ CD8 T cells that were Eomes+ was a striking modifier of the likelihood of CR as nearly all observed CRs occurred when the percent Ki67+GzmB+ in PD1+ CD8 T cells was high but the relative size of the PD1+Eomes+ exhausted population was not (Figure 5a). Similar relationships existed with
the CD8/Treg ratio, and prediction using T cells from peripheral blood yielded highly similar results (Figure 13c-e).

In order to assess whether immune predictors discovered in mice could be shared with patients, I examined peripheral T cells and tumor biopsies from patients on the clinical trial of RT + anti-CTLA4. For all 10 patients with available pre- and post-treatment blood, two had PRs in unirradiated tumors and PFS significantly longer than the median. For both of these patients, the percentages of Ki67⁺GzmB⁺ increased in PD1⁺Eomes⁺ CD8 T cells after treatment while the proportion of PD1⁺Eomes⁺ T cells remained at or below the mean (Figure 5b). In contrast, patients with a high percentage of PD1⁺Eomes⁺ T cells post-treatment did not have PRs and had a short PFS, regardless of reinvigoration. Comparison of patient PT-402, who had extended PFS/PR (Figure 2a-b), with patient PT-102, who had short PFS/PD, demonstrates how reinvigoration is associated with response to RT + anti-CTLA4 as it is in mice (Figure 5c vs. Figure 13f-g and Figure 4e-f). Examination of pre-treatment tumor biopsies from patients PT-402 and PT-102 (Figure 5d), and from all patients with available biopsy (Table 5), revealed that PD-L1lo intensity on melanoma cells (Figure 14a) was associated with reinvigoration of PD1⁺Eomes⁺ and of PD1⁺ CD8 T cells after RT + anti-CTLA4, while PD-L1hi status was associated with persistent exhaustion (Figure 5e, Figure 14b). None of the patients with PD-L1hi on melanoma cells had a CR/PR, and all rapidly progressed and died (Figure 5f-g). PD-L1 status on macrophages was neither associated with reinvigoration nor
independently predictive of PFS (Figure 14c-d). Thus, collective results from patients and mice suggest that elevated PD-L1 on melanoma cells inhibits T cell function and tumor response to RT + anti-CTLA4.

**Discussion**

In collaboration with Dr. Twyman Saint Victor and others, I investigated RT + anti-CTLA4 in mice and patients to understand mechanisms of both response and resistance (Figure 14e). Anti-CTLA4 predominantly inhibits regulatory T cells, increasing the CD8/Treg ratio as previously described, (Curran et al. 2010) and results in modest peripheral expansion of TCR clonotypes in the tumor, also consistent with other reports (Cha et al. 2014, Robert et al. 2014). RT diversifies the TCR repertoire of TILs and shapes the repertoire of expanded clones. My observations suggest that the favorable immune changes in TILs after immune checkpoint blockade promote their peripheral clonal expansion. When combined with increased TCR repertoire diversity afforded by RT, selection and oligo-clonal peripheral expansion of clones with distinct TCR traits are favored. Although the cause and consequence of these repertoire changes remain to be defined, RT can alter peptide presentation (Reits et al. 2006), and CDR3 changes after *M. tuberculosis* infection have been hypothesized to be antigen-driven (Thomas et al. 2014). Resistance to RT + anti-CTLA4 can ensue due to elevated PD-L1 on cancer cells driving T cell exhaustion, a process that can be antagonized by PD-L1 blockade. I additionally
demonstrated that immune parameters from peripheral T cells that relate the size of the exhausted T cell population, reinvigoration, and the CD8/Treg ratio can predict response to RT combined with immune checkpoint blockade. However, severely exhausted T cells may regain only limited function after reinvigoration (Paley et al. 2012, Wherry 2011), explaining why the correlation between reinvigoration and response declines when the exhausted T cell pool is large. Although tumors with genetic elimination of PD-L1 in melanoma cells can still relapse, suggesting resistance through other pathways and/or PD-L1 on non-tumor cells, the upregulation of PD-L1 by cancer cells is a dominant resistance mechanism. Furthermore, data from the murine KPC model suggest that combinatorial therapy with anti-CD40 sensitizes PDA tumors to RT + dual checkpoint blockade. Thus, RT + dual checkpoint blockade, in combination with additional immune priming, can elicit curative, systemic antitumor T cell responses even in refractory tumor types. Lastly, the shared findings between mice and patients predict that addition of PD-L1/PD1 blockade to RT + anti-CTLA4 may show significant efficacy in clinical trials.
# Tables

Table 2. Demographics and baseline characteristics for patients on phase I clinical trial of radiation 1 anti-CTLA4 for metastatic melanoma.

<table>
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Table 3. Grade 3 and 4 toxicities from phase I clinical trial of radiation 1 anti-CTLA4 for metastatic melanoma.

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<th>6Gy x 2 n=6</th>
<th>8Gy x 2 n=6</th>
<th>6Gy x 3 n=6</th>
<th>8Gy x 3 n=4</th>
<th>Total</th>
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<td>4</td>
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<td>4</td>
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</tbody>
</table>

* No Grade 4 toxicities were observed

Two dose levels in two strata were tested. Stratum 1 (lung/bone) used 8 Gy 3 2 or 8 Gy 3 3. Stratum 2 (liver/subcutaneous) used 6 Gy 3 2 or 6 Gy 3 3. Six patients for each dose level were planned. All dose levels met accrual except 8 Gy 3 3 before trial closure.
Table 4. Stratum, irradiated sites, and response for patients on clinical trial.

| ID | Strat | Irradiated Site         | RECIST | PERCIST | POD New | POD Clin | Local CT | Local PET | Dist CT | Dist PET |
|----|-------|-------------------------|--------|---------|---------|----------|----------|-----------|----------|----------|----------|
| 1  | Lung  | Rt lower lung           | 15%    | NA      | no      | no       | PD       | CMR      | SD       | PMD      |
| 2  | Sub-Q | Lt upper abdomen        | no     | yes     | no      | no       | PR       | PMR      | PR       | PMR      |
| 3  | Lung  | Lung                    | 13%    | 6%      | yes     | no       | PR       | CMR      | PD       | PMD      |
| 4  | Sub-Q | Rt gluteal region       | -68%   | -76%    | no      | no       | PD       | PD       | PD       | PD       |
| 5  | Sub-Q | Lt axilla               | 30%    | 93%     | yes     | no       | SD       | PMR      | PD       | PMD      |
| 6  | Lung  | Rt middle lung          | 96%    | 96%     | yes     | no       | PD       | PD       | PD       | PD       |
| 7  | Lung  | Lt middle lung          | 96%    | 96%     | yes     | no       | PD       | PD       | PD       | PD       |
| 8  | Lung  | Rt middle lung          | 5%     | -14%    | yes     | no       | SD       | CMR      | PD       | PMD      |
| 9  | Lung  | Rt middle lung          | -20%   | 0%      | yes     | no       | PD       | PMR      | PD       | PMD      |
| 10 | Liver | Rt lateral liver        | -50%   | -100%   | no      | no       | SD       | CMR      | PR       | CMR      |
| 11 | Liver | Posterior liver         | 77%    | 42%     | yes     | no       | SD       | SMD      | PD       | PMD      |
| 12 | Sub-Q | Lt posterior abdomen    | 49%    | yes     | no      | no       | PR       | PD       |          |          |
| 13 | Lung  | Rt posterior lung       | no     | yes     |         |          |          |          |          |          |
| 14 | Sub-Q | Lymph node              | 69%    | NA      | yes     | no       | SD       | SMD      | PD       | PMD      |
| 15 | Sub-Q | Lt axilla               | -59%   | -100%   | no      | no       | SD       | SMD      | PR       | CMR      |
| 16 | Lung  | Lt lung hilum           | -49%   |         | no      | no       | PR       |          |          |          |
| 17 | Sub-Q | Lt axilla               | 38%    | 29%     | yes     | no       | SD       | PMR      | PD       | PMD      |
| 18 | Sub-Q | Rt inguinal region      | -21%   | -25%    | no      | no       | SD       | PMR      | SD       | PMR      |
| 19 | Liver | Middle liver            | 71%    |         | yes     | no       | SD       |          |          |          |
| 20 | Lung  | Rt middle lung          | -19%   |         | no      | no       | PR       |          |          |          |
| 21 | Sub-Q | Lt SCV node             | 153%   |         | yes     | no       | PR       |          |          |          |
| 22 | Lung  | Lt lower lobe           | -7%    |         | no      | no       | PR       |          |          |          |

Response of the local irradiated site (Local) and distant unirradiated sites (Dist) were determined by CT and PET/CT. Percent change from baseline for distant lesions measured by CT are indicated using RECIST and change measured by PET/CT are indicated by PERCIST. The irradiated Tumor was not included in RECIST measurements per RECIST guidelines due to radiation-related effects precluding accurate CT measurements (for example, patient ID 1 and 9). NA indicates the value was not measurable based on criteria. Progression of disease due to new lesion(s) before re-imaging (POD New) or due to clinical progression (POD Clin) is also indicated. PD, progression of disease; PR, partial response; SD, stable disease; CMR, complete metabolic
response; PMR, partial metabolic response; PMD, progressive metabolic disease. Patient ID 3 is patient PT-102, and patient ID 4 is patient PT-402.
Table 5. Melanoma biopsy sites and PD-L1(hi) status of melanoma cells from patients on clinical trial.

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<tbody>
<tr>
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<tr>
<td>3</td>
<td>Skin</td>
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<td>4</td>
<td>Bowel</td>
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<tr>
<td>19</td>
<td>Bowel</td>
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</table>

Recent biopsy was optional for enrolment on the clinical trial. Tumor tissue from all patients with recent biopsy was used. PD-L1 status was determined by examination of membrane staining on melanoma cells. PD-L1(hi) was classified as 21 on at least 1% of cells. Patient ID 3 is patient PT-102, and patient ID 4 is patient PT-402.
Figure 2. RT + anti-CTLA4 promotes regression of irradiated and unirradiated tumors and is inhibited by PD-L1 on tumor cells
a) Waterfall plot of unirradiated tumors after RT to a single index lesion with anti-CTLA4. Dashed lines are thresholds for PD (red) and PR (blue). * Patients with new lesions. ** Clinical progression without imaging. b) PET/CT images of irradiated (white arrows) and unirradiated (yellow arrows) tumors from patient PT-402. c) PFS and OS for all patients (dashed lines: 95% CI). d) B16-F10 tumor growth after RT to the index tumor (n=8), anti-CTLA4 (C4) (n=9), anti-CTLA4 and RT to the index tumor (n=18), or no (control) treatment (n=9). The p-values are comparisons with control. Pie chart shows %CRs (yellow). See Figure 3d for survival. e) Heat map showing relative abundance of immune cells or their ratios from tumors that are resistant (black hatch) or sensitive to RT + anti-CTLA4. Boxplot shows bootstrap importance scores for each variable. Higher values (red) are more predictive. f) Change in T cell subsets or their ratio after RT + anti-CTLA4 for sensitive parental (Sen) or resistant (Res) tumors. Values are subtracted from average of untreated controls. Red line is mean. g) Heat map of resistance gene signature and PD-L1 across human melanoma. p < 0.001 by gene set enrichment analysis. h) Expression of PD-L1 on Res 499 compared to B16-F10 melanoma cells and of Res 237 compared to TSA breast cancer cells. Isotype control (IgG). i) Total tumor volume from PD-L1 knockout (KO) or control (WT) Res 499 and corresponding survival.
Figure 3. Addition of PD-L1 blockade reinvigorates exhausted T cells and improves response to RT + anti-CTLA4.

a) Representative contour plot of CD8 effector T cells from B16-F10 or Res 499 tumors after RT and anti-CTLA4 (C4) +/- anti-PD-L1 (P1) examined for PD1 and Eomes.
(top row), followed by examination of the PD1\textsuperscript{+}Eomes\textsuperscript{+} subset for Ki67 and GzmB (bottom row). Schema shows exhaustion and reinvigoration markers. **b)** Proportion of PD1\textsuperscript{+}Eomes\textsuperscript{+} CD8 T cells that are either Ki67\textsuperscript{+}GzmB\textsuperscript{+} or Ki67\textsuperscript{+}GzmB\textsuperscript{+}. **c)** Changes in T cell subsets and their ratio from Res 499 tumors. **d)** Survival of mice with B16-F10 tumors (n=18 for RT+C4, n=5 for others). Shown are overall p-values.
Figure 4. RT, anti-CTLA4, and anti-PD-L1 have distinct effects on the TCR repertoire, regulatory T cells, and T cell exhaustion.

a) Heat map of changes in the frequency of immune cells or their ratios from B16-F10 tumors. Black hatches indicate treatment. Bar plots show bootstrap importance scores (mean +/- SE) that assess changes in immune parameters predicted by treatment type (read row-wise). Higher values (yellow) represent stronger association. b) T cell subsets and their ratios. c) Frequency distribution (dashed line is 0.5%) and d) boxplot of diversity index (0: clonal, 1: fully diverse) for most frequent TCR clonotypes found in TILs of unirradiated B16-F10 tumors after RT and/or anti-CTLA4. Boxplot summarizes data for mice treated with anti-CTLA4 (NoRT) or RT +/- anti-CTLA4 (+RT). e) Representative contour plots and f) ratios examining PD1+Eomes+ splenic CD8 T cells from mice with B16-F10 tumors for Ki67+GzmB+ (reinvigorated) or Ki67 GzmB− (exhausted) subsets. g) TCR clonal frequency in post-treatment blood vs. TILs (top row) or vs. pre-treatment blood (bottom row). Quadrant boundaries are top 5% quantiles from the control. Clones below detection in pre-treatment blood are assigned upper bounds (blue). h) Maximum clonal frequency in post-treatment blood (dot) of the most frequent TCR clonotypes found in TILs. i) Distances to cluster centroids for the average CDR3 amino acid features of the five most frequent clones in pre- or post-treatment blood from mice treated with (red) or without (orange) RT. Membership into two clusters (circles and squares) determined by k-means.
Figure 5. Tumor PD-L1 and T cell exhaustion and reinvigoration can predict response in mice and patients.
a) Percent PD1⁺ CD8 T cells that are Eomes⁺ vs. Ki67⁺GzmB⁺ after RT combined with checkpoint blockade. Values are subtracted from average of untreated controls. Each circle represents a mouse. Probability of CR (proportional to circle size), prediction error rate, and quadrant boundaries are estimated from an RF model. b) Percent Eomes⁺PD1⁺ CD8 T cells in post-treatment blood vs. change in % PD1⁺Eomes⁺ CD8 T cells that are Ki67⁺GzmB⁺ after treatment. Each circle represents a patient. PFS is proportional to circle size and quadrant boundaries are average values for patients under the mean PFS. Concordance index of the RF model is 0.59. c) Contour plot of peripheral blood CD8 T cells from patients PT-102 and PT-402 examined for PD1 and Eomes (top row), followed by examination of the PD1⁺Eomes⁺ subset for Ki67 and GzmB (bottom row). d) PD-L1 staining from corresponding tumor biopsies. e) Change in % Ki67⁺GzmB⁺ in PD1⁺Eomes⁺ CD8 T cells vs. PD-L1 status of melanoma cells from all patients with available pre- and post-treatment blood. f) RECIST response, g) PFS, and OS stratified by PD-L1 status of melanoma cells.
Figure 6. Patients and mice treated with RT + anti-CTLA4 for melanoma.

a) Twenty-two stage IV melanoma patients (M stage indicated) were stratified by treatment site of a single index metastasis, which was the irradiated tumor. Two dosing levels (DL) for stereotactic body radiation (SBRT) were in each stratum. b) Waterfall plot of the RECIST % change from baseline of unirradiated tumors annotated to indicate metabolic responses by PET/CT (hatches above plot) and response of the irradiated index tumor as measured by CT and PET/CT (hatches below plot). RECIST criteria do not include irradiated tumors. Legend shows color-codes for response after CT or PET/CT.
(parenthesis). PMD: progressive metabolic disease; SMD: stable metabolic disease; PMR: partial metabolic response; CMR: complete metabolic response. White hatches indicate no imaging obtained. See Table 3. Grade 3 and 4 toxicities from phase I clinical trial of radiation 1 anti-CTLA4 for metastatic melanoma.
c) Survival (right) and total tumor growth (bottom) after RT with either concurrent or sequential anti-CTLA4 compared to anti-CTLA4 (C4) or RT alone. The regimens and the standard regimen used for all other melanoma experiments are illustrated (left). The p-values for tumor growth are compared to anti-CTLA4.
d) Survival after RT and/or anti-CTLA4 with or without T cell depletion (n=5-10) using anti-CD8 (CD8). Shown are overall p-values. The p-value for RT + anti-CTLA4 with and without anti-CD8 is p=0.005. Control is an isotype-matched antibody.
e) Three mice with CRs were rechallenged with B16-F10 tumors. Shown is a representative mouse. Arrow indicates location of regressed tumor and vitiligo-like condition represented by non-pigmented fur (observed in approximately 50% of mice with CRs). Time line starts from original tumor implantation (day 0) and values above marks are days after first rechallenge. Recurrence occurred only after anti-CD8 treatment and second rechallenge.
Figure 7. Tumor cells resistant to RT + anti-CTLA4 upregulate PD-L1 but not other candidate inhibitory receptor pathways.

a) Unirradiated tumor growth (left: normalized, right: raw values) for mice implanted with Res 177 (n=21), Res 499 (n=25), and B16-F10 (n=18) melanoma cells and treated with RT + anti-CTLA4. For normalization, volumes were divided by average of untreated controls (V/V_{cont}) to account for differences in growth between untreated
tumor types. The p-values are for comparisons with B16-F10 tumors. b) Corresponding tumor volumes of unirradiated or irradiated index tumors at day 21 (blue line is mean). c) Clonogenic survival for Res 499 and B16-F10 cells (n=2). d) Selection of immune variables that robustly predict resistance to RT + anti-CTLA4 using minimal depth (MD). A variable was selected if its MD was less than a threshold value for significance. Shown are bootstrap distributions of MD values (left) and % bootstrap models for which the MD for the indicated variable was significant (right). Bootstrap mean +/- SD for the out-of-bag prediction error rate is listed on top. e) Volcano plot of differentially expressed genes from resistant tumors. Horizontal black line is 5% false-discovery rate and dotted green line is fold-change cut-off. Ligands for select inhibitory receptors are indicated. See SI Table 1. f) Unirradiated tumor volumes (day 26-29) and g) survival after RT + anti-CTLA4 for mice with bilateral tumors from TSA breast cancer cells (n=25) or from the Res 237 subline selected to be resistant (n=21). h) Expression of candidate T cell inhibitory receptor ligands on B16-F10 and Res 499. Interferon-gamma (Interferon-γ) responsiveness was tested. i) Boxplots show distribution of % positive CD8⁺CD44⁺ T cells for the indicated inhibitory receptor compared to IgG control. j) PD-L1 surface expression for CRISPR PD-L1 homozygous knockout Res 499 and wild type control cells. interferon-γ was used to induce PD-L1 and confirm abrogated response.
Figure 8. Addition of PD-L1/PD1 blockade antagonizes resistance to RT + anti-CTLA4, and optimal response to checkpoint blockade requires RT.

a) Change in % CD8+CD44+ T cells after RT and checkpoint blockade vs. change in the degree of reinvigoration of exhausted T cells measured by % PD1+Eomes+ T cells that are Ki67+GzmB+. Values are subtracted from average of untreated control. b) Growth of Res 499 tumors after RT + anti-CTLA4 (C4) with and without addition of anti-PD-L1
(PDL1). Shown are index and unirradiated tumors from n=25 mice in each group. The p-value is for comparison to RT + anti-CTLA4. c) Proportion of CRs (yellow) for mice with Res 499 tumors. d) Total tumor growth (index + unirradiated) for B16-F10 tumors after the indicated treatment that includes anti-PD1 (PD1) or anti-PD-L1. The p-values are for comparisons to RT + anti-CTLA4 (n=18, n=5 for others). Pie charts show % CRs (yellow). e) Survival of mice after RT + anti-CTLA4 + anti-PD1. Shown is the overall p-value, and f) the two-way comparisons that include those from Figure 3d. g) Proportion of mice with CRs (yellow) after RT + anti-PD-L1 or anti-PD1 that survived 90+ days after tumor rechallenge at day 60 (n=12). h) Survival of mice with bilateral Res 237 breast cancer tumors treated with RT + anti-CTLA4 with (n=16) or without (n=21) anti-PD-L1. i) Proportion of CRs (yellow) for mice with Res 237 or TSA breast cancer tumors. j) Survival of mice with pancreatic tumors from a cell line derived from KPC mice (Kras<sup>LSL-<sub>G12D</sub></sup>/;p53<sup>LSL-R172H</sup>/;Pdx-1-Cre) (n=5 for each group). Select treatment groups are labeled on the plot for clarity. Overall p-value is shown.
Figure 9. Unirradiated pancreatic tumor resistance to RT + anti-CTLA4 + anti-PD1 is overcome by the addition of anti-CD40.

(a) Experimental schema. In contrast to Figure 8, a second, unirradiated tumor was implanted on the opposite flank, similar to Figure 2. b) Growth of PDA tumors from a cell line derived from KPC mice (Kras^{LSL-G12D/+};p53^{LSL-R172H/+};Pdx-1-Cre) after no treatment, RT, or RT + anti-CTLA4 + anti-PD1. Shown is unirradiated tumor growth from n=5 mice per group (overall p < 0.001). (c- d) Survival of mice after RT and combinatorial antibody therapy. P values are for RT + anti-CTLA + anti-PD1 + anti-CD40 vs. other groups (c) or as indicated (d) (n = 5-10 mice per group).
Figure 10. Early response to RT + anti-CTLA4 + anti-PD1 + anti-CD40 is T cell independent.

(a-b) Total tumor growth (index + unirradiated) for pancreatic tumors from a cell line derived from KPC mice (Kras<sup>LSL-G12D/</sup>;p53<sup>LSL-R172H/+</sup>;Pdx-1-Cre) after the indicated treatment for the index (a) or unirradiated (b) tumor. P value shown in (b) is for Tx vs. Tx + depleting antibody groups (black arrows): no treatment; 8depl: CD8-depleting antibody; 4depl: CD4-depleting antibody; Tx: RT + anti-CTLA4 + anti-PD1 + anti-CD40. (c) Survival of mice with pancreatic tumors from the experiment shown in (a-b). As indicated, all p values for treatment groups separated by the blank lines were less than 0.01.
Figure 11. Adaptive and innate immune composition distinguishes treated vs. untreated pancreatic tumors.

(a-b) Top: parallel coordinate plot showing proportion (y-axis, standard deviation from mean) of the indicated immune population (x-axis) across individual mice (lines). Red vs. blue color indicates treatment group as indicated in the legend. Bottom: importance (minimal depth) of the immune population to the randomforest model prediction of treatment group. Minimal depth is a statistic to measure predictiveness; accuracy of the overall models for (a) and (b) were greater than 90%.
Figure 12. TCR clonotypes associated with RT are not observed in random clones from post-treatment blood and have distinct CDR3 features.
a) Boxplot of the bootstrap variance explained by multivariable RF regression model for effect of RT, anti-CTLA4, and/or anti-PD-L1 on immune variables from TILs.

b) K-means clustering ($k=2$) was used on the average CDR3 amino acid features of randomly sampled clones from post-treatment blood after anti-CTLA4, anti-PD-L1, and/or RT. Membership into each cluster was determined and the p-value for separation into treatment groups with and without RT was calculated. Boxplot shows log10 p-values from 1000 random iterations. Comparison to the p-value from the observed data (red dotted line) gives a simulated p < 0.001.

c) Log10 p-values for separation into treatment groups with and without RT vs. cut-off value used to select the most frequent clones. The 0.05 significance level is indicated (red dotted line).

d) Average % occupancy in the CDR3 of the most frequent T cell clonotypes after RT +/- checkpoint blockade (+RT, red line) or checkpoint blockade alone (NoRT, orange line) by contiguous short amino acid sequences of length three (3-tuples) belonging to e) subsets with distinct treatment-related amino acid properties. These properties are characterized by Atchley factors, which measure 1) PAH: accessibility, polarity, and hydrophobicity, 2) PSS: propensity for secondary structure, 3) MS: molecular size, 4) CC: codon composition, and 5) EC: electrostatic charge. Shown (right) are the average values of each Atchley factor for amino acids that comprise the 3-tuples from the indicated subset (red) compared to all unselected 3-tuples (blue). Boxplots (left) show the proportion of 3-tuples from each of these subsets that are found in the CDR3s of the five most frequent clones after treatment. Compared to pre-treatment samples (Pre-tx), subset 6 is associated with RT +/-
checkpoint blockade (+RT) or checkpoint blockade alone (NoRT). Subset 1 is primarily associated with checkpoint blockade alone, and subset 16 is primarily associated with RT +/- checkpoint blockade.
Figure 13. Peripheral T cell exhaustion, reinvigoration, CD8/Treg ratio, and tumor PD-L1 predict response to RT + immune checkpoint blockade.

a) Heat map showing the relative proportions of PD1+ CD8 T cells that are Ki67‘GzmB+ or Eomes+ and the CD8/Treg ratio for each sample (columns) subtracted from the average values of untreated controls. Black hatches indicated CR and treatment with RT + anti-CTLA4 (C4) +/- anti-PD-L1 (P1). From these data, a multivariable RF predictor for CR was developed. Boxplot shows bootstrap distributions of variable importance scores (more predictive variables have higher values), and of b) minimal
depth (MD), a statistic to measure predictiveness. Bar plot shows % bootstrap models for which the MD for the indicated variable was significant. Bootstrap mean +/- SD for the out-of-bag prediction error rate is listed on top. c) Probability of CR vs. change (treated vs. untreated control) in CD8/Treg ratio for mice with a high (blue dots) or low (red dots) change in % PD1+ splenic CD8 T cells that are Eomes+. d) Heat map similar to (a) except using T cells from peripheral blood. e) Percent peripheral blood PD1+ CD8 T cells that are Eomes+ vs. Ki67+GzmB+ after RT + checkpoint blockade. Values are subtracted from average of untreated controls. Each circle represents a mouse. Probability of CR (proportional to circle size), prediction error rate, and quadrant boundaries are estimated from the RF model. f) Representative contour plots examining splenic CD8 T cells from B16-F10 or Res 499 tumors for PD1 and Eomes (top), followed by examination of the PD1+Eomes+ subset for Ki67 and GzmB (bottom). g) Ratios of PD1+Eomes+ splenic CD8 T cells that are Ki67+GzmB+ (reinvigorated) compared to Ki67 GzmB- (exhausted) from mice with Res 499 tumors.
Figure 14. Melanoma PD-L1 is associated with T cell exhaustion, response, and survival for patients treated on clinical trial of RT + anti-CTLA4.

a) Representative images (right) for patients with biopsies showing PD-L1 staining on tumor cells classified as PD-L1<sup>lo</sup> (top), 2+ (middle), or 3+ (bottom). Scores of 2+ and 3+ are classified as PD-L1<sup>hi</sup>. The arrow indicates PD-L1 staining on macrophages. An isotype antibody negative control and positive controls are shown (left).

b) Changes
in % Ki67^ GzmB^ in PD1^ CD8 T cells after RT + anti-CTLA4 vs. PD-L1 status on melanoma cells from all patients with available pre- and post-treatment blood. c) Changes in % Ki67^ GzmB^ in PD1^ Eomes^ CD8 T cells (left) or in PD1^ CD8 T cells (right) vs. macrophage PD-L1 status. d) Hazard ratio and 95% CI for PFS from a Cox regression model using PD-L1 status on tumor cells and macrophages. e) Model for non-redundant mechanisms and resistance to RT and immune checkpoint blockade.
CHAPTER 4 – Distinct immune cytolytic activity and neoepitope load in human pancreatic cancer

Much of Chapter 4 has been published, see Manuscripts (page xi).

Introduction

Pancreatic adenocarcinoma (PDA) is the third most common cause of death from cancer – with an overall 5-year survival rate of less than 5% – and is predicted to become the second leading cause of cancer mortality in the United States by 2030 (Hidalgo 2010, Rahib et al. 2014). The American Cancer Society predicts that for the first time, more patients will die annually of pancreatic cancer than breast cancer beginning in 2016 in the United States (Siegel et al. 2016). Recently approved combination chemotherapies for metastatic PDA modestly impact patient outcomes and durable remissions are rare (Conroy et al. 2011, Hoff et al. 2013). Several recent studies have identified distinct genetic and transcriptional PDA tumor and stromal subtypes, which may present opportunities to identify individual patients likely to respond to targeted therapies (Bailey
et al. 2016b, Collisson et al. 2011, Moffitt et al. 2015, Waddell et al. 2015, Witkiewicz et al. 2015). Immune modulation is a particularly attractive approach to treatment because of its potential to generate durable clinical responses in the proper setting (Foley et al. 2015, Vonderheide & Bayne 2013). Although single-agent immunotherapies targeting the immune checkpoint pathways PD1/PD-L1 and CTLA4 have shown striking efficacy in multiple tumor types, such approaches have failed to show clinical benefit in the overwhelming majority of patients with PDA (Brahmer et al. 2012, Royal et al. 2010).

Immunologically, PDA is characterized by a highly suppressive tumor microenvironment and a dense desmoplastic stroma, (Vonderheide & Bayne 2013, Wörmann et al. 2013) and for most patients there is scant intratumoral infiltration of effector T cells (Bailey et al. 2016b, Beatty et al. 2013). A small fraction of human PDA tumors do exhibit an immunogenic profile, (Bailey et al. 2016b, Fukunaga et al. 2004) and there is provocative evidence that survival is improved in resectable PDA patients whose tumors have higher-than-average or unusual tumor T cell infiltration (Fukunaga et al. 2004, Hiraoka et al. 2015, Ino et al. 2013). At present, the determinants of immune activation in PDA are poorly understood, providing little therapeutic guidance.

Here, in collaboration with Drs. Balli and Stanger, I contextualize immune activity in PDA by expanding previous analyses to multiple MHC class I and II neoepitope types across 9928 human cancer samples belonging to 35 solid tumor types. Using a highly-optimized, open source discovery pipeline, I evaluated over 400 million peptides.
generated from over 1 million missense mutations. To characterize immune correlates, this analysis assessed multiple immune indices, immune- and cancer-related gene sets, and gene mutational status for associations with neoepitope load both in PDA and then across human cancer. I further assessed the overall characteristics of neoepitope load, type and quality in human cancer at the global level and employed random forest machine learning to understand complex relationships in these data and build predictive models at the sample and disease levels.
Results

Stratification of human PDA based on cytolytic T-cell activity

Using publicly available data from 134 primary tumor resection samples, I profiled the genomic and transcriptional landscape of human PDA in the context of the immune microenvironment. I focused on a validated gene expression signature of granzyme A (GZMA) and perforin-1 (PRF1) to assess intratumoral cytolytic T cell activity (cytolytic index; CYT) (Rooney et al. 2015). GZMA is a tryptase that induces caspase-independent programmed cell death and PRF1 is a pore-forming enzyme that mediates entry of granzymes into target cells, both produced by activated cytolytic CD8 T cells and upregulated following response to immunotherapy (Chowdhury & Lieberman 2008, Herbst et al. 2014, Johnson et al. 2003, Keefe et al. 2005). Although Rooney and colleagues pioneered the utility of this cytolytic index broadly across many human cancers in their initial report, pancreatic cancer was omitted (Rooney et al. 2015). Here, to assess cytolytic index in PDA, I obtained RNA sequencing data from The Cancer Genome Atlas (TCGA) for multiple tumor types including recently released data for PDA samples (Network et al. 2013). Consistent with previous findings, I found that cytolytic index was highest in kidney, lung and cervical cancers and lowest in glioblastoma, ovarian, and prostate adenocarcinoma (Figure 15A) (Rooney et al. 2015). The median cytolytic index of PDA samples was comparable to that of other cancer types, including lung squamous cell
carcinoma and stomach adenocarcinoma (Figure 15A) and cytolytic activity in PDA was significantly higher than activity in normal pancreas. (Figure 15A, inset) Interestingly, while PDA has a median cytolytic index similar to stomach adenocarcinoma (8.59±7.5 vs. 8.01±11.9 CYT index), the distribution of cytolytic index is significantly narrower in PDA (Kolmogorov-Smirnov test, Figure 23).

**Cytolytic activity differs across established PDA subtypes**

To classify the subpopulations of PDA tumors with high versus low cytolytic activity, I stratified the PDA dataset by defining tumors in the top 10th percentile by cytolytic index as cytolytic-high (CYT High) and tumors in the bottom 25th percentile as cytolytic-low (CYT Low) (Figure 15B). Cytolytic-high PDA tumors were enriched for gene sets associated with activated CD8+, PD1\textsuperscript{high} T cells, (Duraiswamy et al. 2011, Parish et al. 2009) confirming that expression of GZMA and PRF1 correlated with immune response and infiltration of CD8 cytolytic T cells (Figure 15C, Figure 24).

As these data suggest that stratification based on cytolytic T cell infiltration, as measured by the cytolytic index, may be associated with distinct PDA subtypes, I determined if cytolytic activity is associated with genomic and transcriptional metrics of PDA tumor biology. Recent studies from multiple groups have demonstrated the
extensive genetic and transcriptional diversity of PDA tumors (Bailey et al. 2016b, Collisson et al. 2011, Moffitt et al. 2015, Waddell et al. 2015, Witkiewicz et al. 2015). PDA can be stratified into at least three tumor subtypes based on gene expression profiling: (1) Classical/Pancreatic Progenitor, (2) Squamous/Quasi-mesenchymal/Basal-like, and (3) ADEX (aberrantly differentiated endocrine exocrine)/Exocrine-like (Bailey et al. 2016b, Collisson et al. 2011, Moffitt et al. 2015). Furthermore, these PDA tumors types can overlap with gene programs associated with distinct stroma populations: (1) activated stroma, (2) normal stroma, and (3) immune gene signatures (Bailey et al. 2016b, Collisson et al. 2011, Moffitt et al. 2015). I assessed enrichment of gene programs defining PDA subtypes using the TCGA PDA dataset (Figure 16A) and their association with cytolytic index (Figure 16B). Cytolytic-high tumors were statistically enriched for the immune gene programs (GP7 and GP8) from Bailey and colleagues and the normal stroma gene program from Moffitt and colleagues (Figure 16B) (Bailey et al. 2016b, Collisson et al. 2011, Moffitt et al. 2015). Immune gene programs GP7 and GP8 contain markers for macrophages and T cell co-inhibition (GP7) and CD8 T cells and B cells (GP8) (Bailey et al. 2016b), and the normal stroma gene signature contains markers of pancreatic stellate cells (Moffitt et al. 2015). Pancreatic stellate cells have been linked to an immune suppressive tumor microenvironment through PTX3 regulation of immune escape by blocking antigen presentation (Baruah et al. 2006). Expression of genes defining the normal stroma gene program were increased in cytolytic-high tumors, suggesting a relationship between stromal microenvironment and T cell infiltration (Figure 16C).
Cytolytic-low PDA tumors had statistical enrichment of gene programs associated with the Classical/Pancreatic Progenitor tumor subtypes (Figure 16B). Genes involved in pancreatic differentiation were increased in cytolytic-low tumors suggesting an inverse relationship between differentiation status and immune reactivity (Figure 16D). Moreover, using a previously defined “immunome” gene signature of 28 distinct immune-cell specific markers, (Bindea et al. 2013) I found that cytolytic-high tumors were associated with multiple other immune cell signatures (Figure 26A). These data suggest that stratification of patients with PDA based on transcriptional profiling can differentiate between tumors with strong cytolytic T cell response and tumors for which a privileged immune microenvironment precludes such responses (Vonderheide & Bayne 2013).

**Cytolytic activity correlates with distinct mutational events in PDA**

I next sought to determine if cytolytic activity correlated with distinct mutational profiles characterized for PDA (Alioto et al. 2015, Bindea et al. 2013, Waddell et al. 2015). Curated mutational data for PDA was obtained from TCGA and I identified significantly mutated genes occurring in cytolytic high and low PDA tumors. Cytolytic-high tumors had a statistically significant association with mutations in TGFbetaR1/TGFbetaR2 as well as HMGB3 (Figure 27A). Cytolytic-low tumors were associated with non-silent mutations in CDKN2A, ANKRD36, NCOA3, and HIST1H1B. Most mutations across the dataset
were associated with G>A and C>T transitions, and the frequency of specific substitutions did not differ between cytolytic high and low tumors (Figure 27B). Likewise, there was no association between cytolytic index and KRAS mutation type (Figure 27C).

PDA is characterized by increased genomic instability with extensive copy number alterations in both human patients and genetically engineered mouse models (Hingorani et al. 2005, Waddell et al. 2015, Witkiewicz et al. 2015). I next obtained the GISTIC2.0 analysis for PDA from TCGA and assessed copy number alterations between cytolytic subtypes. Cytolytic-low (but not high) tumors had recurrent copy number alterations at loci important in PDA, (Witkiewicz et al. 2015) including MYC, NOTCH2, and FGFR1 (Figure 27D, right). Consistent with increased copy number alteration and MYC amplification, cytolytic-low tumors had increased expression of gene signatures associated with increased genomic instability and MYC target genes (Figure 27) (Zeller et al. 2003). Recurrent deletions were observed in cytolytic-low tumors at loci containing CDKN2A/B and SMAD4. MYC amplification has been observed in mouse models of hepatocellular carcinoma and human melanoma tumors and associated with reduced T cell infiltration and cytolytic activity, suggesting that genomic events may modulate inflammatory response in PDA (Linsley et al. 2014). Cytolytic-high PDA tumors did not have recurrent copy number losses but rather had amplifications at 4q13.1 (TECRL), 9p13.3 (CA9, TPM2, C9orf100), and 18q11.2 (IMPACT, OSBPL1A) (Figure 27D, left).
Assessed globally, cytolytic-high tumors had significantly fewer somatic copy number alterations (SCNA events) than cytolytic-low tumors (Figure 27E-F).

Mutational analysis of tumor samples can be hampered due to tumor cellularity because this reduces the ability to confidently detect somatic mutation and copy number alterations. To ensure that the TCGA PAAD cohort has sufficient cellularity for mutational analysis, cellularity estimates were calculated using ABSOLUTE (Carter et al. 2012) and Sequenza (Favero et al. 2014). Tumor cellularity estimates did not correlate with either total mutation load or total copy number events in the TCGA PAAD cohort (Figure 30). Furthermore, TCGA PAAD cellularity estimates (59±16.9%) were comparable to the lung adenocarcinoma (LUAD, 52±11.8%) and stomach adenocarcinoma (STAD, 53±12.8%) tumor cohorts (Figure 29). Moreover, there was no difference in cellularity estimates between cytolytic subtypes, suggesting that observed differences in copy number and mutational load were not a result of variable tumor cellularity (Figure 30). Thus, distinct mutational and structural changes in the genome distinguish those PDAs with low vs. high cytolytic activity.

*High cytolytic activity in PDA does not correlate with increased neoepitope load*

To investigate the landscape of neoepitopes in human cancer, I developed a highly optimized pipeline for predicting neoepitopes that integrates TCGA DNA variants, gene
expression and raw DNA exome reads (Figure 34, see also Neoepitope analysis). Poor tumor sample purity can reduce the ability to confidently determine somatic variants from whole exome sequencing, therefore I estimated tumor DNA fraction for each sample analyzed using ABSOLUTE (Carter et al. 2012) and confirmed results using Sequenza (Waring 2006). I found wide variation across TCGA (0.79 ± 0.08) but no correlation between tumor DNA fraction and missense mutation burden (Figure 35). MHC class I and II alleles were determined using OptiType and HLAreporter, respectively. I validated these methods to have > 95% (MHC class I) and >90% (MHC class II) 4-digit accuracy compared to standard clinical typing, consistent with other reports, and sample HLA alleles paralleled frequencies reported for the general population. Using tools provided by the Immune Epitope Database and Analysis Resource (IEDB), I then analyzed all possible peptides generated from missense mutations in expressed genes for ability to bind MHC class I and II. High affinity binders were classified as predicted neoepitopes (median half-maximum inhibitory concentration of less than 50nM for MHC class I, percentile rank of less than 1 for MHC class II).

I then determined if cytolytic activity correlated with neoepitope load in PDA, as has been widely suggested for cancers in general (Rooney et al. 2015). Neoepitopes, derived from peptides encoded by somatic tumor mutations and thus not subject to central tolerance in the thymus, have been demonstrated to preferentially drive T cell recognition of tumor cells (McGranahan et al. 2016, Schumacher & Schreiber 2015). A
total of $1.1 \times 10^4$ unique variants leading to $5.1 \times 10^6$ potential peptides were evaluated for the PDA dataset. This analysis revealed no correlation between total mutations per individual tumor and cytolytic index, although when viewed as a group the cytolytic-low tumors exhibited a slight increase in the number of mutations per tumor compared to cytolytic-high tumors (Figure 18A). Neoepitope load did not correlate with cytolytic activity in PDA, with striking examples of cytolytic-low tumors with multiple predicted neoepitopes and cytolytic-high tumors with few neoepitopes (Figure 18B-E). Consistent with the findings from the overall mutation rate, the tumor cellularity estimates did not correlate with the number of MHC Class I or II neoepitopes in PDA (Figure 30). In contrast, as expected, (Rooney et al. 2015) both lung adenocarcinoma and stomach adenocarcinoma showed a strong correlation between total number of mutations, predicted MHC class I neoepitope load, and number of mutations generating one or more neoepitopes in cytolytic-high tumors (top 10th percentile versus bottom 25th percentile ranked by cytolytic activity) (Figure 31). Stratification of patients with PDA based on established gene signatures (Bailey et al. 2016b, Moffitt et al. 2015) also did not associate with increased neoepitope load in any PDA transcriptional subtype (Figure 32). Taken together, these data suggest that cytolytic activity in PDA, in contrast to other tumor types, is not driven by increased mutation or neoepitope load.
Cytokine and immune checkpoint gene expression patterns differ in PDA tumors with high vs. low cytolytic activity

The tumor microenvironment in PDA contains a rich cytokine milieu with both pro- and anti-inflammatory factors that can regulate tumorigenesis (Delitto et al. 2015). I therefore hypothesized that the expression of these cytokines and chemokines would be increased in cytolytic-high tumors across all TCGA tumor types. Consistent with this notion, I found that the expression of numerous pro- and anti-inflammatory cytokines and immune checkpoint molecules was significantly increased in cytolytic-high tumors across TCGA, including PDA (Figure 18F). Specifically, cytokines previously shown to correlate with cytolytic index – including \( \text{C1QA, C1QB, C1QC, CXCL10, and CXCL9} \) were differentially expressed in cytolytic-high TCGA tumors, including cytolytic-high PDA (Rooney et al. 2015). The expression of regulatory T cell markers were also significantly increased in cytolytic-high PDA tumors (Figure 18I).

I next assessed whether cytolytic-high PDA tumors exhibit increased expression of immune checkpoint pathways. An inhibitory checkpoint index was created to assess the expression of key checkpoint molecules across patients with PDA (Figure 18G). The inhibitory checkpoint index was generated by taking the log-average expression in TPM of the following molecules: \( \text{ADORA2A (A2AR), CD274 (PD-L1), PDCD1 (PD1), CTLA4, HAVCR2 (TIM3), IDO1, IDO2, PDCD1LG2 (PD-L2), TIGIT, VISTA (C10orf54), and VTCN1 (B7-H4)} \). Using this index, I found a strong correlation between cytolytic activity
and the expression of inhibitory checkpoint genes in patients with PDA, suggesting that as in melanoma, (Spranger et al. 2013) immune response in cytolytic-high tumors elicits multiple host and tumor mechanisms of immune suppression in the tumor microenvironment (Figure 18H). However, \textit{CD274 (PD-L1)} expression was uniformly low in PDA and was not differentially expressed between cytolytic subsets in PDA despite being increased in cytolytic-high tumors in the other TCGA datasets examined (Figure 18F arrow). Multiple other inhibitory checkpoint molecules were expressed at markedly higher levels in cytolytic-high tumors (Figure 4J). While \textit{PD-L1} expression was not changed between cytolytic subsets in PDA, the expression of other immune checkpoint molecules, including \textit{IDO1, IDO2, CTLA4}, and \textit{PD-L2}, were differentially expressed in cytolytic-high PDA tumors (Figure 18J). When compared individually, the expression levels of \textit{PDCD1, PDCD1LG2, CTLA4, IDO2, A2AR, TIGIT, and LAG3} have the highest correlation with the cytolytic index and, conversely, \textit{PD-L1} and \textit{VTCN1 (B7-H4)} have the lowest correlation (Figure 33). These data suggest that multiple immune checkpoint pathways, other than the \textit{PD1} axis, may mediate peripheral tolerance and immune escape in PDA; combinatorial targeting of these pathways may expand clinical benefit for patients with PDA.
Contextualizing PDA within the neoepitope landscape of human cancer

To contextualize these findings in PDA with the larger landscape of neoepitopes across human cancer, I expanded this analytical pipeline to all available TCGA solid disease types. I noted significant differences in rate of neoepitope generation (percent predicted neoepitopes among all candidate peptides) across HLA alleles for all samples analyzed (Figure 36). Common class I HLA alleles A*02:01, A*11:01, B*44:02, C*05:01, and C*12:03 had significantly higher rates of neoepitope generation (p adj. < 0.005); A*01:01, A*03:01, A*24:02, B*08:01, C*04:01, and C*06:02 had significantly lower rates (p adj. < 0.005). For class II HLA alleles, common alleles DPA1*01/DPB1*04:01, DQA1*05:01/DQB1*02:01, DRB1*03:01, and DRB3*01:01 had higher rates (p adj. < 0.005); no alleles had significantly lower rates (Figure 36). From a total of 9928 samples with genome-wide sequencing, paired normal and tumor somatic variant calling was available for 7776 samples. Whole exome sequencing required to infer HLA alleles was available for 7358 samples. Seven disease types contained less than 15 samples with these required data at the time of ingestion and were excluded from analysis: cholangiocarcinoma, kidney chromophobe, mesothelioma, ovarian serous cystadenocarcinoma, rectum adenocarcinoma, thymoma, uterine carcinosarcoma.

Combining variant calling for these samples resulted in a total of 3,658,044 somatic variants, of which 29.8% were missense mutations of interest, resulting in a total of 230,636,069 generated 8-15-mer peptides (Figure 19A). Integrating RNA expression data,
I found that 69.2% of these variants occurred in genes that were at least minimally expressed in tumor samples and therefore potentially presented on MHC. Overall, after evaluating binding affinity, I identified 486,341 (0.25%) predicted MHC class I and 638,107 (1.59%) predicted MHC class II neoepitopes from 7115 samples across 27 disease types (Table 9). This difference in percent neoepitopes is consistent with previous reports and the greater flexibility of peptide binding in the groove of MHC class II vs. MHC class I (Kreiter et al. 2015, Wang et al. 2008).

As expected, I observed a close correlation between number of missense mutations and neoepitopes. Samples with the most to least number of missense mutations were found almost uniformly to have the most or least predicted MHC class I neoepitopes, with greater variability in the case of class II (Figure 19B, R-squared of 0.852 and 0.452, respectively). When I stratified predicted neoepitopes by disease type, I found a wide distribution of neoepitope load (Figure 19C). The highest mean class I neoepitope load occurred in colon (291 ± 556), metastatic (321 ± 848) and primary (183 ± 222) melanoma and stomach (183 ± 296) disease types. Results for MHC class II neoepitopes were similar (colon: 1014 ± 3288, metastatic melanoma: 316 ± 513, primary melanoma: 314 ± 531, stomach: 361 ± 864). HLA alleles possess variable binding stringency for peptide, likely determined by differences in binding groove characteristics across alleles. Accordingly, I note small but significant differences in rate of neoepitope generation across disease types for MHC class I, MHC class II or the combined rate (Figure 19D).
Disease types with the greatest deviations from the mean pan-cancer neoepitope rate for MHC class I were UVM (higher, p adj. = 0.083, see Figure 19C for abbreviations used throughout), TNBC (higher, p adj. = 0.118), and CESC (lower, p adj. = 0.083). For MHC class II, significant outliers were THCA (higher), PCPG (higher), ACC (lower), and PCPG (lower) (p adj. < 0.01).

Recent reports suggest that genes commonly mutated in cancer may result in frequently reoccurring neoepitopes that could be targeted therapeutically. Targeting widely expressed neoepitopes derived from common driver mutations may be therapeutically advantageous even if these targets are only conserved across a small number of patients. To assess the feasibility of this, I determined the number of shared neoepitopes across all samples analyzed. I found a total of 1,872,298 peptides (< 1%) shared between at least two samples across all disease types, resulting in 2337 shared MHC class I neoepitopes and 4501 shared MHC class II neoepitopes (Figure 19E). While a sizable fraction of samples analyzed contained at least one such neoepitope (17% of samples for MHC class I, 70% of samples for MHC class II), only 11 neoepitopes were identified as shared between >5 samples across all disease types (Table 10). I found no significant enrichment for neoepitopes occurring in 373 genes identified to be significantly mutated in cancer, (Lawrence et al. 2013) and none of the 11 shared neoepitopes occurred in these genes.
**Immune suppression associations with cytolytic index**

Rooney and colleagues have recently developed an RNA-based metric to assess cytolytic T-cell activity (cytolytic index) based on expression of Granzyme A (GZMA) and Perforin-1 (PRF1). This index accurately correlates with active CD8 cytolytic T-cells and other metrics of adaptive antitumor T cell immunity. I compared enrichment of cytolytic and immune suppression indices across TCGA disease types (Figure 20A). Consistent with previous findings reported in this dissertation for PDA, cytolytic T-cell activity was highly correlated with expression of immune suppression pathways (Figure 20A, Figure 38). When I compared expression of individual genes comprising the immune suppression index across disease types, C10orf54 (VISTA), IDO1, ADORA2A (A2AR) and HAVCR2 had the highest expression across disease types compared to other molecules (Figure 20B, Figure 39). VISTA, IDO1 and other checkpoint genes may contribute to potential mechanisms of immune evasion (Restifo et al. 2016). TIGIT, PDCD1 (PD1), and CTLA4 are the genes within the immune suppression index with highest association with cytolytic index across TCGA. TIGIT contributes to an immune suppressive microenvironment by modulating dendritic cell cytokine production.

Consistent with previous findings, (Rooney et al. 2015) the top genes associated with cytolytic index are CXCL8, CXCL10, and CASP8 (Figure 20C). Across all disease types examined, immune suppression index and normalized expression of CD8A were both highly correlated with cytolytic index (R-squared = 0.65, p adj. = 4.11e-7 and R-squared = 0.65, p adj. = 4.11e-7).
0.43, p adj. = 9.33d-6, respectively), suggesting that immune signatures are defined at the disease level and not at the sample level. There were no significant disease type outliers for the ratio of immune suppression index to cytolytic index, indicating that cytolytic activity and suppression increase proportionally across disease types from ‘cold’ to ‘hot’. Significant disease type outliers for ratio of CD8A expression to immune suppression index were DLBC (higher), SKCMm (higher), PRAD (lower), and LGG (lower) (p adj. < 0.01).

**Immune environment predicts neoepitope load across human cancer**

I next assessed relationships between immune signatures and neoepitope load across disease types and patients. Overall, I found a significant correlation between mean neoepitope burden and mean CD8A expression or cytolytic index across disease types (Figure 21A-B). Significant disease type outliers for ratio of predicted MHC class I neoepitopes to CD8A expression were ACC, BLCA, and SKCMm, which are characterized by higher than average neoepitope load given mean CD8A expression compared to other disease types (p adj. < 0.01), and BRCA, CESC, DLBC, GBM, HNSC, KIRC, KIRP, LUAD, LUSC, PAAD, PCPG, PRAD, STAD, TGCT, THCA, TNBC, and UCS (p adj. < 0.01), characterized by lower load. For predicted MHC class II neoepitopes, disease types with higher than average ratio were ACC, BLCA, and LGG (p adj. < 0.01). Disease types with lower than average ratio were BRCA, CESC, DLBC, KIRP, LUAD,
LUSC, PAAD, PCPG, PRAD, TGCT, THCA, TNBC, and UCS (p adj. < 0.01). There were no significant outliers for ratios of cytolytic or immune suppression index to predicted MHC class I or MHC class II neoepitope load. Within each disease type I found significant correlations between neoepitope load and cytolytic index across samples in a minority of disease types; for MHC class I predictions, LGG, KIRC, SKCMm, and THCA, (Figure 40), as well as BRCA, TNBC, CESC, GBM, STAD, and THCA for MHC class II predictions (Figure 41). These data suggest that, in parallel to the relationship between cytolytic index and immune suppressive index, the relationship between cytolytic index and neoepitope load is best defined at the disease type level and is tightly coupled, as opposed to that between CD8A expression and neoepitope load.

Taken together, these analysis shows a high degree of variability in neoepitope load and immune signature within and across tumor types. To best understand if associations between these variables can be discerned, I stratified samples within each tumor type on the basis of neoepitope load into neoepitope-high (NH) and neoepitope-low (NL) cohorts (top and bottom decile within each disease type). I hypothesized that, while differences in immune environment on the basis of neoepitope load likely occur in a spectrum, these cohorts are most likely to represent distinct subclasses of disease for comparison. For instance, the size of the NH cohort roughly parallels that of tumor subtypes with evidently distinct immune environment, such as those responsive to checkpoint blockade. Similarly, many tumor types have a small, transcriptomically-
identified subtype consisting of ‘immunogenic’ tumors (Bailey et al. 2016b, Cancer Genome Atlas Network 2015, Cancer Genome Atlas Research Network 2014, Guinney et al. 2015, Kandoth et al. 2013, Lehmann et al. 2011). I then used random forest machine learning analysis to determine whether immune or cancer related gene sets could distinguish these cohorts and if so, what gene set members were responsible for this predictive ability. Random forest analysis is a multivariable non-parametric ensemble partitioning tree method applicable to classification problems and can be used to determine the effect of multiple input variables on a response variable of interest (Breiman 2001, Chen & Ishwaran 2012, Ishwaran et al. 2014, Twyman-Saint Victor et al. 2015). I use this methodology for two purposes: constructing a prediction rule for a response variable and then ranking input variables based on their contribution to predictiveness. Advantages of this approach over other methods are excellent discriminatory ability in high dimensional space, resistance to noise, missing data and overfitting, as well as built in error estimates (Boulesteix et al. 2012). I found both immune and cancer-related gene sets to predict classification of NH vs. NL for both class I and II neoeptopes. In agreement with findings reported in this dissertation that immune context is largely disease-specific, I noted low-to-modest pan-TCGA predictive ability for all gene sets examined, with T cell activity and T cell exhaustion gene sets performing best (Figure 21B-C, left bar plot). For predicted MHC class I and II neoeptopes, 17/25 and 14/27 disease types contained predictive models for the gene sets examined, respectively. T cell PD1-related, Reactome cytokine signaling, and T cell
exhaustion-related genes recently identified by (Schietinger et al. 2016) best distinguished NH vs. NL cohorts (Figure 21B-C, right heatmap). Figure 21D shows genes responsible for accurate prediction for selected models from Figure 21B. Genes were identified on the basis of minimal depth distance from the threshold for significance, a dimensionless statistic that measures variable predictiveness in tree-based models. Notably, immune suppression index genes were predictive of neoepitope load in both stomach adenocarcinoma and kidney renal clear cell carcinoma, but the gene set members important for this predictive ability were distinct, with IDO1 vs. PDCD1 ranked as the most predictive immune suppressive gene member, respectively.

**Superior survival of high predicted neoepitope groups**

Previous evidence suggests that increased load of immunogenic mutations is associated with significantly improved survival outcomes using samples from 512 patients across six disease types (Brown et al. 2014). This association existed when classifying neoepitopes to be any peptide with < 500nM binding affinity to MHC class I and also required samples to have high median HLA expression. No significant survival advantage was detected in high missense mutation burden patients or when fitting a model that contained an interaction between cancer type and immunogenic mutations. To extend this analysis, I stratified each disease type in TCGA into top or bottom deciles ranked by total missense mutations or neoepitopes and compared survival outcomes using available
TCGA clinical data. Age, gender and disease stage were not significantly imbalanced between cohorts and disease types with less than 15 samples or inadequately power were excluded from analysis.

For 7 of 15 disease types examined, survival was not different between samples ranked in the top or bottom decile for each metric of interest (Figure 22A). For bladder adenocarcinoma, lung squamous cell carcinoma, metastatic melanoma, and stomach adenocarcinoma, samples ranked in the top decile of neoepitope metrics had significantly improved clinical outcomes (Figure 22A-B). Interestingly, a subset of disease types had improved survival when ranked in the bottom decile for neoepitope metrics. Samples in the bottom decile in glioma, head and neck squamous cell carcinoma, liver hepatocellular carcinoma, and sarcoma were associated with significantly improved survival. These data suggest that in disease types with high overall mutation rate, neoepitope load is associated with clinical utility and suggests the potential efficacy of targeting neoepitopes for therapeutic gain.
Discussion

Sophisticated approaches continue to provide unprecedented resolution of the immunobiology of human cancer. In this study, I performed an extensive integrated analysis of the transcriptional and genetic landscape of PDA in the context of cytolytic immune activity in collaboration with Drs. Balli and Stanger. By stratifying patients with PDA based on a validated cytolytic gene expression signature (not previously applied to PDA), I identified a small subset of patients with evidence of prominent T cell reactivity. Beyond strong associations between cytolytic index and recently established transcriptional and genetic subtypes of this disease, these analyses revealed that low cytolytic activity tracked with increased genomic structural variation, most notably prominent and recurrent MYC amplifications and non-silent mutations and/or deletions in CDKN2A/B. Other distinct chromosomal aberrations were associated with cytolytic high PDA tumors. These data point to an underappreciated link between genomic alteration and immune activation in PDA, suggesting that genomic structural variation implicated in PDA progression may also fundamentally influence de novo or therapeutic antitumor immune activation, independently of host immune factors.

I also report the first characterization of neoepitope load in PDA, finding in a large sample size encompassing the entire TCGA dataset for PDA that high cytolytic activity failed to correlate with increased load of nonsynonymous mutations or predicted neoepitopes. This is in striking contrast to the correlation between cytolytic index and
mutational burden in other tumor types such as lung and stomach adenocarcinoma, (Rooney et al. 2015) in which PD1 antibodies trigger clinically significant tumor regression. Indeed, recent studies have highlighted the primacy of tumor neoepitopes in T cell recognition of tumor cells, (Schumacher & Schreiber 2015) renewing interest in patient-specific approaches such as personalized vaccines (Vonderheide & Nathanson 2013). Accordingly, the emerging paradigm is that neoepitope load determines sensitivity to immune checkpoint blockade (Rizvi et al. 2015, Snyder et al. 2014, Van Allen et al. 2015). The lack of an association between neoepitope load and cytolytic index in PDA may reflect a tumor immunobiology that is distinct from that present in checkpoint blockade-sensitive tumors. Consequently, the assumption that neoepitope load is invariably associated with greater adaptive immunity may need to be reassessed, especially as it applies to patient selection in future PDA clinical trials. This lack of association in PDA may be due to extreme Kras-driven immunosuppression resulting in neoepitope-specific T cells that are not triggered, fail to expand, fail to infiltrate the tumor, or all of the above (Pylayeva-Gupta et al. 2012, Vonderheide 2014). Alternatively, the inherently low mutation rate seen in PDA, compared to lung and stomach adenocarcinoma may contribute to the lack of association between cytolytic reactivity and neoepitope load. These insights may explain the lack of clinical response to single-agent anti-PD1 therapy in patients with PDA and also indicate a need to vaccinate against tumor antigens and generate robust antitumor T cells in order to sensitize patients to checkpoint blockade. However, if intrinsic tumor suppression can be overcome by these
combinatorial methods, potent neoepitope-directed responses may yet be possible in the majority of PDA patients whose tumors contain dozens or more of these potential high-affinity targets.

Our findings suggest that intrinsic oncogenic processes, rather than the availability of favorable immune targets, perhaps due to low mutation rate in PDA, may be the primary driver of immune activity in human PDA. MYC amplification is associated with decreased T cell infiltration in mouse models of hepatocellular carcinoma and human melanoma tumors with low cytolytic activity, suggesting that genomic alterations besides neoepitopes can modulate inflammatory response (Casey et al. 2016, Linsley et al. 2014). My comparison of the cytolytic index to established signatures of distinct PDA tumor and stroma types revealed a strong association with a gene signature representing a “normal stroma” phenotype (Moffitt et al. 2015), suggesting that the stromal microenvironment in PDA plays an important role in modulating inflammatory response. In PDA, oncogenic processes appear to enforce a state of immune privilege that precludes host T cell infiltration. Thus, immune checkpoint molecule upregulation in PDA may not be evidence of preceding T cell immunity, as is likely the case in melanoma and lung adenocarcinoma.

I found that tumors with high cytolytic activity exhibited increased expression of multiple immune checkpoint genes such as CTLA4, TIGIT, TIM3, and VISTA. In contrast, PD-L1 expression was uniformly low. Whether therapeutic vaccines can
generate sufficient cytokine-producing T cells that infiltrate PDA tumors and upregulate
PD-L1 remains to be seen in clinical trials; regardless, my data provide a rationale for
prioritizing immune checkpoints other than only PD-L1/PD1 as therapeutic targets.
While redundant suppressive mechanisms may undermine efforts to target any single
immune checkpoint pathway, it may be possible to use expression profiling on a patient-
specific basis to accomplish “immune precision medicine.” In addition, the lack of
immune activation in response to high neoepitope load additionally suggests that
immune checkpoint blockade may be insufficient in a relatively low-mutation rate tumor
type like PDA, and could be more effectively paired with immune-activating strategies
that have shown promise in murine models (Soares et al. 2015, Vonderheide et al. 2013).
In summary, my findings suggest that it will be important to look beyond standard
neoepitope-based strategies for immunotherapy in PDA and to focus instead on other
tumor-intrinsic features that render these tumors immune privileged. The extent to
which this immunobiology characteristic of human PDA also manifests in other types of
carcinoma requires further investigation.

To determine how PDA fits in the larger landscape of neoepitopes across human
cancer, I evaluated the high dimensional TCGA dataset, exploring relationships across 35
diseases types and evaluating over 400 million peptides as potential neoepitopes. My
analyses demonstrate that neoepitopes are abundant and tightly correlated with missense
mutation load. Consistent with a previous report finding few shared neoepitopes in
patients with NSCLC, (Karasaki et al. 2015) I identify only a handful of neoepitopes shared between more than 5 samples out of more than 7000 samples analyzed, none of which occurred in genes significantly mutated in cancer (Lawrence et al. 2013). I find that neoepitope generation in human cancer is, as expected, a probabilistic process and therefore largely restricted to random passenger mutations expressed by tumors.
Tables

Table 6. Patient information for TCGA PAAD dataset.
   See electronic supplementary materials.

Table 7. GISTIC2.0 data for cytolytic TCGA PAAD dataset.
   See electronic supplementary materials.

Table 8. MuSiC Genome significantly mutated gene (SMG) information for TCGA PAAD dataset.
   See electronic supplementary materials.

Table 9. Pan-TCGA sample information, neoepitope load, and cytolytic index.
   See electronic supplementary materials.

Table 10. Pan-TCGA shared neoepitopes.
   See electronic supplementary materials.
Figures

A

Cytolytic activity: GZMA, PRF1 expression in TCGA datasets

B

Expression of GZMA and PRF1 in Pancreatic Adenocarcinoma

Log2(TPM + 1)
Figure 15. Stratification of human PDA based on cytolytic index.

(A) Cytolytic index (geometric mean of expression of GZMA and PRF1 in transcripts per million (tpm)) across TCGA tumor types. Kidney renal clear cell carcinoma (KIRC, n = 606), lung adenocarcinoma (LUAD, n = 116), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n = 309), lung squamous cell carcinoma (LUSC, n = 553), pancreatic adenocarcinoma (PAAD, n = 134, red underline), stomach adenocarcinoma (STAD, n = 418), head and neck squamous cell carcinoma (HNSC, n = 566), colon adenocarcinoma (COAD, n = 328), skin cutaneous melanoma (SKCM, n = 105), bladder urothelial carcinoma (BLCA, n = 427), esophageal carcinoma (ESCA, n = 196), liver hepatocellular carcinoma (LIHC, n = 371), thyroid carcinoma (THCA, n = 572), ovarian serous cystadenocarcinoma (OV, n = 309), glioblastoma multiforme (GBM, n = 169), and prostate adenocarcinoma (PRAD, n = 555). Inset, cytolytic index between normal pancreas expression levels obtained from Genotype-Tissue Expression (GTEx) project and TCGA PAAD. (B) Distribution of cytolytic genes within pancreatic adenocarcinoma. Gene set variation analysis (GSVA) signature scores for cytolytic index distinguished top decile (orange) and bottom quartile (green) samples for cytolytic-high (CYT High) and low (CYT Low) tumors, respectively.
Figure 16. Cytolytic index correlates with classifiers of PDA subtypes.

(A) Hierarchical clustering of GSVA signature scores for gene programs defining PDA subtypes from (Bailey et al. 2016a, Collisson et al. 2011, Moffitt et al. 2015) (B) Distribution of GSVA signature scores for each PDA subtype program between cytolytic-high and low tumors. ** = FDR adjusted P-values ≤ 0.05 and N.S. = Not statistically significant. (C) Hierarchical clustering of Moffitt Normal Stroma gene expression between cytolytic-high and low tumors showing enrichment in cytolytic-high tumors.
(D) Hierarchical clustering of Bailey GP1 Pancreatic Progenitor gene expression between cytolytic-high and low tumors showing enrichment in cytolytic-low tumors.
Figure 17. Low cytolytic index is associated with increased copy number alterations in
PDA.

(A) Co-mutation plot showing significantly mutated genes (SMGs, FDR < 0.1) in cytolytic subsets in the PAAD dataset. Red boxes indicate mutation. SMGs that correlate with cytolytic subtypes (p < 0.05) are highlighted by green or orange circles in the left column. Genome MuSiC (v0.4) FDR p-values for SMGs are plotted in –log10 on the right. (B) Nonsynonymous mutation spectra across PDA cytolytic subsets. (C) KRAS mutation types across PAAD dataset and association with cytolytic index, showing no statistically significant correlation between KRAS mutations and cytolytic subsets. (D) GISTIC2.0 analysis identified recurrent somatic copy number alterations (SCNA) in cytolytic-low tumors. Recurrent amplifications at 8q24.21 (MYC), 1p12 (NOTCH2), 8p11.22 (FGFR1), and deletions at 9p21.3 (CDKN2A/B), 18q21.2 (SMAD4) in CYT low tumors. (E) Total SCNA were calculated for each TCGA PAAD patient and were significantly increased in cytolytic-low tumors (Mann-Whitney). (F) Co-mutation plot of copy number alterations and non-silent SNVs/INDELs in genes amplified or lost in cytolytic-low PDA tumors.
Figure 18. Inhibitory checkpoint molecules, but not neoepitope load, are associated with cytolytic index in PDA.

(A) Local regression curves (Spearman rank correlation) between cytolytic index and total mutation count and boxplot distributions between cytolytic subsets (Mann-Whitney). (B) Local regression curves and boxplot distributions between cytolytic subsets for cytolytic index and total MHC class I neoepitopes (50nM predicted binding affinity) and (C) cytolytic index and number of mutations generating ≥1 neoepitopes to MHC class I. (D) Local regression curves and boxplot distributions between cytolytic subsets for cytolytic index and total MHC class II neoepitopes (<1% rank) and (E) cytolytic index and number of mutations generating ≥1 neoepitopes to MHC class II. (F) Differentially expressed chemokines, cytokines, and inhibitory checkpoint molecules between cytolytic-high (top decile) and low (bottom quantile) samples across TCGA. Fold change between subtypes indicated by color. Size of circle indicates statistical significance (-log10(Adjusted P value)). Arrow and box indicate no differential expression of PD-L1 (CD274) in between cytolytic subtypes in TCGA PAAD dataset. (G) Distribution of inhibitory immune checkpoint index (geometric mean of TPM values) across PAAD TCGA. Checkpoint molecules: CD274 (PD-L1), IDO2, PDCD1LG2 (PD-L2), CTLA4, IDO1, ADA2A2A (A2AR), LAG3, PDCD1 (PD1), TIGIT, HAVCR2 (TIM3), VISTA (C10orf54), VTCN1 (B7-H4). (H) Local regression curve showing statistical significant relationship between cytolytic index and inhibitory immune checkpoint index in PDA (Spearman rank correlation). (I) Expression of differentially expressed Treg markers in
PDA subsets. (J) Expression of differentially expressed inhibitory checkpoint molecules.

N.S = not statistically significant, ** = FDR adjusted P-values ≤ 0.1.
Figure 19. Neoepitope landscape in human cancer.

(A) Pipeline overview of data types (grey), intermediate pipeline steps (tan), important computational steps (orange) and results (red). See Figure 34 for a detailed pipeline. (B) Parallel coordinate plot showing relationship between frequency of mutations, MHC class I neoepitopes, and MHC class II neoepitopes in all samples analyzed. Lines are individual samples in the top (red) or bottom (blue) decile by MHC class I neoepitope load and are normalized on each node. (C) Summary of missense mutations, predicted MHC class I neoepitopes (<50nM affinity), predicted MHC class II
neoepitopes (<1% rank) and cytolytic index (geometric mean GZMA and PRF1 normalized expression) by disease type. Disease types are ordered from top to bottom by mean missense mutations. Sample numbers shown are for MHC class I neoepitope load. See Figure 37 for individual samples by disease type. (D) Predicted neoepitope rate across all disease types analyzed. Blue box and line indicate the 75th to 25th percentile and median, respectively. Disease subtypes are ordered from left to right by mean. P value is by ANOVA. (E) Shared MHC class I and II neoepitopes across all samples analyzed. Neoepitopes were considered duplicates across samples if the same peptide sequence met neoepitope criteria for sample-specific HLA. Calculation of duplicated peptides and neoepitopes is independent of sample HLA allele.
Figure 20. Immune suppression associations with cytolytic index.

(A) Cytolytic index (blue) and immune suppression index (orange) across all disease types analyzed. Cytolytic index is expressed as the gene set variation analysis.
(GSVA) score of normalized expression of genes GZMA and PRF1. Immune suppression index is the gene set variation analysis (GSVA) score of normalized expression for genes ADORA2A (A2AR), CD274 (PD-L1), PDCD1 (PD1), CTLA4, HAVCR2 (TIM3), IDO1, IDO2, PDCD1LG2 (PD-L2), TIGIT, VISTA (C10orf54), and VTCN1 (B7-H4). Box and line indicate the 75th to 25th percentile and median, respectively. Disease subtypes are ordered from left to right by mean cytolytic index. P value is by ANOVA. (B) Heatmap of normalized log10 immune suppression index member gene expression across disease types. Columns and rows are unscaled and ordered by hierarchical clustering. (C) Mean immune suppression (light blue) and overall top (dark blue) gene associations with cytolytic index across all disease types analyzed. (D-E) Mean immune suppression index (D) or CD8A normalized expression (E) vs. cytolytic index by disease type as labeled. R-squared values are for linear regression and P values are for Spearman’s rho. Dotted line is Bonferroni-adjusted cutoff for significance.
Figure 21. Immune environment predicts neoepitope load.
(A-B) Mean MHC class I and II predicted neoepitope load vs. CD8A expression (A) and cytolytic index (B), by disease type as labeled. See Figure 40 and Figure 41 for sample scatterplots by disease type. R-squared values are for linear regression and P values are for Spearman’s rho. (C-D) Predictability of top immune-related gene sets for MHC class I neoepitope load (C) and MHC class II neoepitope load (D) across all tumor types (left, bar plot) and by disease type (right, heatmap). Classification models were for the top and bottom decile ranked within each disease subtype. Relative Brier score is the normalized Brier score relative to negative control for the re-fit forest after top variable selection, averaged across bootstrap iterations. A relative Brier score equal to 1 denotes equivalency to negative control (equal to random guessing) and a relative Brier score equal to 0.9 denotes a 10% improvement over negative control. A Brier score equal to 0 denotes perfect predictive ability. Barplot is for all disease types. Heatmap is unscaled and ordered form left to right alphabetically. (E) Minimal depth plots for selected predicted MHC class I neoepitope models from (C). Listed genes are top variables from the analyzed gene set used to predict high vs. low class membership and are ranked from top to bottom by mean minimal depth. Minimal depth is a measure of variable importance derived from individual trees in the forest. Smaller values indicate greater importance and absolute values are model-specific. Minimal depth is defined as the distance between the root node of a tree in the forest and the closest maximal subtree for the indicated gene. Dotted line indicates minimal depth threshold of significance, defined as 1 standard error below the mean across all model variables.
Figure 22. Superior survival of high predicted neoepitope groups.

(A) Summary of Kaplan-Meier p values for missense mutations or the indicated neoepitope class across all disease types analyzed. Comparison is between high (red) and low (blue) neoepitope load patients, defined as the top and bottom decile, respectively. P
values highlighted in yellow are significant. Disease types with low sample number are excluded. (B) Selected Kaplan-Meier curves for the indicated disease subtypes showing survival difference between high (red) and low (blue) neoepitope load patients. Tick marks denote the last time survival status was known for living patients.
Figure 23. Cumulative distribution of cytolytic index between PAAD and STAD cohorts.

Cumulative density (left) and cumulative frequency (right) of the cytolytic index between PDA (PAAD) and stomach adenocarcinoma (STAD) cohorts in TCGA. PDA has a much smaller distribution in comparison with STAD (Kolmogorov-Smirnov test).
Figure 24. Enrichment of selected immune related gene sets in cytolytic-high PDA tumors.

Cytolytic-high tumors show increase enrichment of gene sets from activated, cytolytic CD8 T cell populations (Duraiswamy et al. 2014, Parish et al. 2009).
Figure 25. Enrichment of immune related gene sets in cytolytic-high PDA tumors

Gene set variation analysis (GSVA) of known immune related gene sets show statistically significant increase in tumors identified as cytolytic-high based on expression of GZMA and PRF1.
Figure 26. Cytolytic index associates strongly with expression of Immunome gene sets.

(A) GSVA scores for gene sets comprising 28 immune cell types identified previously. (Immunome, (Bindea et al. 2013)) Cytolytic high and low tumors are designated by orange and dark green boxes, respectively. (B) GSVA of gene sets related to Tight junctions, Argi


gannine/Proline Metabolism, Cholesterol/Steroid synthesis, and glucose metabolism from the REACTOME (R) and KEGG (K) databases. (C) GSVA of gene sets related to Cholesterol/Steroid synthesis, and glucose metabolism from the REACTOME and KEGG databases.
Figure 27. Expression of copy number alteration-associated and MYC target genes are enhanced in pancreatic tumor (A) Gene set enrichment analysis (GSEA) of genes whose expression in pancreatic tumor correlated with copy number gains. (Aguirre et al. 2004). (B) GSEA of known MYC target genes (Zeller et al. 2003). ES = Enrichment Score, NES = Normalized Enrichment Score, FDR = False discovery rate.
Figure 28. Tumor cellularity/purity estimates do not correlate with mutational load.

Tumor purity estimates for TCGA PAAD do not correlate with total mutation count (top left), total copy number events (top right), type I neoepitopes (bottom left), and type II neoepitopes (bottom right).
Figure 29. Tumor purity estimates for PDA, stomach adenocarcinoma (STAD) and lung adenocarcinoma (LUAD).
Figure 30. No difference in tumor cellularity or purity between cytolytic subtypes in TCGA PAAD cohort.
Figure 31. Cytolytic subtypes associate with neoepitope load in stomach adenocarcinoma (STAD) and lung adenocarcinoma (LUAD).
Figure 32. Neoeptope burden does not differ across PDA subtypes.

(A) Hierarchical clustering of TCGA PAAD dataset using subtype classifiers from (Moffitt et al. 2015) (left) and (Bai et al. 2015) (right) and association with cytolytic subtypes. (B) Mutation and neoeptope load as a function of Moffitt subtype classification of TCGA PAAD. Statistical enrichment of total mutation count between Classical and Normal Stroma (one-way ANOVA, Tukey’s post hoc test for multiple comparisons). (C) Same B except using Bailey subtype classification of TCGA PAAD.
Figure 33. Correlation plots of individual Immune suppression index versus cytolytic index.
Figure 34. Neoepitope analysis pipeline.

Schematic of neoepitope analysis pipeline showing TCGA datasets (grey), pipeline steps (tan), MHC affinity prediction (orange), results (red) and analysis (green). The Neoepitopes R package source code is freely available can be obtained from GitHub (https://github.com/andrewrech/Neoepitopes).
Figure 35. TCGA sample cancer DNA fraction.

A. Cancer DNA fraction across all disease types analyzed. B. Relationship between number of missense mutations and cancer DNA fraction across all samples analyzed. R-squared values are for linear regression and P values are for Spearman’s rho. Cancer DNA fraction was determined using ABSOLUTE. (Carter et al. 2012)
Figure 36. TCGA HLA types.
(A) Predicted MHC class I (left) and class II (right) HLA types for all samples analyzed. The area of the box is proportional to the occurrence of the indicated HLA allele across all samples analyzed. (B) Observed neoepitope rate (predicted neoepitopes / total predictions) across all HLA alleles analyzed. Blue box and line indicate the 75th to 25th percentile and median, respectively.
Figure 37. Missense mutations and neoepitopes across disease types.
Missense mutations (A), MHC class I neoepitopes (B), MHC class II neoepitopes (C) across all disease types analyzed. Blue box and line indicate the 75th to 25th percentile and median, respectively. Disease subtypes are ordered from left to right by mean. P values are by ANOVA.
Figure 38. Immune suppression index member gene expression across selected disease types.
Columns (samples) and rows are unscaled and ordered by hierarchical clustering.

Figure 39. Immune suppression index vs. cytolytic index.

Mean class I neoepitope load by disease type as labeled. R-squared values are for linear regression and P values are for Spearman’s rho.
Figure 40. Predicted neoepitopes (class I) vs. cytolytic index.

Predicted MHC class I neoepitopes vs. cytolytic index across all samples for the indicated disease type. R-squared values are for linear regression and P values are for Spearman’s rho. Diseases types are ordered alphabetically.
Figure 41. Predicted classic neoepitope (class II) vs. cytolytic index.

Predicted MHC class II neoepitopes vs. cytolytic index across all samples for the indicated disease type. R-squared values are for linear regression and P values are for Spearman’s rho. Diseases types are ordered alphabetically.
CHAPTER 5 – Discussion

Research over the past two decades demonstrates that the immune system plays a dual role in most forms of cancer. The immune system can suppress tumor growth for long periods of time by eliminating tumor cells, but also promote growth by selecting for resistant tumor cells or establishing a hospitable tumor microenvironment. These protective and deleterious effects are the basis of the cancer immunoediting hypothesis, in which the immune system quantitatively and qualitatively impacts tumor development, at first eliminating tumor cells but eventually becoming ineffective, leading to tumor escape (Schreiber et al. 2011). Our expanded understanding of the immune system’s role in cancer has led to refined attempts to control cancer via immunotherapy. In most experimental systems, effective cancer immunotherapy requires CD8 effector T cells, as confirmed by hallmark murine studies (DuPage et al. 2012, Matsushita et al. 2012). Indeed, the quantity, quality and distribution of CD8 effector T cells correlate positively with patient survival (Erdag et al. 2012, Naito et al. 1998, van Houdt et al. 2008). The distribution of regulatory T cells that suppress CD8 effector T cells correlates negatively with patient survival (Curiel et al. 2004, Knol et al. 2011) and it is increasingly apparent that the escape phase includes tumor-instructed immunosuppression that thwarts productive antitumor immunity. Recent breakthroughs, notably the success of immune checkpoint blockade in patients with metastatic melanoma, (Hodi et al. 2010, Topalian et
al. 2012) demonstrate the relevance of understanding such resistance pathways and broader determinants of immunity.

**Immune vs. tumor origins of therapeutic resistance**

In work investigating the combination of radiation and immune checkpoint blockade (Chapter 3, page 63), I found that RT enhances the diversity of the T cell receptor (TCR) repertoire of intratumoral T cells and anti-CTLA4 predominantly inhibits T regulatory cells (regulatory T cells) to increase the CD8 T cell to Treg (CD8/Treg) ratio. Together, anti-CTLA4 promotes expansion of T cells, while RT shapes the TCR repertoire of the expanded peripheral clones. Addition of PD-L1 blockade reverses T cell exhaustion to mitigate depression in the CD8/Treg ratio and further encourages oligo-clonal T cell expansion. Thus, PD-L1 on melanoma cells allows tumors to escape anti-CTLA4-based therapy, and the combination of RT, anti-CTLA4, and anti-PD-L1 promotes response and immunity through distinct mechanisms.

In contrast to my work demonstrating a tumor cell-intrinsic mechanism of PD-L1 upregulation in melanoma, recent work also in melanoma shows that the induction of immune checkpoint pathways is a consequence of CD8 T cell infiltration (Spranger et al. 2013). In this melanoma model, mechanistic studies demonstrated that upregulation of PD-L1 by tumor cells was dependent on the presence of CD8 T cells and interferon-γ – a
negative feedback loop intrinsic to immune activation and independent of oncogene signaling. Of course, oncogene-driven vs. T cell-driven regulation of tumor PD-L1 are not mutually exclusive, and future studies may resolve the contribution of each mechanism in these malignancies. In a different murine melanoma model, previous work has already demonstrated a role for the EGFR pathway in immune suppression via upregulation of the cytokine CCL27 (Pivarcsi et al. 2007). Nevertheless, there may be important differences between tumors expressing PD-L1 due to oncogenes and those in which PD-L1 is induced due to the infiltration of antitumor T cells. The former is less likely to have undergone selective pressure exerted by the immune system, and may therefore respond more favorably to additional immune therapies. These investigations also generate hypotheses for how to identify patients who are likely to respond to PD1 blocking antibodies. For instance, I can speculate that EGFR-driven NSCLC tumors may benefit from PD1 blockade regardless of preexisting immune infiltrate. Alternatively, melanomas harboring a T cell-inflamed tumor microenvironment may respond regardless of driving mutations such as BRAF, the status of which does not appear to predict response to anti-CTLA4.
Determinants of adaptive immunity in pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDA) is a difficult clinical problem, with poor response to therapy including near universal failure of single-agent immune checkpoint blockade antibodies. This biology is investigated in Chapter 4 (page ). To understand the features that might make some tumors more responsive to immunotherapy, I used publically available expression data from 134 primary resection PDA samples from TCGA to stratify patients according to a cytolytic T-cell activity expression index. I correlated cytolytic immune activity with mutational, structural and neoepitope features of the tumor. I found that high cytolytic activity in PDA does not correlate with increased neoepitope burden. Rather, tumor-intrinsic characteristics such as MYC and NOTCH2 amplifications and recurrent deletions and mutations at CDKN2A/B are linked to the status of intratumoral immune activation. High cytolytic activity is associated with increased expression of multiple immune checkpoints (with the notable exception of PD-L1).

Our emerging understanding suggests that rather than being linked to mutation burden or neoepitope load, immune activation indices in PDA are linked to genomic alterations, suggesting that intrinsic oncogenic processes drive immune activity in human
PDA. My data support the utility of combining genomic and immune profiling for a comprehensive understanding of immune activation in PDA. This approach may help guide the development of effective immune therapy in PDA and other immune therapy-refractory cancers. Furthermore, these data highlight the potential importance of immune checkpoints other than PD-L1/PD1 as therapeutic targets in this lethal disease.
The landscape of neoepitopes in human cancer

Chapter 4 (page 103) builds on previous work by others (Brown et al. 2014, Rooney et al. 2015) and my own initial study of PDA by expanding analysis of neoepitopes to MHC class I and II types across 9928 human cancer samples belonging to 35 solid tumor types. I evaluated over 400 million peptides generated from over 1 million missense mutations and then characterized multiple immune indices, immune- and cancer-related gene sets, and gene mutational status for associations with neoepitope load. I also assessed the overall characteristics of neoepitope load, type and quality in human cancer at the global level and employed machine learning to understand complex relationships in these data and build predictive models. Due to the probabilistic nature of neoepitope generation and high MHC allele diversity in humans, I find there are nearly zero neoepitopes shared across patients in human cancer at frequencies amenable to therapeutic targeting. Though previous reports have found deviations in the expected vs. observed number of neoepitopes in some disease types, (Rooney et al. 2015) I note few meaningful differences in my analysis. Higher resolution exome sequencing of human tumors, improved prediction tools, and appreciation of newly-identified sources of neoepitopes (Liepe et al. 2016) may be required to confirm evidence of population-scale immunoediting.

Our results suggest that predicted neoepitope load correlates closely with rate of missense mutations, in agreement with previous findings (Rooney et al. 2015). Recent
work has identified a non-synonymous mutation cutoff of 192, based on published response rates to checkpoint inhibitors, and hypothesized that this discriminates patients likely to respond to checkpoint blockade. This includes 30% of bladder, colon, gastric and endometrial cancers. I note that while these patients may be more likely to respond, a small number of neoepitopes can drive therapeutically effective antitumor T cell responses, (Linnemann et al. 2013, Lu et al. 2013, Matsushita et al. 2012, Robbins et al. 2013) and I find few strong correlations within these tumor types between cytolytic activity and missense mutation or neoepitope load despite large variability in these metrics across samples within each disease type. This suggests that spontaneous immunity in primary adenocarcinomas is unlikely to be limited by a critical neoepitope threshold, consistent with a recently published report (Spranger et al. 2016).

Accordingly, the abundance of neoepitopes across solid tumors in my analysis suggests that immunotherapies capable of improving T cell priming, infiltration or effector function have at least dozens of ‘immunologically visible’ targets to act upon in nearly all solid human tumors. This is especially true given that technical limitations in exome sequencing depth and tumor sample purity, as well as ample additional sources of neoepitopes such as frameshift mutations and gene fusions, (Maby et al. 2016, Zhang et al. 2016) imply that my analyses necessarily underreport absolute neoepitope load. Thus, the potential for T cell immunity with checkpoint blockade or other strategies is unlikely to be limited to high mutation load patients or disease types on the basis of available T
cell targets. Additionally, the effect of greater neoeptope load on likelihood of response is probably disease type-specific. For instance, anti-PD1 may be effective therapy in colon adenocarcinoma patients whose tumors harbor microsatellite instability, (Llosa et al. 2015) in contrast to the majority of colon cancer patients. Yet my results suggest a strong correlation between immunity and neoeptope load in kidney renal clear cell carcinoma, and a landmark study has shown that these patients respond to immune checkpoint blockade despite a median neoeptope load considerably lower than that in colon adenocarcinoma (Brahmer et al. 2012, Topalian et al. 2012).

An alternative explanation of the varied association between neoeptope load and responses to checkpoint blockade across tumor types is that underlying genetic subtypes with greater DNA damage are also characterized by distinct immune profiles, a subset of which may be amenable to checkpoint blockade. For example, several recent reports have identified distinct melanoma or pancreatic adenocarcinoma molecular subtypes associated with immune activity, yet the underlying genetic alternations that comprise these subtypes differ substantially (Bailey et al. 2016b, Cancer Genome Atlas Network 2015). My findings of diverse predictive ability of immune- and cancer-related gene sets for neoeptope load across disease and neoeptope type underscore this heterogeneity. Beyond first approximation correlations with CD8 T cell infiltration or other broad, previously described metrics, the dynamics of adaptive immune activity with respect to
neoepitope load or patient outcome must be contextualized in a disease type-specific manner.

The diverse pattern of immune suppressive molecules observed at the disease type level further suggest that tailoring the blockade of inhibitory pathways in a disease or patient-specific manner, rather than focusing on overall mutational and/or neoepitope burden, is likely required to overcome suppressive barriers within the tumor microenvironment. My data indicate that tumor-specific neoepitopes alone are not sufficient to elicit a positive immune response: several disease types with high mutation rates were not associated with improved survival outcomes in patients belonging to the top decile by neoepitope load. In fact, several disease types have negative association between survival outcome and high neoepitope/mutation load. I have shown in Chapter 4 (page 103) that cytolytic response can be disconnected from elevated neoepitope load in pancreatic ductal adenocarcinoma (Balli et al. 2016). I find here that expression of immune suppressive index gene members is heterogeneous across TCGA disease types. Thus, my data suggest that immune- or tumor-intrinsic sequelae of increased cytolytic activity within the tumor microenvironment, that vary dramatically across human cancer, determine potential neoepitope immunogenicity. My finding that cytolytic index, but not overall CD8A expression, is coupled to immune suppression index and neoepitope load at the disease level across 35 human cancer types suggests that immune-intrinsic regulation
tightly controls cytolytic activity in the tumor microenvironment regardless of tumor etiology.
CHAPTER 6 – Future directions

Combinatorial radiotherapy and immunotherapy

More than half of cancer patients receive RT during the course of treatment (Delaney et al. 2005). RT achieves local control of disease and improvements in survival in lung, prostate, and head and neck cancer and is used palliatively in many other disease types. Yet a major limitation of RT, and the primary mechanism of treatment resistance, is disease reoccurrence outside the treatment field. For instance, most pancreatic cancer patients, regardless of whether their primary tumor is resectable, develop distant metastases and die within two years of diagnosis (Hidalgo 2010). My data add to increasing evidence that tumor-intrinsic pathways alter the tumor microenvironment to promote immune escape. Cellular immune responses to radiation and adaptive immunity intersect at the level of proinflammatory signals, antigen-presenting cell maturation, T cell priming and tumor cell recognition. Dissecting the crosstalk between oncogene-driven networks of immune suppression and the dynamic regulation of tumor-infiltrating T cells will no doubt be a critical area of investigation in the future. In particular, whether immune modalities can limit abscopal reoccurrence, and the extent of overlap between tumor and immune-intrinsic resistance pathways to RT and immune-modulating antibodies, will require careful mechanistic insight. The PDA tumor
microenvironment is a profoundly immune suppressive one, where immune resistance similarly overlaps with pathways of radioresistance. Successful combinatorial therapy in PDA will require a greater understanding of these intersection points, especially in regard to regulatory T cells and myeloid-derived suppressor cells that are shown to facilitate pancreatic immune tolerance.

A critical additional future direction is how radiation may augment the creation or processing of tumor rejection antigens; unaccounted for neoantigens that arise after RT may play a crucial role in tumor rejection, especially in solid adenocarcinomas with the lowest baseline availability of neoantigens. Radiotherapy has been shown to induce T cell responses to peptides that are upregulated in response to radiation-induced cell damage, (Reits et al. 2006) which occurs due to increased protein translation induced by activation of the mammalian target of rapamycin pathway. The extent that this, or other mechanisms by which RT directly contributes to loss of acquired immune privilege, are operative in human cancer has not been investigated. Future work should determine the relative importance of these factors vs. indirect effects of RT on proinflammatory milieu, which will have implications for therapy design.

Future clinical trials will need to carefully investigate a role for RT with combinatorial immune checkpoint blockade and other immune-modulating antibodies. First, RT may enhance what is in some cases, notably combinatorial therapy with anti-CTLA4 and anti-PD1, an already severe toxicity profile (Silk et al. 2013). Second, immune
checkpoint blockade results in a unique pattern of response in which tumor T cell infiltration may appear radiologically as progression in some patients, necessitating use of novel response criteria that must be factored into design of clinical trials incorporating RT (Bohnsack et al. 2014, Wolchok et al. 2009). Third, due to the risk of inducing clinically significant lymphopenia that may negatively impact antitumor responses,(Grossman et al. 2015) an important design criteria will be choice of RT approach and regimen. The tolerance of circulating lymphocytes to radiation is dependent on fractionation and irradiated surface volume, (Yovino et al. 2013) therefore stereotactic delivery over fewer fractions to smaller tissue volumes may be preferable. Preclinical models have yielded conflicting data regarding whether hypofractionated protocols are required or yield superior results as an immune adjuvant. Clinically, an initial retrospective analysis of melanoma patients with brain metastases treated with a single high-dose fraction vs. conventional RT suggests the former may be superior, (Silk et al. 2013) and many ongoing clinical trials with diverse fractionation schemes may confirm this (Spiotto et al. 2016). A related basic question is whether irradiation of all vs. part of a tumor is required for optimal immunity. In summary, it is likely the precise nature of RT delivery will have profound impact immune biology and treatment efficacy, warranting a systematized approach to investigation that does not currently exist.
Emerging classes of neoepitopes

I have established that predicted neoepitope formation in human cancer is essentially a probabilistic process. Yet it is possible that certain classes of neoepitopes are more advantageous than others as targets for therapeutic gain or as correlative markers to understand immunity. For instance, neoepitopes derived from mutations in oncogenic driver genes may be important targets of adaptive antitumor immunity due to the resistance to selective pressure these offer. Additionally, gene mutation status, especially mutations resulting in greater genomic instability or deficiency in DNA repair, may correlate with neoepitope load or response to immune therapies that augment neoepitope-directed T cell responses.

Recent investigations have led to additional factors to consider when identifying and prioritizing neoepitopes. To date, essentially all neoepitope analysis pipelines, including my own, have relied on missense mutations as the single source of somatic differences that could result in novel peptides for which no self-tolerance likely exists. These are chosen because identification is computationally straightforward and error-resistant; however, this is an obvious simplification of the genomic variation present in tumors that can result in novel peptides. Next generation neoepitope prediction pipelines will also consider gene fusions, which may be of particular importance in tumor types with very few somatic missense mutations such as many pediatric tumors. Excellent tools exist for identifying gene fusions and predicting which lead to fusion peptides that could
be processed and presented by MHC, (Kumar et al. 2016) though higher quality RNA sequencing than is generally available in large databases of tumor samples may be required for accurate analysis.

Prioritizing neoepitopes by likelihood of immunogenicity, for experimental validation or inclusion in peptide vaccines, is a major future challenge. Currently, despite significant enrichment by computational methods for high-affinity peptides that generate T cell reactivity (Castle et al. 2012), only a minority of identified neoepitopes can be validated to generate immunity in vivo (McGranahan et al. 2016). An additional factor to consider for prioritization in the future is neoepitope clonality, as recent work has found that highly clonal neoepitopes are associated with an inflamed tumor microenvironment in lung adenocarcinoma (McGranahan et al. 2016). PD-L1 and IL-6 were significantly upregulated in tumors with low intratumoral heterogeneity and high neoepitope clonality in this analysis. An enticing hypothesis is that high neoepitope clonality can be used as selection criteria across human cancer. Clonal neoepitopes are more widely shared across tumor cells and may therefore be particularly advantageous for adoptive therapy approaches that subject a handful immune targets to extreme selective pressure (Fisher et al. 2013).

Many neoepitopes have wildtype counterparts that also bind MHC class I with high-affinity. In these cases, unless missense mutations occur on TCR contact residues or substantially alter peptide structural characteristics, T cells may be similarly reactive.
against normal and mutant peptides and thus subjected to central tolerance, a paradigm that does not exist in the case of foreign antigens used to establish affinity criteria for peptides to MHC. Differential agretopicity (DAI) expresses the degree to which the determinants of peptide binding to MHC class I or II differ due to the presence of a missense mutation. Previous work has demonstrated that selection of neoepitopes on the basis of high DAI resulted in a substantially improved rate of experimentally-validated tumor-protective epitopes (Duan et al. 2014). To determine differential agretopicity and identify peptides with high DAI, I could select mutant peptides that at least minimally bind MHC and then compare the binding affinity of these peptides to their normal counterpart. Because these selection criteria differ substantially from those for classic neoepitopes, I would expect these classes of neoepitopes to be largely non-overlapping sets. Indeed, previous work has found that classic vs. high DAI neoepitopes differ substantially in amino acid composition and mutant amino acid position within the MHC binding groove (Duan et al. 2014). Thus, human cancer types demonstrated to have different mutation profiles, (Alexandrov et al. 2013, Ciriello et al. 2013) due to distinct mechanisms of genomic instability, may differ substantially in the availability of classic vs. high DAI neoepitopes for targeting.

Taken together, my identification of MHC class I and class II neoepitopes imply that a larger than currently appreciated pool of potential neoepitopes exists across human tumor types. It will be important for future studies to investigate the relative importance
of these and emerging neoepitope classes. Some recent evidence already suggests MHC class II and high DAI neoepitopes are important. Due to the relatively high promiscuity of peptide binding in the groove of MHC class II vs. class I, I found that on average, missense mutations are more likely to generate MHC class II neoepitopes. Kreiter et al. recently identified MHC class II neoepitopes capable of driving therapeutic responses in murine tumor models, (Kreiter et al. 2015) and mutation-specific CD4 T cells recognize neoepitopes and can mediate tumor regression in humans (Linnemann et al. 2015, Tran et al. 2014). Whether broad class II-restricted T cell responses exist in human cancer and can be augmented therapeutically remains to be determined, and my results indicate that, similar to MHC class I, immune activity against MHC class II neoepitopes is unlikely to be limited on the basis of prevalence in virtually any solid tumor type.

Recent findings are also consistent with an important role for high DAI neoepitopes, identified on the basis of improved MHC binding vs. normal sequence counterparts, in mice and humans. In 2012, one of the first investigations of neoepitopes identified many missense mutation-containing B16-F10 melanoma peptides based on predicted MHC class I affinity, subsequently experimentally verifying the immunogenicity of these peptides (Castle et al. 2012). 11/13 reactive mutant peptides identified were found to have normal counterparts with equal or less reactivity, suggesting that in the B16-F10 model, immunogenic mutations generally do not result in substantial changes in critical TCR-facing amino positions. Many of these normal
peptides presumably bypassed central tolerance by not binding strongly enough to class I MHC, as hypothesized. In humans, three validated neoepitopes in NSCLC patients all had higher predicted binding for mutant vs. normal peptide. (McGranahan et al. 2016) In 1 out of 3 cases, the generating missense mutation resulted in an anchor position change and would therefore be classified as a high DAI peptide. Thus, a critical future direction is characterizing the landscape of these neoepitope classes in human cancer. Functional validation investigating the extent to which high DAI peptides are predominant tumor rejection antigens in contexts other than B16-F10 melanoma will further determine the important of this neoepitope class. In summary, it will be important to evaluate neoepitope type and disease-specific immune context when considering therapies designed to augment T cell responses directed against neoepitopes.
CHAPTER 7 – Software

Main functions from the Neoepitopes R package.

Table 11. *neoepitope_predictions* function specification from the Neoepitopes R package.

**Performs epitope prediction.**

```
neoepitope_predictions(mhc_intake_df, class_I, class_II, analysis_id = "TCGA",
exclusion_id = NULL, sample_number = NULL, peptide_length = 9:14,
mhc_df_class_I, mhc_df_class_II, intake_type, query_database, MHC,
expression_dir, skip_no_expr = FALSE, omit_expr = FALSE, assemble,
variant_selection = NULL, normal = FALSE, generate, calculate,
log_dir = getwd(), storage_dir = getwd())
```

**Arguments**

- `mhc_intake_df`: Data frame. Input data frame from intake_NGS. Must contain three columns: sample_id, transcript_affected (ensembl_transcript_id), and aa_mutation (single letter abbreviations).
- `class_I`: Logical. Perform class I predictions?
- `class_II`: Logical. Perform class II predictions?
- `analysis_id`: Character. Text string used to select pipeline files.
- `exclusion_id`: Character. Connection read with an imported column ‘Sample_id’ containing samples to exclude. Can be output from previous class I or class II predictions.
- `sample_number`: Numeric. Index of input samples found to use.
- `peptide_length`: Numeric. Length of peptides to make for MHC class I predictions.
- `MHC_df_class_I`: Data frame. Column sample_id specifies sample ID and remaining columns specify MHC types to test.
- `MHC_df_class_II`: Data frame. Column sample_id specifies sample ID and remaining columns specify MHC types to test.
- `intake_type`: Character. Intake data source, one of ‘vcf’ (Sippef processed vcf files) or ‘df’ (standard input data frame described above).
- `query_database`: Character. Protein database, one of ‘GRCh38’, ‘GRCh37’, or ‘GRChm38’.
- `MHC`: Character. Directory with MHC types.
- `expression_dir`: Character. Directory with expression data.
- `skip_no_expr`: Logical. Whether to skip samples without associated RNA expression data.
- `omit_expr`: Logical. Whether to skip mutations whose expression value is unknown.
- `assemble`: Logical. Assemble intake data?
- `variant_selection`: Numeric vector. Index of intake variants to generate on.
- `normal`: Logical. Run normal instead of mutant peptide sequences?
- `generate`: Logical. Generate peptides and commands?
- `calculate`: Logical. Calculate binding affinities?
- `log_dir`: Character. Path to directory for log files.
- `storage_dir`: Character. Path to directory for storage of output files.
Table 12. `neoepitope_predictions_worker` function specification from the Neoepitopes R package.

**Parallelized worker function for neoepitope_predictions**

```
neoepitope_predictions_worker(mhc_df, class_I, class_II, analysis_id,
query_database, peptide_length = 8:34, expression_dir, MHC,
MHC_df_class_I = NULL, MHC_df_class_II = NULL, variants_to_select, normal,
log_dir, storage_dir)
```

### Arguments

- **mhc_df**: Data frame, input data frame from `neoepitope_predictions`.
- **class_I**: Logical, Perform class I predictions?
- **class_II**: Logical, Perform class II predictions?
- **analysis_id**: Character, String to identify pipeline files.
- **query_database**: Character, Protein database; one of 'GRCh38', 'GRCh37', or 'GRCh36'.
- **peptide_length**: Numeric, Length of peptides to make for MHC class I predictions.
- **expression_dir**: Character, Directory with expression data.
- **MHC**: Character, Directory with MHC types.
- **MHC_df_class_I**: Data frame. Column sample_id specifies sample ID and remaining columns specify MHC types to test.
- **MHC_df_class_II**: Data frame. Column sample_id specifies sample ID and remaining columns specify MHC types to test.
- **variants_to_select**: Numeric, Index of missense variants to generate peptides with.
- **normal**: Logical, Run normal instead of mutant peptide sequences?
- **log_dir**: Character, Path to directory for log files.
- **storage_dir**: Character, Path to directory for storage of output files.
Table 13. *neoepitopes_output* function specification from the Neoeptopes R package.

Collates results from epitope prediction.

```r
neoepitopes_output(mhc_df = NULL, class_I, class_II, prediction_dir,
peptide_length = 8:16, analysis_id = "predictions.txt",
output_file_name, consensus_score_cutoff = 50, ensemble, summarize,
output_size_x = 8, output_size_y = 8, output_font_size = 12, cores,
storage_dir = getwd())
```

**Arguments**

- **mhc_df**: Entry data frame passed to neoepitope_predictions.
- **class_I**: Logical. Collate class I predictions?
- **class_II**: Logical. Collate class II predictions?
- **prediction_dir**: Character. Path to prediction files.
- **peptide_length**: Numeric. Length of peptides to make for MHC class I predictions.
- **analysis_id**: Character. String to match input .txt prediction files.
- **output_file_name**: Character. Name of output files.
- **consensus_score_cutoff**: Numeric. Class I consensus nM binding affinity threshold for classification as a potential neoepitope.
- **assemble**: Logical. Assemble basic output metrics for each sample?
- **summarize**: Logical. Create a summary file for each sample cohort?
- **output_size_x**: Numeric. Size of analysis graph pdf x dimension in inches.
- **output_size_y**: Numeric. Size of analysis graph pdf y dimension in inches.
- **output_font_size**: Numeric. Relative ggplot2 font size.
- **cores**: Numeric. Number of CPU cores to use for parallelization.
- **storage_dir**: Character. Path to directory for storage of output files.
CHAPTER 8 – References


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