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# Evaluating Targeted And Immunomodulatory Therapies For Melanoma In A Genetically Engineered Mouse Melanoma Model

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Evaluating targeted and immunomodulatory therapies for melanoma in a  
genetically engineered mouse melanoma model

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment  
of the Requirements for the Degree of Doctor of Medicine

By Billy J. Lockhart

2015

## **Abstract**

### **EVALUATING TARGETED AND IMMUNOMODULATORY THERAPIES FOR MELANOMA IN A GENETICALLY ENGINEERED MOUSE MELANOMA MODEL.**

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Melanoma therapy has changed rapidly due to the emergence of new therapies: MAPK-pathway targeted drugs and immunomodulatory agents. Given the relative success of these new individual drugs, this work set out to evaluate and develop effective melanoma treatments using combination therapies in a preclinical mouse melanoma models. Therapies tested include BRAF kinase inhibition in combination with: immune checkpoint inhibitors anti-CTLA4, anti-PDL1, and with the topical TLR7/8 agonist imiquimod. Drugs efficacies were tested in established melanomas in a conditional inducible mouse melanoma model based on activation of Braf and beta catenin and loss of Pten. BRAF inhibition in combination with anti-CTLA-4/anti-PD-L1 was not more effective than BRAF inhibition alone in retarding tumor growth or prolonging survival in these studies. Treatment with imiquimod significantly retarded tumor growth and increased survival. Imiquimod-treated tumors show increased macrophage infiltration, but not increased intratumoral T lymphocytes. Further work remains to identify effective, synergistic drug combinations in preclinical models.

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## **Introduction**

Melanoma, a cancer that arises from melanocytes, among the skin cancer possesses the highest morbidity and mortality. In the United States, it is estimated that 76,690 cases of melanoma and 9,480 deaths due to melanoma occurred in 2013 (1). Melanoma incidence predominates in countries with conjunction of fair-skinned ethnic populations and high UV light exposure, and global incidence of melanoma continues to rise (2): 132,00 new cases are diagnosed world-wide and an estimated 48,000 persons die from advanced melanoma across the globe each year (3) (4). From 1950 to 2000, a national cancer database documented increases of 619 percent in annual diagnoses of cutaneous melanoma and 165 percent in annual mortality from 1950 to 2000 (5).

At the initial diagnosis of a primary melanoma, the depth of the primary lesion is used to predict patient survival. For instance, survival at 10 years for a melanoma less than 1.00 mm in depth is greater than 90% (6). However, once melanoma has metastasized, the standard of care treatments offer little in the way of long term benefit: a 2009 report estimated one-year survival based on location of melanoma spread as high as 62% and as low as 33% (6). Thus, high mortality rates associated with unresectable or metastatic melanoma persists, and given the high mortality rates and the relatively young age at which disease often

occurs, melanoma skin cancer was the second leading causes of lost productive years among cancers (5).

#### Current therapeutic landscape

##### *Local Excision of Primary:*

If melanoma is diagnosed early, standard treatment involves wide local excision +/- sentinel lymph node biopsy based on thickness, ulceration status and a melanoma specific depth level known as the Clark level (5). High dose administration of Interferon alfa-2b, a type1 interferon that activates both innate and adaptive immune responses, has been studied as an adjuvant therapy in patients who have high-risk prognostic factors upon local melanoma excision. Multiple studies documented improvement in relapse-free survival, and two studies showed significantly improved overall survival among patients receiving high-dose interferon alfa-2b, and these reports lead to FDA approval of this treatment for patients whose initial primary lesions are greater than 4 mm in thickness (i.e., stage IIB or IIC) or for melanoma that involves a regional lymph node(s) excised during sentinel lymph node biopsy (i.e., stage III) (5). However, upon longer follow-up of these clinical trials, a significant long-term overall survival due to adjuvant interferon alfa-2b was not observed (7).

### *Targeted Cancer Therapies*

Note again that a relatively small tumor of a depth slightly larger than 1 mm has a high metastatic potential. It is this metastatic disease that causes nearly all melanoma-associated mortality. Until recently, no therapy for advanced melanoma resulted in a significant increase in median overall survival.

Dacarbazine, an alkylating agent first approved in 1975, is associated with overall survival of 5.6 to 7.8 months, and had been the mainstay of chemotherapy treatment until recently (8) (9). However, recent translational research in the field of melanoma therapeutics has yielded a string of new FDA approvals and large center clinical trials of several new agents.

Building on the success of rational drug design in other cancers, a new class of protein inhibitors that target key signaling cascades essential to melanoma growth has emerged. The BRAF gene encodes an intracellular serine/threonine kinase in the Raf family, B-raf, which has a primary role in mitogen signaling pathways. The BRAF gene is mutated in approximately 50% (40-60%) of human melanomas, and these mutations are predominately of the activating substitution type, the majority characterized as BRAF<sup>V600E</sup> (~90%) (10). This mutation results in constitutive downstream activation of the mitogen-activated protein kinase (MAPK) pathway, contributing to melanoma development and growth.

Vemurafenib was the first of the new class of BRAF inhibitor approved in 2011 for advanced melanoma with BRAF<sup>V600</sup> mutations. The drug is able to induce potent,

specific inhibition of mutated BRAF protein, providing for significant, often dramatic, responses in treated patients and significant increases in progression free survival and overall survival. Dabrafenib is a newer BRAF inhibitor and has also been shown to improve response rates, progression-free and overall survival, significantly compared to chemotherapy (11, 12). Still others are in development. However, in the vast majority of patients, the initial response to this targeted inhibition is followed by the successive development of a total BRAF inhibitor resistance; it appears regularly in approximately six to nine months (12, 13). Some evidence suggests that that reactivation of the MAPK pathway may play a key role in the overcoming of BRAF inhibition in patients treated with these drugs (12).

Not surprisingly, another new class of targeted protein inhibitors target MEK, a kinase that is downstream of Raf kinases in the MAPK pathway. Trametinib, a MEK inhibitor, has recently been FDA approved as a monotherapy in melanoma, and was associated with a survival advantage as compared with chemotherapy (14). In results that bear similarity to those of the BRAF inhibitors class of agents, objective response rates are initially high with MEK inhibitors, but the response is not durable, and resistance to therapy emerges within 6-18 months in most cases (14).



Two phase 3 trials published in 2014 tested whether the combination of these two classes--the MEK and BRAF inhibitors, i.e. dabrafenib or vemurafenib plus trametinib--would improve outcomes and response duration compared with BRAF inhibition, vemurafenib or dabrafenib, monotherapy (12, 15). Robert et al. described evidence that the combination of dabrafenib plus trametinib significantly improved overall survival in previously untreated patients with BRAF V600E or V600K mutation positive metastatic melanoma without increased overall toxicity: the median progression free survival was 11.4 months in the combination vs. 7.3 in vemurafenib only group (15). The authors concluded that by combining trametinib with dabrafenib “results in a significant delay in the emergence of resistant, and longer progression-free survival, with a longer median progression-free survival than with dabrafenib alone... and is superior to vemurafenib monotherapy with regard to all efficacy end points, including overall survival, with no additional overall toxicity (15).” Long et al describe a similar trial comparing the same combination to dabrafenib plus placebo: the median progression-free survival 9.9 vs 8.8 months respectively, and the two groups had nearly identical objective response rates to the Robert et al study (12, 15). While the data supported the authors’ claim that the combination has better response rates than anti-BRAF monotherapy, ~40% of patients w/BRAFV600 mutation did not benefit from the combination and the median improvement in progression free survival was only four months versus vemurafenib monotherapy and only one month versus dabrafenib monotherapy. This evidence also demonstrates

that resistance to BRAF/MEK inhibition occurs in the nearly all cases, indicating that this approach will not cure a significant proportion of melanoma patients.

### *Immune therapies*

Small molecule inhibitors targeted to driver mutations or essential pro-growth signaling pathways are one branch of the new therapies to emerge in the clinical arsenal against advanced melanoma in the last four years. The other branch can be broadly classified as immune-based therapies that harness the immune response to clear the aberrant cells. This branch is comprised of a cell-based therapy, monoclonal antibody-based immunomodulatory therapies, and Toll Like Receptor (TLR) agonists.

### *TIL-based therapy*

The first, Adoptive T-cell therapy (ACT), consist of the isolation and then adoptive transfer of the autologous tumor-infiltrating lymphocytes (TILs) after significant ex-vivo expansion using various treated culture media. Long-term follow-up of patients who have been treated with tumor-infiltrating lymphocytes (TILs) for metastatic melanoma has demonstrated that a significant portion of those treated with this cell-based therapy experienced complete, durable tumor regression (16). A recent review cited a growing body of evidence that mutated gene products may act as the primary immunological targets of TILs that have been extracted and from melanomas and re-administered (16). Several recent clinical

trials studying TIL administration have taken place or are underway. One resulted in objective clinical responses in about 56% of the patients, and importantly with 40% of those responders having complete response to the therapy (16, 17). The authors reported that 95% of the complete responders were long-lasting “i.e. ongoing after 64– 109 months of follow-up (16, 17).” Similar results have been achieved at other centers across the globe (16, 18, 19). ACT of TILs is therefore a life-saving therapy for those patients who respond to therapy, many of which have been purportedly cured. However, ~40-45% of patients do not respond, and the process of T cell maturation is time and labor intensive. In addition, only a subset of metastatic melanoma patients can complete ACT, as many patients may not have a tumor suitable for extraction of TILs or the TILs do not grow adequately. As research centers continue to improve TIL treatments for melanoma, they will also test this platform in other types of cancer.

### *Immunomodulatory Therapeutic Antibodies*

New advances in immunomodulatory therapeutic antibodies continue to redefine the landscape of melanoma therapy. Ipilimumab was the first in its class of immunomodulatory antibodies to be studied, and the first to show a survival benefit in advanced melanoma; it was first to be approved by the FDA in 2010. Ipilimumab is a monoclonal antibody that blocks the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 is a protein receptor found on the surface of T cells that binds to CD80 or 86 on antigen presenting cells; thus CTLA-4 signaling competes with CD28 signaling, and leads to T Cell inhibition,

thereby acting as a potent checkpoint in the immune response. Blocking CTLA-4 leads to enhancement of antitumor activity. Ipilimumab has been shown to improve the rate of survival at 1 and at 2 years, when compared to a peptide vaccine control, among previously treated patients with metastatic melanoma as well as among previously untreated patients who also received dacarbazine (9, 20). Furthermore, long-term follow up of three recent Ipilimumab treatment groups reported 5 year survival rates between 13-25% with one the group following one protocol reporting a 17% complete response rate (21). However, the immune checkpoint blockade is a double-edged sword: high grade autoimmune side effects (immune-mediated enterocolitis, hepatitis, dermatitis, and endocrinopathies) occur in approximately 1-6% of patients and in some cases result in discontinuation of the therapy (22). Ipilimumab monotherapy demonstrated the promise of immunomodulatory antibody therapies in the treatment of melanoma, but also suggested that there was room for improvement.

Another pathway in the immune system has seen application as a cancer therapeutic: the programmed death 1 signaling cascade. Expressed predominantly by T cells, programmed death-1 (PD-1) is a co-receptor. PD-1 binds to its ligands, PD-L1 or PD-L2, in a process essential to the physiologic regulation of the immune system by acting as a negative signal that regulates T-cell activation and proliferation. Thus, a major function of the PD-1 signaling pathway is the inhibition of self-reactive T cells activity, which serves to guard against autoimmune diseases (8). Removal of the PD-1 pathway can

consequently produce a failure of immune tolerance, which ultimately could lead to the development of pathogenic autoimmunity (23). Conversely, tumor cells have co-opted the PD-1 pathway to evade immune surveillance (24, 25).

Therefore, PD-1 pathway has become an attractive disruption target in cancer therapy. Several agents targeted to this pathway have been the subjects of recent experimental study: nivolumab, pembrolizumab, and lambrolizumab will be briefly discussed herein.

Pembrolizumab, is an anti-PD-1 antibody that was FDA approved on the basis of an objective response rate of ~ 25% among patients with advanced melanoma (26). Lambrolizumab, an anti-PD-L1 is currently in Phase 3 trials after a successful phase 2 trial in 2013 that showed response rates ~35%, with a durable response in the majority of patients and an overall median progression-free survival that was longer than 7 months (27).

The third agent, nivolumab, was the subject of several recent reports. Nivolumab is a fully human antibody also directed at inhibition of the programmed death 1 (PD-1). In ipilimumab-refractory melanoma, nivolumab had higher rate of objective response than chemotherapy with dacarbazine (32% vs 11%): the nivolumab treated group had a one-year survival rate of 72.9% compared with 42.1% with dacarbazine. This stage 3 study compared these two drugs in previously untreated BRAF-negative advanced melanoma. The median progression free survival was 5.1 months vs 2.2 and an objective response was

documented in 40% vs 14% of patients in the nivolumab and dacarbazine groups respectively (8).

Nivolumab was also used in a Phase 1 trial in combination with ipilimumab with both a concurrent therapy treatment arm and a sequenced treatment arm (28). Reporting using strict response criteria, the objective-response rate in the concurrent-regimen group was 40% (with the authors stating that any clinical activity was observed in 65% of patient), while the objective response rate in the sequenced treatment arm was 20% (28). Given the serious auto-immune based adverse effects with ipilimumab alone, it is perhaps not surprising that high-grade adverse events (3 or 4) were reported in 53% of the patients who received concurrent therapy vs 18% in the sequenced group (28). Of note, at the highest dosages with an acceptable level of adverse events, 53% of patients had an objective response that consisted of significant tumor reduction of  $\geq 80\%$  (28). The two-year survival of the combined therapy cohort was 80%, far greater than historical non-treatment controls (~25%).

TIL and Immunomodulatory therapeutic antibodies that target immune checkpoints have been established as clinically effective and for some may represent long-term remission (and possibly cure) from disease progression: a triumph of modern medicine in the battle against cancer. A similar pattern to the previously described new therapeutic options is reflected in these results: a subset of patients has significant improvement, especially as compared to previous standard of care chemotherapy. But substantial ground remains to be

covered: TIL, anti-CTLA-4, and anti-PD-1 inhibitor studies reveal large proportions of patients who do not respond to therapy, and to date no predictive biomarkers have been identified and some patients lack the appropriate T cells in their tumors to be candidates for TIL therapy. Current opinion is that response biomarkers may someday help to predict candidates who would benefit from therapy, but to date no such markers have been validated, e.g. prognostic role of for instance PD-L1 status has yet to be determined(8).

#### *Toll-like receptor agonists*

Chemical agents that exhibit the ability to potently activate the innate immune response have been tested and used as therapeutics to treat skin cancers. Particular attention has been focused upon Toll-like receptor (TLR) agonists in the family of compounds known as the imidazoquinolines, e.g. imiquimod and resiquimod, which activate TLR 7 and TLR 8 and consequently, induction of nuclear factor-kappa B (NF- $\kappa$ B) (29). Imiquimod, an agonist thought to act predominately at TLR 7, was originally approved for the treatment of actinic keratosis and external genital warts. The compound has been found to be efficacious for basal cell and squamous cell cancers, and the FDA subsequently approved the use of topical imiquimod for the treatment of superficial basal cell carcinoma and squamous cell carcinoma (30). Further studies, mostly case reports, have evaluated the potential usefulness of imiquimod as a topical treatment for dermal metastases of melanoma that are not readily managed with

surgery (29). However many of these reports included other treatment modalities, complicating a pure assessment of the efficacy of imiquimod.

For instance A phase I/II clinical trial suggests that imiquimod effectively treats many superficial dermal and subcutaneous melanoma metastases (31). In point of fact, Imiquimod was combined with intralesional Bacille Calmette-Guerin (ILBCG) in order to induce regressions of a portion of lesions in patients with mixed dermal and subcutaneous disease. The authors' conclusion was that imiquimod alone could be used to effectively control dermal disease, but subcutaneous disease was better treated in combination with ILBCG(31).

Alternative mechanisms of actions of imiquimod, besides a TLR-mediated response, have been proposed. Several groups have published evidence that argues for high dose imiquimod directly triggering apoptosis induction of apoptosis via Bcl-2 and caspase activation (32, 33). Another report looked at the vehicle for Aldara, the non-generic formulation of imiquimod, which is known to cause psoriatic-like inflammation when applied to murine epidermis (33). The authors of this study concluded that the vehicle for the drug, citing evidence pinpointing the isostearic acid component, was sufficient to induce inflammation in cultured keratinocytes and drawing into question whether or not imiquimod was solely responsible for all the therapeutic activity seen clinically (34). However, multiple vehicle-controlled, double blind studies have shown imiquimod efficacy in treating non-melanoma skin cancers (35, 36).



## A mouse model of melanoma

In order to further explore research questions about the genetic basis of melanoma development, the formation of melanoma metastasis, and potential therapeutics, a novel mouse model has been developed to investigate specific genetic mutations within the melanocytes of mice by the Bosenberg lab. The melanoma mouse model referred to has been constructed from a transgenic mouse line, which was previously engineered and characterized to express a Cre recombinase-estrogen receptor fusion protein controlled by a melanocyte-specific tyrosinase promoter ( $Tyr::CreER^T$ ) (37). The mouse model's genetically engineered system allows for the inducible recombination of genes that contain lox sites flanking targeting the relevant DNA sequences of known melanoma genetic hits. Targeted DNA excision is reproducibly induced by the application of topical 4-hydroxytamoxifen (4-OHT), an estrogen agonist that binds to the estrogen receptor fusion protein in a spatially and temporally restricted manner (38). Following the demonstration of functionality of the  $Tyr::CreER^T$  lines, lox knock-in and knockout lines of genes relevant to human melanoma were acquired including *Braf*, *Pten* and *Bcat*.

As previously described, the B-raf serine/threonine kinase is one of the most commonly mutated genes known in human melanoma, with about 50% of melanomas containing activating mutations of BRAF (usually *Braf* V600E) (39). Martin McMahon's lab at UCSF produced a knock-in allele of activated BRAF

(BRAF<sup>CA</sup>) that is phenotypically wild type but after Cre-mediated recombination produces a constitutively active allele that contains the V600E point mutation found in human melanomas. This recombinant gene product retains a single intronic 34bp loxP site and the activating point mutation, but otherwise resembles the wild type chromosome exactly (38).

Studies of human melanoma tumor samples have revealed that the Pten tumor suppressor is markedly reduced in about 30% of advance melanoma, but the Pten gene itself is only mutated in a small proportion of melanomas (40). The Bosenberg lab acquired a Pten allele which has lox sites inserted so as to flank exon 5 (38). Subsequently, various mouse genotypes have been actively generated: previous work in the lab analyzed 4-OHT-treated mouse cohorts in which these targeted genes, Braf and Pten, as well as others key melanoma genes (Cdkn2a, p53, etc.) have be manipulated individually and in combination(s). When the inductions of genetic recombination are performed with a relatively high concentration of topical 4-OHT (50mg/ml in DMSO) applied to the mouse on days 3, 5, and 7 of life, the inductions result in a widespread recombination of lox-containing alleles specifically in melanocytes. Induction of constitutively active Braf<sup>V600E</sup> construct in melanocytes results in formation of small melanocytic nevi that stop growing after 3-4 weeks (37). Other genetic hits that have been experimentally examined in this system have not yielded a markedly abnormal melanocytic phenotype when altered individually. In contrast, when combinations of key genes have been recombined using 4OHT induction,

these combinations have yielded useful tumorigenic and metastatic phenotypes. Induction of  $\text{Braf}^{\text{V600E}}$  combined with loss of Pten yields a dramatic melanoma phenotype (Braf/Pten tumor model). When recombination is induced in melanocytes throughout the mice within the first week of postnatal life, greater than 10,000 melanomas form without detectable latency. In contrast, when recombination is induced locally by application of 2  $\mu\text{L}$  4-OHT solution on flank skin after weaning, localized melanomas form and grow to 1  $\text{cm}^3$  in 6-8 weeks without fail. This model allows for the production of melanoma driven by mutations relevant to human melanoma in an immune competent setting.

In addition to these two key gene targets in the melanoma mouse model, the Bosenberg lab developed a novel genetically engineered model based on the Braf/Pten model with the addition of an inducible gene product that results in the stabilization of beta catenin ( $\text{Ctnnb1}^{\text{loxex3}}$ ), resulting in constitutive activation of Wnt pathway signaling (41). This particular model, Braf/Pten/Ctnnb1, adds the activation of Wnt-pathway signaling in the context of Braf activation and Pten loss. As demonstrated by previous work in the Bosenberg lab, this triple gene combination results in >20 fold increase in lung and lymph node metastases after induction, and a 100 to 1000 fold increase in the expression of canonical melanoma differentiation antigens relative to the Braf/Pten model (41).

## Preliminary Data

The Braf/Pten mouse model therefore presents an opportunity to study the Braf inhibition in a preclinical animal model. Most *BRAF*<sup>V600E</sup>-mutant human melanomas respond to vemurafenib (10). The mechanism of either intrinsic or acquired resistance in BRAF-mutated melanomas to vemurafenib is variable, but includes activation of PDGFR signaling, IGF-1R signaling, or acquisition of mutations and other mechanisms that activate MAPK pathway signaling (42, 43). Loss of PTEN or increased PI3K activity has been hypothesized to be a mechanism of resistance to BRAF inhibitors. This was evaluated in the mouse melanomas by determining the dose response *in vitro* of Braf/Pten melanoma cell line—cell lines generated from tumor samples derived from induced mouse tumors—to the Braf inhibitor vemurafenib. The IC<sub>50</sub>s ranged between 200 and 800nM in three lines, demonstrating that Pten loss does not necessarily mediate intrinsic resistance in this system (unpublished data). Furthermore, several resistant mouse melanoma lines were generated by chronic *in vitro* exposure to vemurafenib, with IC<sub>50</sub> concentrations of >10 μM in resistant clones (unpublished data). This supports the hypothesis that the Bosenberg mouse Braf-driven melanoma models are responsive to BRAF inhibitors and that resistance to BRAF inhibitors which develops has features that resemble those seen in human vemurafenib-resistant melanomas; this melanoma mouse model represent a useful preclinical system for studying the effects of BRAF inhibitors.

In vivo experiments in the Bosenberg lab, in which the Braf inhibitor compound PLX4720 was administered in mouse chow, demonstrated that Braf inhibition resulted in reduced tumor growth. Overall survival endpoints (primary tumor volume  $>1 \text{ cm}^3$  at the site of topical 4OHT induction, or secondary spread), were not reached in treated mice in greater than 12 months, versus rapid development of induced tumors to endpoint ( $\sim 2$  months) in untreated animal controls. In addition, histological evidence of growth arrest (reduced Ki67) and pathway inhibition (reduced pMek and pErk) was present in treated tumors following 4 and 8 days of treatment (unpublished data).

In addition to testing Braf inhibitors in this model, this system has also been the subject of immunomodulatory therapeutic antibody studies. This author's predecessor in the Bosenberg lab, Laura Huang, conducted initial experiments on anti-mouse CTLA-4 antibodies as a treatment for induced tumor growth in the Bcat/Braf/Pten melanoma mouse model. Her analysis of this treatment showed no significant difference between untreated controls ( $n=12$ ) and those receiving the antibody ( $n=5$ ) in terms of percentage survival as measured to tumor size endpoint (44). Subsequently, a pilot study by Laura Huang consisting of a similar trial with anti-mouse PD-L1 antibodies on a cohort of mice ( $n=7$ ) failed to show decreased tumor growth (unpublished data).

Thus BRAF inhibition successfully treats mice with induced Braf/Pten/Bcat tumor growth, but single agent immune checkpoint blockade failed to stop or slow

Braf/Pten/Bcat tumor growth. This later preliminary data presented an experimental challenge to determining approaches to therapeutically effective immune checkpoint blockade; this observation was explored further in the experiments presented herein.

### Statement of Purpose

BRAF inhibitors and immune therapies, including anti-CTLA4 and anti-PD1, have shown great clinical promise. Additionally, there is encouraging data about the ability of TLR agonists, e.g. imiquimod, to promote an effective immune response to tumors. Given the encouraging data from new human clinical therapeutic trials, there is a clear need to develop approaches that combine these treatment strategies. This is an obvious approach and despite a lack of preclinical data, human clinical trials of some of these combination therapies are underway, as described above (28). These trials will likely determine the efficacy of specific individual combination therapies; however, even with biopsy and biomarker correlation, the ability to evaluate the mechanism of why the combined therapies are effective (or not) is likely to be suboptimal. Prior and ongoing immunotherapy trials suggest that partial or complete responses will occur in a subset of patients, however to date it has been difficult to prospectively predict which patients will respond. In particular, it is difficult to make meaningful conclusions about the characteristics of melanomas that respond, as performing sequential biopsies or

controlling for tumor or immune genetics are not possible in human patients.

Understanding the mechanisms of effective combination therapies and rational optimization of future therapies will require testing in a pre-clinical animal model of Braf<sup>V600E</sup>-driven melanoma in the setting of an intact immune system.

In order to better understand the mechanisms of action of these combined therapies and to better inform future combination therapy clinical trials, I measured responses to combined therapies involving PLX4720, anti-CTLA4, anti-PDL1, and imiquimod. I hypothesized that effective melanoma treatments can be developed using synergistic combination therapies in mouse melanoma models. To this end experiments were undertaken using the Braf/Pten/Bcat conditional inducible mouse melanoma model to develop combination therapies with PLX4720, a tool compound with similar features to vemurafenib, as well as immune therapies that include mouse analogues of anti-CTLA4 and/or PD1 immune checkpoint blockade and/or topical imiquimod. The next step of analysis would then be based on the mechanistic insights derived from these mouse studies. The purpose of this work was to translate the findings into applications in future human clinical trials of combination therapies that result in more frequent and more durable responses.

### **Specific Aims:**

These experiments evaluated the effects of combinations of PLX4720 Braf

Inhibitor chow, 5% topical imiquimod, and Anti-CTLA-4/Anti-PD-L1 treatments on Bcat/Braf/Pten mouse melanoma's overall tumor size and survival (as measured by an endpoint of 2 cm<sup>3</sup>). The treated mice were followed for up to a maximum of an additional 90 days post treatment. Significant effects on overall survival will be determined using Kaplan-Meier survival curves and the log-rank statistic.

### **Methods:**

The work herein was completed by Billy Lockhart (BJL) unless otherwise noted.

#### Animal Model

The mouse colony was maintained at the Yale University School of Medicine Animal Facility and the Yale University Animal Care and Use Committee approved all animal protocols. In the facility, the mouse colony was maintained under normal parameters, including unrestricted access to fresh water and chow, frequent bedding changes, and were checked daily by animal facility staff or by this author. Mice were housed in cages in a secured room with the lighting set to a 12-hour on/off cycle.

All of the mice in this study were obtained from breeding congenic Tyr::CreER

Pten<sup>flox/flox</sup> Bcat<sup>sta/sta</sup> mice with Braf<sup>V600E/V600E</sup> Pten<sup>flox/flox</sup> mice from the

Bosenberg Lab. All strains were on a congenic C57Bl/6 background. Verification of the mouse strain genotypes was completed prior to recombinant breeding and induction. The Tyr-CreER, Braf, Pten and BCAT mouse strains were genotyped



and assayed for recombination as previously described by the Bosenberg Lab (41).

For tumor inductions, 4-hydroxytamoxifen (4-OHT) (#H6278, 70% z-isomer from Sigma Aldrich) was dissolved in DMSO to a concentration of 50 mg/ml. For localized tumor inductions, a mouse was selected at postnatal day 21 and the hair between the shoulder blades was removed with a topical depilatory. Next, 1 $\mu$ L of 4-OHT at 8.3 mg/ml (dissolved in 1 part DMSO and 5 parts 100 percent Ethanol) was directly applied to the skin of the mouse in the hairless area.

Tumor measurement and necropsy methodology:

The mice were monitored daily for health status and tumor growth. Tumor size was captured using a digital caliper measuring three dimensions of the length (L), the width (W), and the height (H). The tumors were measured every 7 to 10 days. The volume of the tumor was calculated using a formula for the volume of a half sphere:  $0.523598 \times L \times W \times H$ . If mice developed secondary tumors, the initial tumor was designated as the primary tumor and was measured separately from any other latent tumor development, which were subsequently labeled by ordinal number, i.e. 2<sup>nd</sup>, 3<sup>rd</sup>, etc.; in the end only primary tumors were included in analysis.

Tumor endpoints were established by animal protocol as tumor volume as 1 cm<sup>3</sup> or ulcerated tumors, or marked decompensating in animal's health status (severe weight loss, markedly decreased movement, inability to self-care). For these experiments, the tumor volume endpoint was approved up to 2 cm<sup>3</sup>. Mice were anesthetized and sacrificed according to Yale University Animal Care and Use Committee protocols. Tumors were resected and samples for genetic analysis were frozen in -80 C or were stored in formalin and sent for slide preparation in the Yale Dermatopathology Department for later immunohistochemical processing.

## Treatments

Male mice were selected at weaning and rechecked at outset of the treatment. The mouse's treatment cohort was randomly selected, and the experimenter, this author, was aware of the treatment group of each cohort and administered all drugs. Mice were candidates for inclusion if their primary tumor size was between 2-6 mm in length or width or 1.5 mm in height, usually ~3 weeks after induction. Mouse IgG2b Anti-murine CTLA-4 (clone 9D9) and Rat IgG2b Anti-Mouse PDL-1 (clone 10F.9G2) were administered via intraperitoneal injection in sterile PBS once a week at dosages equivalent to 5 mg/kg according to the mouse's weight at time of administration. The mice that received combination therapy of anti-CTLA-4 and anti-PD-L-1 (combo) received both intraperitoneal injections on the

same day. Both of these antibodies were purchased from BioXCell. The Vemurafenib analogue chow, known as PLX4720, was provided to respective cohorts as their sole nutrition source (~100mg/kg per day) and was replaced weekly; Plexxicon provided the chow to the lab. Imiquimod 5% (generic by Fougera) topical cream was administered by small measuring spatula directly to the superficial surface of the tumor covering it completely in one layer, 5 days per week; the total amount of imiquimod applied was therefore proportional to the tumor's surface area. The mice that received combination therapy of anti-CTLA-4 and anti-PD-L-1 (combo) received both intraperitoneal injections on the same day. Table 1 lists the treatment cohorts.

Table 1: Treatment Cohorts

Drug	Dosing schedule	Dosage (route)
Anti-CTLA-4 & anti-PD-L1 (combo)	1x per wk	5mg/kg IP for each drug
PLX4720	chow - sole food source	~100mg/kg per day
PLX4720 + Combo	1x per wk + chow	As above
Imiquimod	5x week	~25mg (topical)
Imiquimod plus Combo	5x week + 1x per wk	As above

## Immunohistochemistry

IHC was performed on formalin-fixed, paraffin embedded tumor sections. Slides were processed using Vectastain ABC –Alkaline Phosphatase kit (Vector, #PK-5000) and developed with a red alkaline phosphatase substrate kit (Vector, #sk-5100), according to the manufacturer's instructions. Responsible investigator: BJL with assistance from Katrina Meeth and Goran Micevic.

## Antibodies used

Anti-F4/80 antibody [Cl:A3-1] (ab6640) from abcam

Anti-CD45 antibody (ab10558) from abcam

## Statistical Analysis

Graphpad Prism statistical analysis software was used for all analyses. Kaplan-Meier survival curves were constructed and significance was determined using the log-rank (mantel cox) test. For comparison of pooled data with two data sets unpaired t-test were used.

## Results:

A cohort of 7 untreated male  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice from two different litters had tumors induced on day 22. The Tumor growth was tracked over four months until the tumor sized reached endpoint of 2 cm<sup>3</sup>. Figure 1 shows the measurements of tumor volume over this period of time.

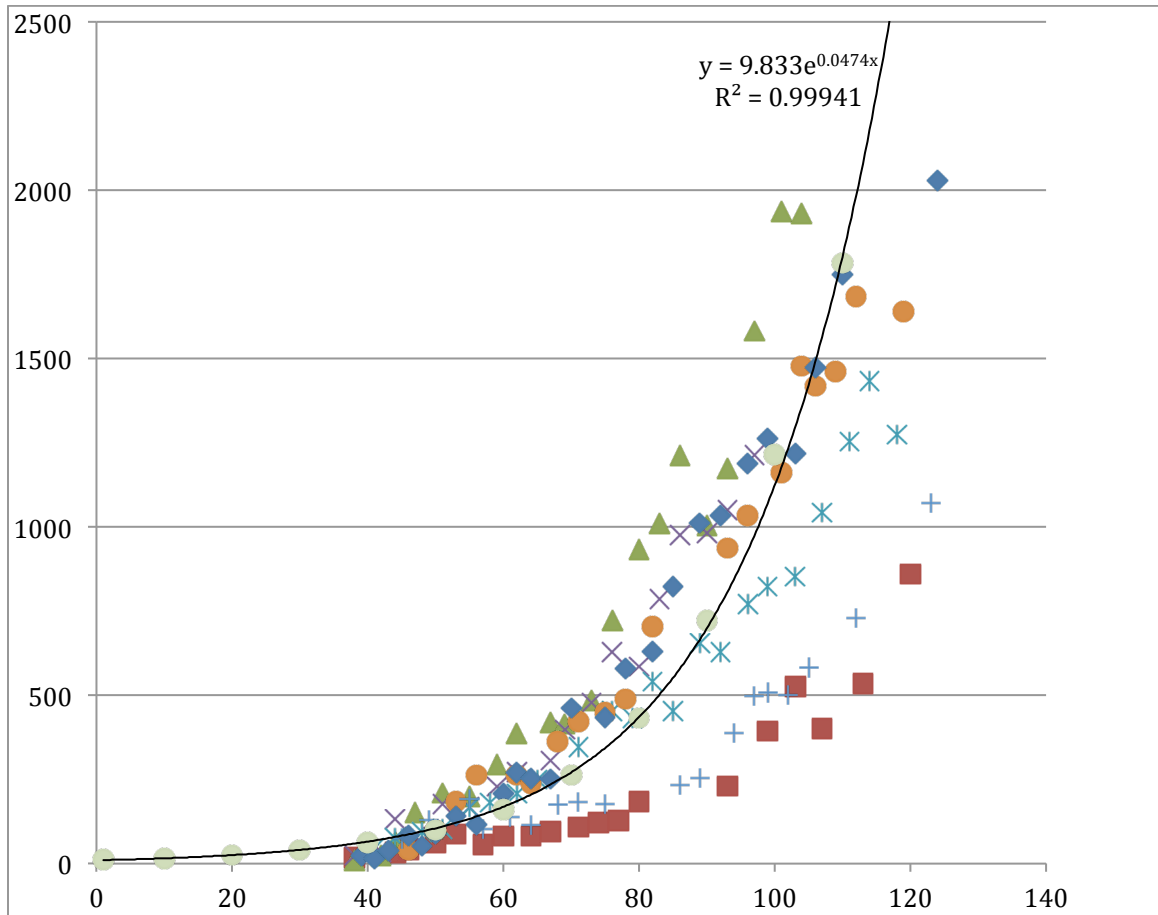


Figure 1: Average tumor growth velocity. X-axis represents size of the tumor in millimeters cubed and y-axis represents day since birth. 7 untreated male mice with tumors induced on day 22, where were recorded over a period of 4 months. Using Excel software, a best fit line was calculated for each line and the resulting functions where used to calculate a trend line at precise 10 day intervals. Each different symbol represents a unique animal's tumor growth curve.

The chart shows the range of tumor size at various time points, and the trend line provides a rough approximation of when the average tumor size at various points in time. By this calculation, the trend line shows that a given tumor's volume would most likely reach  $1 \text{ cm}^3$  in approximately 76 days post-induction, and the

same tumor would reach 2cm<sup>3</sup> volume in 90 days. The fastest growing tumors in this cohort grew to 1cm<sup>3</sup> in ~52 days and the slowest grew to 1cm<sup>3</sup> in ~100 days.

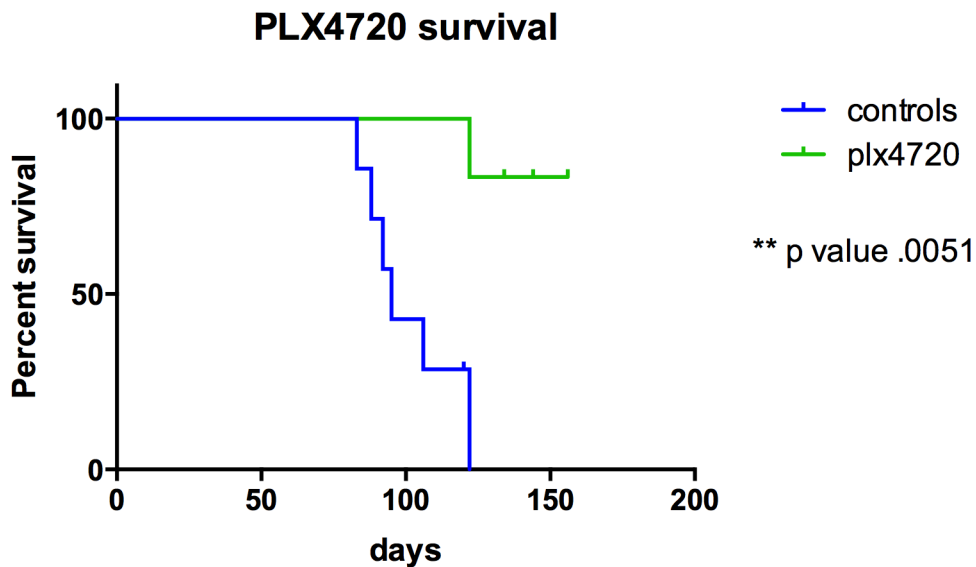


Figure 2: Kaplan-Meier analysis of  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice that received Plx4720 chow (n=6) and those mice that received no treatment (n=7) starting 3 weeks post induction (day 45) of melanomas.

The 6 animals treated with the PLX4720 received their treatment continuously, as past experience indicated that after stopping the chow tumor growth would restart. The survival curve in figure 2 shows the difference in survival past 100 days. For animals not receiving treatment median length of survival was ~100 days. PLX4720 treated cohort did not reach median survival point. There is a significant difference in time to endpoint when the  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice received Braf inhibitor therapy versus controls ( $p=0.0051$ ).

### Survival proportions: Survival of combo survival

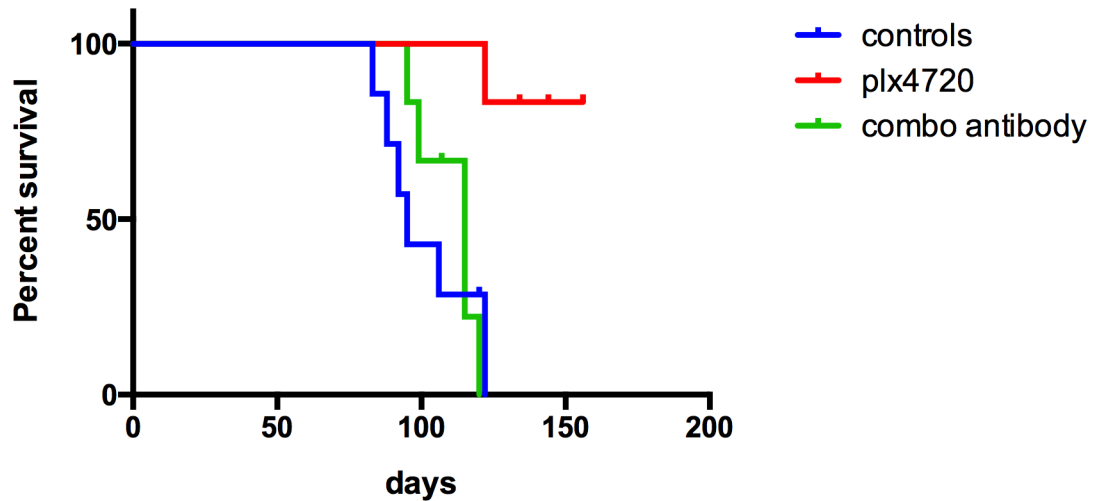


Figure 3: Kaplan-Meier analysis comparing percent survival of  $\text{Braf}^{\text{wt}/V600E} \text{Pten}^{-/-} \text{Bcat}^{\text{stab}/\text{stab}}$  mice treated with the Braf inhibitor Plx4720 chow (n=6), the treatment arm receiving weekly combined Anti-CTLA-4/Anti-PD-L1 treatment (n=6), and those with no treatment (n=7) starting 3 weeks post induction (day 45) of melanomas.

The treatment arm (n=6) receiving combination immune checkpoint point blockade antibodies, anti-CTLA-4 and anti-PD-L1, did not vary significantly from controls in terms of time to endpoint, i.e. survival (p=0.77). Figure 3 compares both the survival percentage of the combination checkpoint blockade group and Braf inhibitor group (plx4720) to untreated controls with induced tumors.

Additionally, a cohort of mice (n=4) received the two checkpoint blockade antibodies in addition to chow with PLX4720 (Figure 4). The percent survival of this cohort differed significantly from the untreated group (p=0.42), but this difference is attenuated when comparing this treatment group to the mice that

received the PLX4720 Braf inhibitor chow only (Figure 5); when compared to Braf inhibition alone, there was not a significant difference in overall percentage survival (Figure 5).

### PLX4720 + Anti-CTLA-4/Anti-PD-L1 Combination

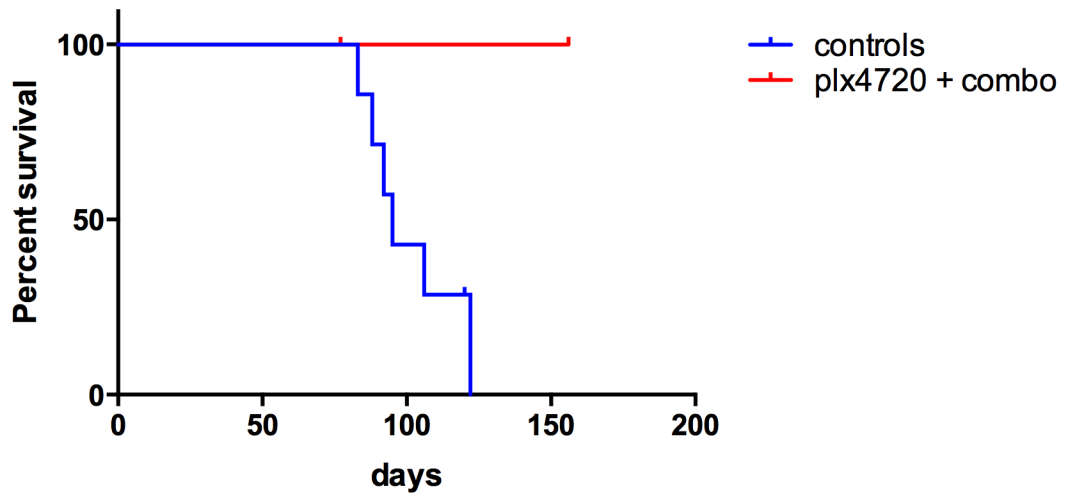


Figure 4: Kaplan-Meier analysis comparing percent survival of  $\text{Braf}^{\text{wt}/\text{V600E}} \text{Pten}^{-/-} \text{Bcat}^{\text{stab}/\text{stab}}$  mice treated with the Braf inhibitor Plx4720 chow in addition to Anti-CTLA-4 and Anti-PD-L1 (n=4), and those with no treatment (n=7) starting 3 weeks post induction (day 45) of melanomas.



### Survival proportions: PLX4720 +/- AntiCTLA-4/AntiPD-L1

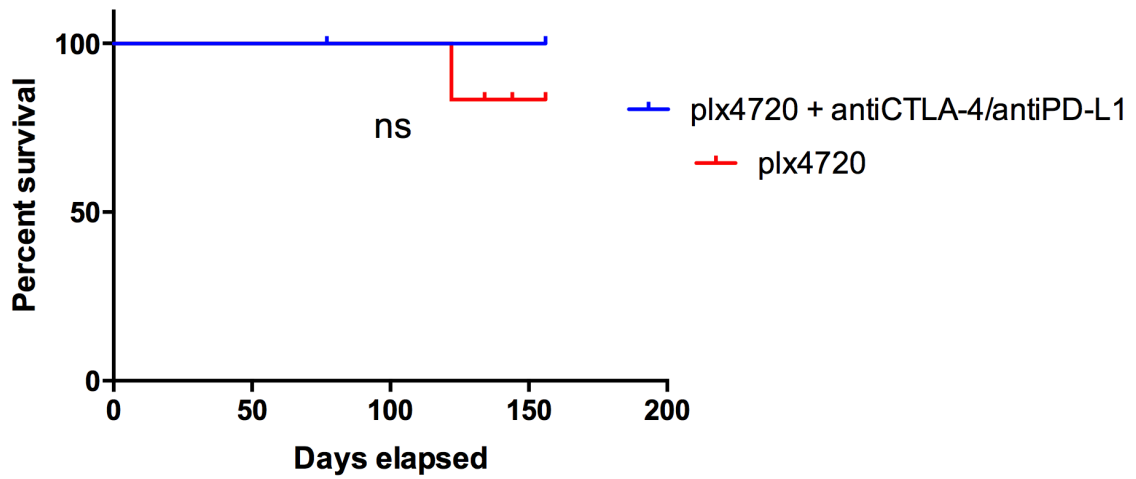


Figure 5: Kaplan-Meier analysis comparing percent survival of  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice treated with the Braf inhibitor Plx4720 chow in addition to Anti-CTLA-4 and Anti-PD-L1 (n=4), as compared to the percent survival of the group treated only with Plx4720 (n=6) starting 3 weeks post induction (day 45) of melanomas.

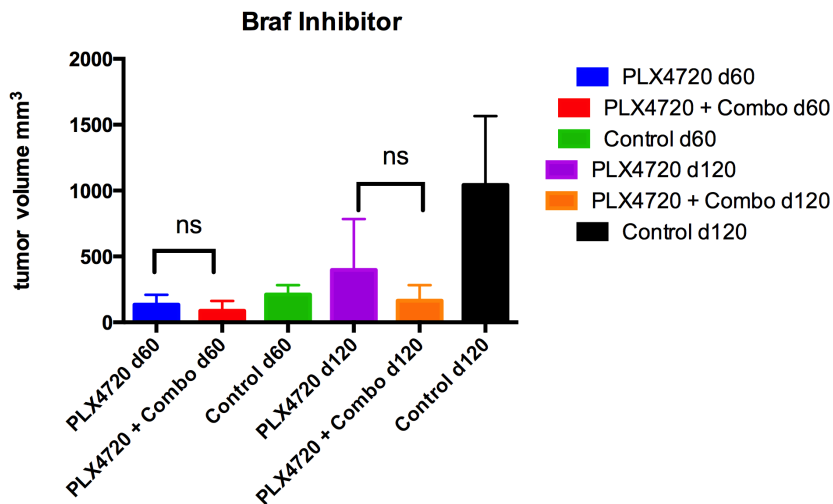


Figure 6: Unpaired t-test analysis comparing mean tumor volume (mm<sup>3</sup>) per treatment cohort at approximate day 60 (5.4 weeks of treatment) and day 120 (14 wks of treatment).

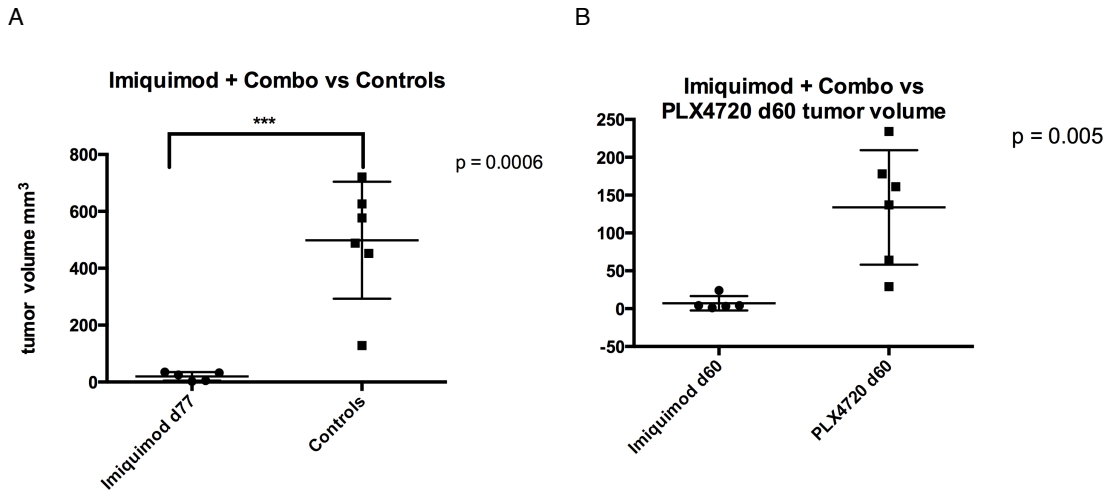


Figure 7. A) An unpaired comparison analysis of the individual tumor volumes (mm<sup>3</sup>) in the plx4720 only versus plx4720 plus combination immune checkpoint blockade antibodies at d60, ~38 days post induction. Panel B shows the same analysis done with tumor volumes at d120, ~98 days post induction.

Figure 6 depicts results of statistical comparison between the mean tumor size at early and late time points for each treatment arm (PLX4720 alone, with Anti-CTLA-4/Anti-PD-L1, and controls). The difference in tumor size when comparing the two treatment arms was not significant at either time point. Figure 7A and 7B shows a similar analysis with the spread of tumors sizes for the two treatment arms at the respective time points.

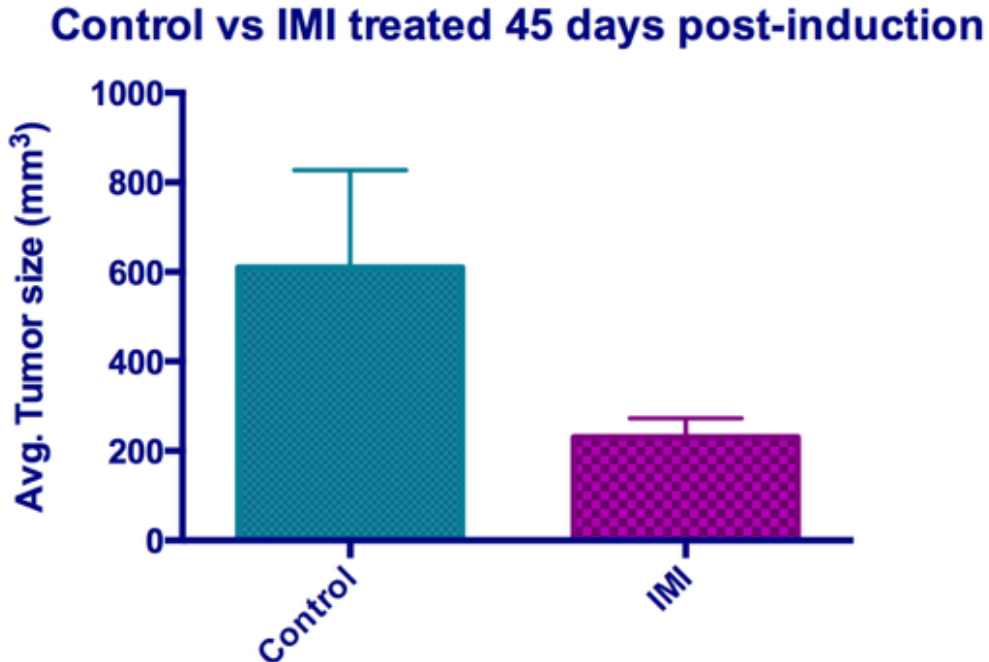


Figure 9:  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice (n=6) were treated with 5% topical imiquimod and their mean tumor volumes (mm<sup>3</sup>) were compared 45 days post induction to a group of control mice (n=6) with untreated tumors. This experiment was completed by Katrina Meeth

Imiquimod demonstrated a significant effect on the inhibition of tumor volume (mm<sup>3</sup>) in mice (n=6) after 4 weeks of treatment when compared to untreated control mice with induced melanomas (n=6) (Figure 9). When imiquimod was applied to tumors and the treated mice were administered the combination anti-CTLA4/anti-PDL1 (Figure 10, 11), the treatment was highly effective in retarding tumor growth.

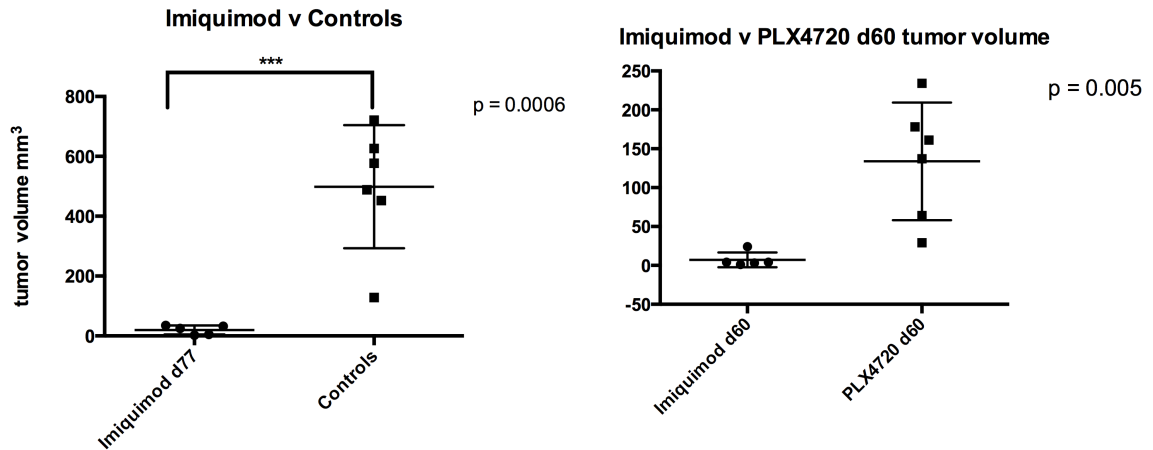
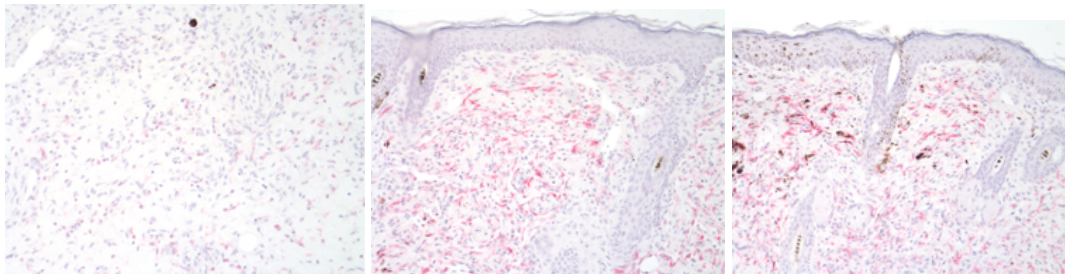


Figure 10:  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mice (n=5) were treated with 5% topical imiquimod and combination Anti-CTLA-4/Anti-PD-L1 and their mean tumor volumes (mm<sup>3</sup>) were compared to a group of control mice (n=6) with untreated tumors at day 77. Figure 11: the same imiquimod treatment cohort compared to a plx4720 cohort (n=6) at day 60.

Untreated F4/80 staining      Imiquimod tx'd F4/80 stained



Untreated CD45 staining      Imiquimod tx'd CD45 stained

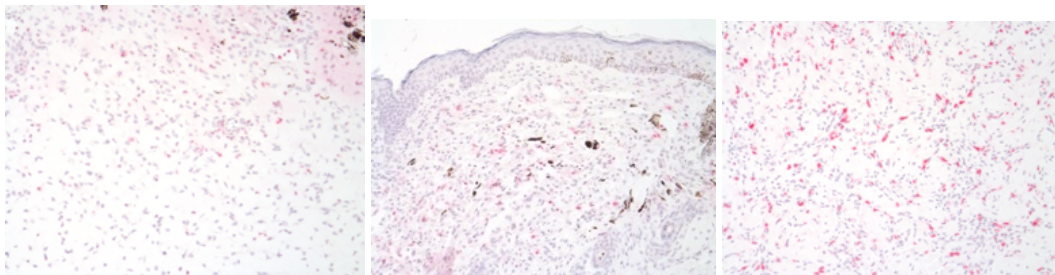


Figure 12: Immunohistochemical staining of tumor samples taken from untreated  $Braf^{wt/V600E} Pten^{-/-} Bcat^{stab/stab}$  mouse tumors compared to imiquimod treated tumors. The top row shows staining for F4/80 and the bottom row show staining for CD45.

Samples from imiquimod treated tumors were embedded and sectioned for histology. Immunohistochemical assays demonstrated that treated tumors had a increased F4/80 staining increased CD45 staining (Figure 12 Additional results from preliminary, but not fully validate, immunohistochemistry suggest that CD3 staining in these imiquimod treated samples appears to be decreased.

#### Discussion:

This worked examined various combinations of cancer drugs in a preclinical mouse model. The mouse model offers many advantages; particularly as it has been genetically engineered to share driver mutations identical to human melanomas and the mouse maintain their native, intact immune system. In these therapeutic trails, the primary endpoints were directly measured, and only this author measured the tumor dimensions to avoid inter-operator variability.

During the initial pilot phases of these experiments, a measurement optimization was undertaken by using triplicate measurements by the same observer averaging the values to ensure best possible accuracy and to established rough parameters for measurement error (estimated at +/- 1mm for any given dimension measurement). The spread of the tumors sizes for given time points are quite large, and their growth curve velocity can differ significantly. This large spread has implications on the detection of difference in tumor response (Figure

1). Previous experimenters working on the same model assessed tumor dimensions bi-weekly. However, considering the initially slower pace of tumor growth after induction (Figure 1), bi-weekly measurements were deemed to easily fall within the +/- 1mm error range, and as such the tumors measurements were spaced out to every 7 to 10 days for these trials in order to better capture actual changes in tumor size. Furthermore, variability in the growth characteristics of a given tumor, including growth rate and shape, and this lack of uniformity in tumor physical characteristics were in part due to the difficulty in administering the 4-hydroxy-tamoxifen due to uneven spread of the liquid over the non-flat surface of the animal's hind quarters and the rapid evaporation rate. Previous experiments in the lab established that less spread produced a smaller initial induction area and a tumor with decreased growth velocity when compared to larger spread/larger induction areas (44). Initial attempts to optimize this induction procedure were tried, including injection of induction material intradermal as well as subcutaneously, but the piloted results were not adequately consistent. Larger sample sizes may have improved the statistical comparisons and in future iterations of these trials in this system; these observation supports using more animals per treatment arm.

Another possible explanation for growth velocity variation may have to do with uncharacterized genetic factors. Note that un-induced mice have a 20-25% chance of developing spontaneous melanomas within 6 months in the absence of

4-OHT induction, an observation attributed to “leaky” Cre expression. In this author’s experience, these spontaneous tumors are unpigmented, uninodular, and dermal and can appear at any surface point of the affected mouse. Once they appear, these tumors grow quite rapidly.

Experimental data at present suggests that anti-CTLA-4 therapy is not better than control in the *Braf/Pten/Bcat* mouse model. Similarly, neither single agent anti-PD-L1, nor the combination of the two antibodies appears to effect survival significantly versus controls (figure 3). The results of the trial testing BRAF inhibition in combination with anti-CTLA-4/anti-PD-L1 have not yielded significant results to date (figure 5). This begs the interesting question: why? Are the mouse analogues of the human antibodies not as effective at inhibiting their target? Mice lacking CTLA-4 die at an age of 2–3 weeks secondary to massive lymphoproliferation (45), clearly the protein plays a significant role in down-regulating the immune system in mice. In 1995, Kearney et al showed that blocking CTLA-4 in mice greatly enhanced antigen specific clonal expansion, but those experiments used a specially engineered antibody in a Fab form “as described by Wassau” (46). Severe immune side effects in human patients on anti-CTLA-4 therapy have been documented (22), yet no severe side effects were noted in these trials (5mg) or in previous trials in the labs at twice the dose (10mg/kg the highest dose given) in humans during phase II trials. Furthermore animals were dosed weekly as opposed to every third week. A future experiment designed such that multiple mouse anti-CTLA-4 sourced from different

manufacturers is administered in increasing doses until side effects are observed may help characterize how the mouse immune system responds to the anti-body. Or perhaps the Wassau method should be resumed for anti-CTLA-4 antibody production.

Another compelling difference between the mouse model and human melanomas is how few mutations they possess – melanomas are known to contain a relatively high number of mutations and also to be relatively antigenic (2). The three induced mutations in the model suffice for melanoma growth but may be sub-threshold to activate the immune system's surveillance. It's worth noting that when immune checkpoint inhibitors are administered to humans in clinical trials a substantial fraction have no response, as high as ~85%.

It is possible that specific genetic drivers of melanoma and other cancers can be associated with induction of an immunosuppressive tumor microenvironment. In unpublished work from the Gajewski lab at the University of Chicago, it appears that the Braf/Pten/Cnntb1 model induces potent local tumor microenvironment immunosuppression compared to the Braf/Pten model. These findings may indicate the reason for the relative lack of effect of the combined anti-CTLA-4 and anti-PD-L1 therapy in the Braf/Pten/Cnntb1 model.



As had been supported by prior experiments, results from treatment cohorts of mice receiving the Braf inhibitor chow, PLX4720, demonstrated that the drug effectively inhibits tumor growth for the life of the animal as long as the treatment is continued. Adding Anti-CTLA-4 and Anti-PD-L1 offered no significant benefit in efficacy (figure 5). A human trial that combined vemurafenib and ipilimumab was stopped due to toxicity when the drugs were used in combination (47), an effect not noted in the combination trial in the mouse model. A recent collaboration between the Bosenberg and Kaech laboratories established that T-cells are involved in the Braf-inhibitors mechanism of growth suppression (48). However, based on preliminary analysis of mouse melanoma responses to PLX4720, induction of apoptotic cell death is minimal. Therefore it is possible, that PLX4720 treatment alone may be insufficient to generate effective immune responses in combination with anti-CTLA4 and/or PD1 pathway inhibition.

Another difference between these trials and the standard of care in humans is surgical excision. Humans usually present with a primary melanoma on the skin, which is subsequently almost always surgically removed; the administration of systemic agents is reserved for metastatic disease. Does post-surgical excision wound healing alter the tumor microenvironment in ways fundamental to generating a more effective T-cell response to melanoma cells? Is cell death a prerequisite to step to activating a T-cell response that can be further enhanced by immune checkpoint blocking antibodies? Laura Huang, this author's

predecessor in the Bosenberg lab, has performed experiments administering a brief pulse of treatment with a cytotoxic agent (temozolomide) before initiation of immune therapies in established tumors results in minor improvements in survival of treated mice, but her data show the median end points are identical between chemotherapy alone or with anti-CTLA-4 therapy(44). Several pilot experiments in which tumor bearing mice receiving treatment has tumors directly injected with cytotoxic agents or inoculation with cultured melanoma cell lines that had been lysed did not result in encouraging results (unpublished data).

Treatment with 5% topical Imiquimod significantly retards tumor growth and thereby increases time to end point in this model (figures 10,11). Imiquimod causes visible inflammation at application site, massive splenomegaly, and weight loss when applied at high doses in this mouse model, according to previous work by Katrina Meeth. At the doses used in these experiments, no such side effects were noted. Perhaps higher doses of imiquimod combined with immune checkpoint blocking agents would provide the impetus for an effective immune response. Immune cell infiltrate characterization of treated tumor samples reveals increased macrophage subsets. Preliminary FACS analysis of imiquimod treated samples does not yet show a consistent pattern of increased T cell populations. RNA Seq Gene expression analysis could yield interesting leads to follow up in future experiments that would in form ongoing and future human

clinical trials involving these agents and are likely to be critical to the success of the proposed experiments.

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