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Modulation of Antitumor Immunity by the Mek inhibitor Trametinib: Implications for Targeted Therapy of Cancer

Michael J. Allegrezza
University of Pennsylvania, mallegrezza23@gmail.com

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Abstract
Through rational drug design, much progress has been made to develop small molecules that specifically inhibit the oncogenic signaling pathways driving malignant growth. However, the normal function of immune cells depends upon many of the same pathways inhibited by such targeted cancer therapies. Because the immune system can influence the growth of many cancers, I hypothesized that most small molecule inhibitors would have activity on leukocytes relevant in cancer, and this activity would contribute to their antitumor mechanisms. In order to test this hypothesis, I first screened a panel of over 40 small molecule inhibitors for their activity on proliferating human cancer cells and human T cells. Almost every small molecule inhibitor I tested had detrimental activity on human T cells at the concentrations required for limiting tumor cell proliferation. However, when I focused on the FDA approved MEK inhibitor trametinib, I found that some common γ-chain cytokines were able to rescue T-cell functions blunted by trametinib. Notably, an IL-15 agonist, ALT-803, could rescue the in vivo proliferation of tumor-antigen specific T cells in mice treated with trametinib. I developed a p53-/-KrasG12D+Myristoylated-p110α+ murine breast cancer model to perform tumor challenge experiments in a model only weakly sensitive to trametinib, a setting where combination with immunotherapy may be clinically useful. In this tumor model, ALT-803 synergized with trametinib, even leading to tumor rejection in several mice. Trametinib treatment alone was able to limit tumor growth, but this activity actually depended upon the presence of CD8+ T cells. Upon further investigation I found that trametinib reduced the expansion of monocytic myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice, a finding also recapitulated in vitro during the expansion of MDSCs from mouse and human bone marrow. These results suggest the inhibitory activity of trametinib on T cells in vivo is overcome by a corresponding reduction in immunosuppressive MDSCs and the endogenous presence of common γ-chain cytokines, and that the function of antitumor T cells can be further enhanced by IL-15 agonists administered during trametinib therapy. This work also demonstrates the importance of considering immune-dependent mechanisms of targeted therapies when designing personalized cancer treatments.

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Michael J. Allegrezza

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Supervisor of Dissertation

_____

Jose R. Conejo-Garcia, M.D., Ph.D.

Professor of Tumor Microenvironment and Metastasis

Graduate Group Chairperson

_____

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

Dissertation Committee

Robert H. Vonderheide, M.D., DPhil, Hanna Wise Professor in Cancer Research

Steven M. Albelda, M.D., William Maul Measey Professor of Medicine

Nicola J. Mason, BVetMed, Ph.D., Associate Professor of Medicine and Pathobiology

Martin Peter Carroll, M.D., Associate Professor of Medicine

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IMPLICATIONS FOR TARGETED THERAPY OF CANCER

To the one
with a fine sense of humor for when I'm feeling low down
I come to most days after the sun has gone down
who takes away my trouble and takes away my grief
who takes away my heartache in the night like a thief
To the one
To LAO

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ABSTRACT

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Through rational drug design, much progress has been made to develop small molecules that specifically inhibit the oncogenic signaling pathways driving malignant growth. However, the normal function of immune cells depends upon many of the same pathways inhibited by such targeted cancer therapies. Because the immune system can influence the growth of many cancers, I hypothesized that most small molecule inhibitors would have activity on leukocytes relevant in cancer, and this activity would contribute to their antitumor mechanisms. In order to test this hypothesis, I first screened a panel of over 40 small molecule inhibitors for their activity on proliferating human cancer cells and human T cells. Almost every small molecule inhibitor I tested had detrimental activity on human T cells at the concentrations required for limiting tumor cell proliferation. However, when I focused on the FDA approved MEK inhibitor trametinib, I found that some common γ-chain cytokines were able to rescue T-cell functions blunted by trametinib. Notably, an IL-15 agonist, ALT-803, could rescue the *in vivo* proliferation of tumor-antigen specific T cells in mice treated with trametinib. I developed a *p53*$^{-/-}$ *Kras*$^{G12D+}$*Myristoylated-p110α*$^{+}$ murine breast cancer model to perform tumor challenge experiments in a model only weakly sensitive to trametinib, a setting where combination with immunotherapy may be clinically useful. In this tumor model, ALT-803 synergized with trametinib, even leading to tumor rejection in several mice. Trametinib treatment alone was able to limit tumor growth, but this activity actually depended upon the

presence of $CD8^+$ T cells. Upon further investigation I found that trametinib reduced the expansion of monocytic myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice, a finding also recapitulated *in vitro* during the expansion of MDSCs from mouse and human bone marrow. These results suggest the inhibitory activity of trametinib on T cells *in vivo* is overcome by a corresponding reduction in immunosuppressive MDSCs and the endogenous presence of common γ-chain cytokines, and that the function of antitumor T cells can be further enhanced by IL-15 agonists administered during trametinib therapy. This work also demonstrates the importance of considering immune-dependent mechanisms of targeted therapies when designing personalized cancer treatments.

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CHAPTER 1

INTRODUCTION

Forty-five years ago President Nixon declared the “War on Cancer,” yet we are still struggling to comprehend the complexities of malignant disease in ways that allow us to deliver precise therapies that achieve long-term success in patients. Still every year approximately 580,000 people die from cancer in the US (R. L. Siegel, Miller, and Jemal 2016). Although surgical resection, chemotherapy, and radiation are extremely effective as first line treatments to send cancer into remission, many patients relapse months or years later with inoperable metastases that are resistant to the initial therapies, eventually progressing to terminal disease. One reason for the limited success of conventional therapies is that diverse ranges of malignancies are grouped into the term “cancer.” Although cancers can be categorized according to similarities in tissues of origin, histology, and molecular features, each patient essentially presents with a genetically unique disease (Ogino, Fuchs, and Giovannucci 2012). From this realization, it is understandable that variable results should be expected when large cohorts of patients are treated with the same therapeutic regimen. Thus, interest has expanded in developing personalized treatments where therapies are fine tuned for individual patients. In order for truly personalized cancer therapy, progress is still required to understand the molecular activity of cancer cells and their interactions with the diverse environment of non-malignant host cells that contribute to the growth, metastasis, and pathology of cancer. The development of effective interventions will require better knowledge of how specific therapies influence the biology of tumor-host cell interactions.

TARGETING ONCOGENIC PROTEINS WITH SMALL MOLECULE INHIBITORS

Drug discovery is currently driven by the idea that knowledge of the underlying causes of disease is the most effective guide in designing agents that successfully restore these aberrant mechanisms to homeostasis. For cancer biologists, this rationale leads to a seemingly simple goal: uncover the molecular causes for the ability of tumor cells to continuously survive, proliferate, and invade healthy tissue. In the 1960s and 70s, pioneering researchers began uncovering the molecular mechanisms of tumorigenesis. The first evidence that genes could transform healthy cells into tumors came from the discovery of the *v-src* gene in the Rous Sarcoma Virus (Martin 1970). Oncogene became the term used to describe these genetic elements that could initiate cancer formation. When later a homolog of *v-src* was identified as a normal gene in mammalian cells, termed *c-src* (Stehelin et al. 1976), the understanding emerged that normal, so-called proto-oncogenes in mammalian cells could be converted into oncogenes through either mutations or overexpression. Numerous other oncogenes and their proto-oncogene precursors have been identified in human cancers in the decades since these first discoveries (Croce 2009).

Because the activity of many oncogenes is carried out by their protein products, therapies that interfere with the functions of these proteins can abrogate the molecular events driving tumor growth. Collectively referred to as targeted therapies or molecularly targeted agents, rational design of these therapies aims to manipulate (usually inhibit) proteins specifically utilized by cancer cells (Stegmeier et al. 2010). One class of targeted therapies is small molecule inhibitors, low molecular weight organic compounds that block the signal transduction of their protein targets (Imai and Takaoka 2006). Clinical proof that small molecule inhibitors can be effective against cancer was first demonstrated with the success of Imatinib (Gleevec), an inhibitor of the BCR-ABL kinase that drives chronic myeloid leukemia (Buchdunger et al. 1996; Druker et al. 2001).

The number of small molecule inhibitors entering clinical trials has exploded in the past decade. Although several molecules have shown efficacy, there is a strong selective pressure within tumors to evolve resistance to targeted therapies. Many clinically used small molecules have documented cases of acquired resistance (Sierra, Cepero, and Giordano 2010). For instance, over 40% of metastatic melanomas harbor the mutated *BRAF*^{V600E} oncogene, which encodes a constitutively active B-Raf kinase (Ascierto et al. 2012). The normal Raf kinases (C-Raf, B-Raf, A-Raf) initiate signaling in the mitogen-activated protein kinase (MAPK) cascade by phosphorylating the MEK1 and MEK2 proteins (Matallanas et al. 2011). MEK1/2 are also kinases, and when phosphorylated by Rafs, they can phosphorylate ERK1 and ERK2, which then act on other molecules to induce gene transcription and cellular processes such as mitosis (Schulze et al. 2004). B-Raf^{V600E} is constitutively active, and this chronic signaling leads to melanoma growth (Ascierto et al. 2012). Two clinically successful small molecules have been developed to specifically prevent the activity of B-Raf^{V600E} without inhibiting wild-type B-Raf, vemurafenib and dabrafenib, although around half of the patients treated with either agent individually show disease progression in six months (Sosman et al. 2012; Hauschild et al. 2012). To combat reactivation of the MAPK pathway that occurs in some B-Raf^{V600E} melanomas treated with vemurafenib or dabrafenib (Nazarian et al. 2010; Villanueva et al. 2013), an inhibitor of MEK1/2 (trametinib) was tested in combination with dabrafenib, showing superior efficacy compared to dabrafenib alone, leading to the FDA approval of trametinib plus dabrafenib (Flaherty et al. 2012). This success was only marginal, though, because 1/3 of patients receiving the combo progressed within six months, and MAPK pathway reactivation was observed in 9/10 tumor relapse samples (Long et al. 2014). While efforts to prevent or target the resistance mechanisms in tumors with additional small molecules still persist, another

attractive possibility for combination therapy has emerged from an entirely separate area of cancer research.

IMMUNOSURVEILLANCE OF CANCER

An argument can be made that evidence for the immunosurveillance of cancer was documented well before any of the molecular details of cancer or immunity were described. In 1891, William Coley published several case studies in which he observed the shrinkage of aggressively growing tumors after inoculating patients with live bacteria (Coley 1891). It wasn't until recently however, that solid evidence emerged to demonstrate that the immune system plays a role in preventing malignant growth. In humans, it is well documented that a person's risk of developing cancer increases if they develop acquired immunodeficiency (Boshoff and Weiss 2002; Kirk et al. 2007) or receive an organ transplant and immunosuppressive drugs (Loeffelbein, Szilinski, & Hölzle, 2009; Vajdic et al., 2006). Additionally, the clinical outcome of many cancers is positively correlated with the presence of T cells, effector leukocytes that recognize peptide antigens through the T-cell receptor (TCR), observed in tumor specimens (L. Zhang et al. 2003; Galon et al. 2006; van Houdt et al. 2008). In some instances even, an expansion of tumor-specific T cells in patients has been observed during spontaneous tumor regressions (Ferradini et al. 1993).

Studies in mice have also demonstrated that various types of immunodeficiency predispose mice to spontaneous cancer formation and increase the rate of tumor development after chemical or genetic induction (Vesely et al. 2011). In a series of elegant experiments, Shankaran et al showed that when carcinogen-induced tumors arising in mice lacking adaptive immunity (due to *Rag2* knockout, a gene necessary for proper development of the T-cell and B-cell receptors) were transplanted into immune-

competent hosts, they were rejected by a substantial proportion (40%) of the mice, while the same tumor cells lead to tumor formation in 100% of inoculated *Rag2*$^{-/-}$ mice (Shankaran et al. 2001). These results demonstrated that the adaptive immune system is capable of spontaneously eradicating some tumors that otherwise grow uncontrollably in immune-deficient mice.

Several types of leukocytes are able to mount cytotoxic activity against tumor cells. T cells can be classified into two groups based upon their expression of the co-receptors CD4 and CD8. CD8$^+$ T cells, sometimes called cytotoxic T cells, have the ability to directly kill tumor cells through recognition of peptides presented by MHC-I molecules on the tumor cells (Hanson et al. 2000), while CD4$^+$ T cells, sometimes called helper T cells, are more indirectly involved by recognizing peptides presented by MHC-II molecules, whose expression is generally restricted to antigen-presenting cells (APCs) and B cells, although CD4$^+$ T cells can kill MHC-II expressing tumor cells (Haabeth et al. 2014). CD8$^+$ T cells have been found to limit tumor growth in many models, sometimes even in the absence of CD4$^+$ T cells (Scarlett et al. 2012; Harimoto et al. 2013; Chou and Shu 1987; Kast et al. 1989). In some settings CD4$^+$ T cells lead to tumor killing (Nesbeth et al. 2010; Greenberg, Kern, and Cheever 1985), although they can also promote tumor growth by suppressing CD8$^+$ T cells, because some CD4$^+$ T cells are regulatory cells (Yu et al. 2005) (described more below). Natural killer (NK) cells, an innate effector population that can kill virus infected and tumor cells through non-antigen specific ligand interactions, are also known to restrict the growth of some tumors (Smyth, Crowe, and Godfrey 2001).

The requirement of effector molecules utilized by T cells and NK cells to kill tumors have also been demonstrated experimentally. For instance, the cytotoxic molecules secreted by activated CD8$^+$ T cells, such as perforin, IFN-γ and TNF-α are

utilized to restrict tumor growth (Smyth et al. 2000; Barth et al. 1991). Ligand dependent killing of tumor cells through pathways such as TRAIL and FasL (expressed on T cells and NK cells) are also known to be important for immune control of tumor formation (Cretney et al. 2002; Davidson, Giese, and Fredrickson 1998). The specific subsets of cells and effector pathways critical for restricting tumor growth varies between different models, but there is significant evidence for the overall concept that antitumor immunity is a component of many mouse models of cancer.

A critical factor in determining the quality of the tumor-directed immune response is the nature of antigens expressed in tumor cells. The immune system is naturally adapted to respond to foreign molecules while being tolerant of those originating from the "self." All neoplasms are derived from "self" cells, thus the healthy parental cells are poorly immunogenic. Neoplastic cells become immunogenic through genetic changes that result in unique non-self peptides (termed neoantigens) or the overexpression of self proteins whose expression is normally tightly controlled (termed shared antigens), events which are sometimes directly related to the initial process of transformation. Virus-associated tumors may represent the far end of the immunogenicity spectrum because the foreign viral antigens are present in the tumors. On the other end of the spectrum are tumors where immunity contributes little to tumor progression presumably due to a dearth of novel antigens. For instance, soft-tissue sarcomas initiated in mice by genetic ablation of p53 and activation of the *Kras*^{G12D} oncogene show similar growth in *Rag2*$^{-/-}$ compared to WT mice; however, when the foreign chicken ovalbumin antigen (OVA) is introduced into these tumors, growth is significantly restricted in immune-competent mice relative to *Rag2*$^{-/-}$ (DuPage et al. 2012). While T-cell responses to specific tumor antigens have been described in many human cancer patients, there is

still active investigation into comprehensively characterizing the antigenic repertoire unique to each patient's cancer.

MECHANISMS OF IMMUNE EVASION AND IMMUNE SUPPRESSION IN CANCER

Considering that the immune system has the ability to restrict tumor growth, it's important to understand why so many immune-competent individuals actually develop lethal cancers. On one hand, some tumors may never acquire enough mutations in or overexpression of antigens to elicit T-cell responses (DuPage et al. 2012). Yet even in tumors that do contain sufficient antigens, the presence of antitumor immunity exerts a strong selective pressure for the outgrowth of clones that either evade or actively suppress immunity. Immune evasion, also called immunoediting, has been demonstrated in many circumstances (Dunn et al. 2002). In addition to selecting for the loss of antigens, immune pressure can also favor the growth of tumor cells with decreased expression of antigen presenting molecules (such as MHC-I and TAP) (Seliger, Maeurer, and Ferrone 2000), death receptors (such as TRAIL receptor DR4 and FAS) (Özören and El-Deiry 2003), or proteins involved in responding to IFN-γ (L. H. Wong et al. 1997). Selection may also occur for tumor cells with high surface expression of co-receptors that inhibit the activation of T cells (such as PD-L1) (Saudemont and Quesnel 2004).

Malignant lesions additionally utilize a second mechanism to escape immune-mediated elimination by co-opting the normal regulatory mechanisms within the immune system. Tumor-derived factors drive the expansion and recruitment of regulatory T cells (Tregs) and regulatory myeloid cells into the tumor. Tregs are classified as $CD4^+$ T cells expressing the FOXP3 transcription factor that act to suppress immune responses

through the production of cytokines like TGF-β and IL-10, and their presence in tumors has been negatively correlated with outcome in many human cancers (Mougiakakos et al. 2010). Regulatory myeloid cells are a more diverse subset of suppressor cells and the distinctions among them most likely lie in a spectrum rather than discrete boundaries. Nevertheless, they can be generally classified into three groups. Regulatory macrophages (also called tumor-associated macrophages, TAMs) and regulatory dendritic cells (also called tumor-associated DCs, TADCs) are the immunosuppressive cousins of classical macrophages and dendritic cells (DCs) that function as APCs. The unique factors in the microenvironment of different tumors may determine which of these cells is preferentially recruited, but their presence and ability to suppress immune responses has been described in many human and mouse cancers (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012).

The third group, known as myeloid-derived suppressor cells (MDSCs), is generally defined as a heterogeneous population of immature myeloid cells capable of suppressing immune responses. They are distinguished from macrophages and dendritic cells by their less differentiated phenotype and lack of antigen presenting MHC-II expression (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012). First described in mice as $CD11b^{+}GR\text{-}1^{+}$ cells, these cells were found to greatly increase in the spleen and bone marrow of tumor-bearing mice, and were able to potently suppress antitumor T-cell responses (D. I. Gabrilovich et al. 2001; Kusmartsev, Li, and Chen 2000). The expansion of these cells in cancer is due to chronic inflammatory signals emanating from tumors, such as the cytokines GM-CSF, G-CSF, VEGF, IL-6, and S100A8/9, which act on hematopoietic stem and progenitor cells to promote the production of immature myeloid cells and prevent their maturation into functional APCs (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012).

MDSCs are categorized based upon their morphology, surface molecule expression, and function into two populations. Monocytic MDSCs (M-MDSCs) share the cytological features of monocytes, including a single-lobed nucleus. In mice they are routinely identified by surface markers as $CD11b^{+}MHC\text{-}II^{-/low}Ly6C^{hi}Ly6G^{-}$, but in humans a consensus on standardized phenotyping has not been achieved, although they are generally considered $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}CD14^{+}CD15^{int/low}$ (Solito et al. 2014). M-MDSCs express high amounts of the enzymes ARG1 and NOS2, which deplete arginine and generate NO and reactive nitrogen species (RNS), respectively (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012). Because arginine is an essential amino acid for proliferating T cells, depletion of it from the environment results in proliferative arrest (Rodríguez and Ochoa 2008). NO and RNS can lead to nitrosylation of the TCR and cytokine receptors, reducing the ability of tumor-specific T cells to respond to peptide antigen and receive supporting cytokine signals (Mazzoni et al. 2002; Nagaraj et al. 2007).

Polymorphonuclear MDSCs (PMN-MDSCs) share the multi-lobed nucleus and abundant granules characteristic of granulocytes and neutrophils, and for this reason they are sometimes referred to as granulocytic MDSCs. PMN-MDSCs are distinguished in mice as $CD11b^{+}MHC\text{-}II^{-/low}Ly6C^{int}Ly6G^{+}$, and generally in humans as $CD11b{+}HLA\text{-}DR^{-/low}CD33^{+}CD66b^{+}CD14^{-}CD15^{+}$, although the description of these cells in humans is more variable (Solito et al. 2014). PMN-MDSCs also contribute to arginine depletion and NO/RNS production, but they differ from M-MDSCS in their production of high amounts of reactive oxygen species (ROS) through the enzymatic activity of NADPH oxidase (Raber et al. 2014). High levels of ROS can lead to dysfunctional T-cell activity by disrupting proximal TCR signaling molecules such as CD3ζ and Lck (Cemerski, van Meerwijk, and Romagnoli 2003).

In addition to their ability to directly suppress T cells, MDSCs have other tumor-promoting activities. They can induce the differentiation of Treg cells by producing TGF-β and IL-10 (Huang et al. 2006). They can support the development of new blood vessels to supply growing tumors with nutrients and oxygen (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012). MDSCs additionally secrete factors that promote survival signaling in tumor cells in response to chemotherapy (Ramachandran et al. 2016), and they can contribute to premetastatic niche development to support metastatic spreading of tumor cells (Sceneay et al. 2012).

The ability of MDSCs to suppress antitumor immunity and enhance tumor growth has been shown in many mouse models (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012), and although it is difficult to prove the outcome of their activity in human patients, several lines of evidence suggest their relevance in disease progression. First, increased levels of MDSCs are negatively correlated with clinical outcome in several cancers (Solito et al. 2014), including in the response to the anti-CTLA-4 antibody, ipilimumab, in melanoma (Meyer et al. 2014). Second, their levels are associated with measures of impaired T cells, such as CD3ζ dysfunction (Sippel et al. 2011) and decreased $CD8^+$ T cell frequency (C.-Y. Liu et al. 2010). Lastly, their ability to directly inhibit T cell activity *ex vivo* has been repeatedly demonstrated (Meyer et al. 2014). Considering the ability of MDSCs to potently suppress beneficial T cell responses, these cells have become prime targets for therapies in cancer.

IMMUNOTHERAPIES FOR CANCER

Armed with knowledge about mechanisms of immune suppression in cancer, researchers have tested several types of immunotherapies for both single agent activity

and as combinatorial treatments. Successful outcomes have been achieved most notably in melanoma by delivering monoclonal antibodies to block the co-receptor pathways of CTLA-4 and PD-1 that restrict T-cell activation. These “checkpoint inhibitors” are also being tested in several other cancers (Sharon et al. 2014). Another type of immunotherapy involves the *ex vivo* expansion of tumor-reactive T cells which are then re-administered to the patient. This process, termed adoptive cell therapy, can either use T cells expanded from tumor biopsies (Rosenberg et al. 2011) or peripheral T cells genetically modified to recognize tumor antigens (Porter et al. 2011). Both strategies have shown promise in certain cancers, yet neither has reached a standard of approval from the FDA. An additional strategy to generate therapeutic antitumor immunity is vaccination, which can either be preventative, where vaccination is targeted at viruses like HPV and HCV that drive tumor initiation, or therapeutic, where vaccination occurs after the appearance of cancer. Although preventative vaccinations may be extremely effective, currently their use is restricted to a minority (10-20%) of cancers that are caused by viruses, although people have also advocated their development for non-viral cancers (Lollini et al. 2006). Many therapeutic vaccines have been clinically tested, yet only one (Sipilucel-T) has been approved, and its efficacy is marginal at best (Higano et al. 2009).

Cytokines may also be administered to boost antitumor immunity. Due to their central role in T cell and NK cell proliferation and survival, most clinical trials have focused on cytokines in the common cytokine-receptor γ-chain family. This family of cytokines, which includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, is termed as such because each member signals through the common cytokine-receptor γ-chain (γc). The importance of γc is evidenced by the disease X-linked severe combined immunodeficiency, in which patients with functional knockouts of γc fail to produce T

cells and NK cells (Noguchi et al. 1993). Signaling through γc occurs when members of this cytokine family bind to γc along with one or two other cytokine-specific co-receptors. For instance, IL-7 binds to γc along with the IL-7 receptor α (IL-7Rα) (Leonard et al. 1998), while IL-2 binds to γc along with either IL-2Rβ for intermediate affinity signaling or along with IL-2Rβ and IL-2Rα for high affinity signaling (Rochman, Spolski, and Leonard 2009). IL-15 can also bind γc with IL-2Rβ, however efficient signaling requires IL-15 to be transpresented by another cell expressing IL-15Rα (Schluns, Klonowski, and Lefrançois 2004; Sandau et al. 2004). All γc family cytokines activate signaling through the JAK-STAT pathway, although the specific STAT proteins activated varies slightly among the cytokines. IL-2, IL-7, IL-9, and IL-15 mostly induce phosphorylation (and thus, activation) of STAT5 and STAT3, while IL-4 generally leads to phosphorylation of STAT6 and STAT3, and IL-21 predominantly induces phosphorylation of STAT1 and STAT3 (Johnston et al. 1995; Demoulin et al. 1996; Quelle et al. 1995; Zeng et al. 2007; Foxwell et al. 1995). IL-2, IL-7, and IL-15 signaling can also lead to activation of the phosphatidylinositol 3-kinase (PI3K) pathway and Ras/MAPK signal cascade (Sharfe, Dadi, and Roifman 1995; Fung, Rohwer, and McGuire 2003; Ben Ahmed et al. 2009).

The common γc cytokines play essential roles in the function of T and NK cells. IL-7 signaling is critical for the development of T cells (Leonard et al. 1998) and it also provides trophic survival signals to naïve (antigen-inexperienced) T cells and memory T cells (Rathmell et al. 2001). IL-2 is a major factor enhancing the proliferation of recently activated T cells (Morgan, Ruscetti, and Gallo 1976; D'Souza and Lefrançois 2003). IL-15 also acts to enhance proliferation after activation, although it is more specific for $CD8^+$ T cells than IL-2, which strongly signals in both $CD4^+$ and $CD8^+$ T cells (Rochman, Spolski, and Leonard 2009). Additional important functions for IL-15 are the homeostatic proliferation of memory $CD8^+$ T cells and the activation and expansion of NK cells

(Rochman, Spolski, and Leonard 2009). The differentiation of $CD4^+$ T cells into T-helper-2 (Th2) cells depends on IL-4 (Swain et al. 1990). IL-2 is critical for the maintenance of Tregs, such that IL-2 deficiency actually causes lymphoproliferative and autoimmune diseases (Sakaguchi et al. 2008).

Due to their ability to promote the survival and expansion of T cells, IL-2, IL-7, and IL-15 have been the main common γc cytokines tested in preclinical cancer models and patients. In two clinical trials of IL-7 administration, dose-dependent increases in peripheral $CD4^+$ and $CD8^+$ T cells were observed, but the therapy showed no antitumor activity (Rosenberg et al. 2016; Sportès et al. 2008). For many years, high dose IL-2 has been indicated for use in melanoma and advanced renal cell carcinoma patients (Amin and White 2013). IL-15 could be a more favorable therapy, however, because it lacks the tumor-promoting activity of IL-2 signaling in Tregs. Administration of IL-15 to patients with metastatic disease in a Phase I trial led to increases in NK cells and memory $CD8^+$ T cells, and some encouraging decreases in tumor volumes were observed (Conlon et al. 2015). One challenge in cytokine therapy is the short-half life of the molecules (Stoklasek, Schluns, and Lefrançois 2006). To overcome that limitation, Han et al developed an IL-15 agonist that consists of two $IL\text{-}15^{N72D}$ molecules conjugated to a dimeric IL-15Rα sushi domain and fused to the Fc region of an IgG1 (**Figure 1.1**) (Han et al. 2011). This molecule, ALT-803, showed vastly increased serum half-life in mice and antitumor activity in murine myeloma and glioblastoma models through the activation of $CD8^+$ T cells (Han et al. 2011; Rhode et al. 2015). ALT-803 is currently being tested in multiple clinical trials against solid and hematological tumors either as a single agent or in combination with other therapies (**Table 1.1**).

Because T cells can respond to new antigens and develop memory, they have the potential to completely eradicate tumors and maintain surveillance against the future

emergence of micrometastases. For this reason, immunotherapies could be a useful addition to targeted therapy in order to prevent the emergence of drug-resistant clones. The action of small molecule inhibitors may also synergistically promote tumor destruction by T cells. Towards this goal, several combinations have shown success in preclinical models, such as the combination of B-Raf inhibitors with adoptive cell therapy (Hu-Lieskovan et al. 2015) and HDAC inhibitors with PD-1 blockade (Zheng et al. 2016). In order to develop the most effective combinations, it will first be necessary to understand how small molecule inhibitors impact biochemical signaling in immune cells. Research into this topic has lagged significantly behind that into the effects of small molecule inhibitors on tumor cells, which is partially the result of most preclinical studies of small molecules being performed with xenografts in immune-deficient mice. Although it may seem reasonable to assume that small molecule inhibitors primarily exert activity on tumor cells, cells of the immune system also utilize many of the pathways targeted in cancer.

ONCOGENIC SIGNALING PATHWAYS AND THEIR HOMOLOGS IN LEUKOCYTES

Small molecule inhibitors target either signaling pathways specific to oncogene addicted tumor cells or more conserved pathways important for the proliferation and survival of tumor cells in general. Oncogene addiction refers to the absolute dependence of tumor cells on signaling through specific proteins to the point where blocking this signaling results in either growth arrest and death or acquired resistance via additional mutations that reactive the same pathway (Weinstein and Joe 2008). Two classic examples are BCR-ABL driven chronic myeloid leukemia (CML) cells that acquire resistance to imatinib through additional ABL mutations (Gorre et al. 2001) and *BRAF*^{V600E} melanoma cells that acquire activating mutations in B-Raf's target proteins,

MEK1/2, in response to B-Raf inhibitors (Villanueva et al. 2013). Small molecules may also target wild-type proteins driving oncogenic growth, such as those targeting receptors like HER2 and EGFR (Schroeder, Stevens, and Sridhar 2014). Central cellular signaling not specific to certain oncogenes that may be targeted include pathways for cell division (CDKs), transcription (HDAC), survival (Bcl-2), and autophagy (Atg3/7/10) (J. Zhang, Yang, and Gray 2009; Kang and Reynolds 2009; Khan and La Thangue 2012; Cheong et al. 2012). The PI3K-AKT-mTOR pathway is an interesting target because in addition to some tumors being driven by direct mutations in this pathway, it also integrates signaling events from many other oncogenes (Engelman 2009). In this regard, small molecules may be used to target oncogene-addicted cells (such as PI3K-p110α mutants), to block downstream signaling from other pathways, or to prevent the acquisition of drug resistance. Whatever the intended mechanism for use, the potential for unintended activity on leukocytes appears likely for most small molecule inhibitors due to the homologous presence of these pathways in immunity.

Following activation through TCR engagement, co-stimulatory receptor ligation, and cytokine stimulation, T cells can proliferate at a rate faster than most cancer cells (up to 5.3 hrs per cell division) (Hwang et al. 2006) and through continuous stimulation can be propagated for at least nine months *in vitro* (Boylston and Anderson 1980). Therefore, it is not surprising that many of the pathways driving oncogenic growth of tumor cells are also critical for transducing the activating signals that drive mitosis and survival of T cells. For instance, the binding of peptide-MHC complexes to the TCR causes the intracellular clustering and phosphorylation of the proximal signaling complex proteins, which directly leads to activation of Ras (primarily N-Ras) (Smith-Garvin, Koretzky, and Jordan 2009). Ras initiates the MAPK cascade by activating C-Raf, which activates MEK1/2, which then activates ERK1/2 (Genot and Cantrell 2000). ERK activity

results in the activation of several key transcription factors that orchestrate gene expression changes in T cells. This cascade is critical for transmitting the message of TCR clustering at the surface into the nucleus for the transcriptional switch necessary for cell growth, division, and effector activity. Ligation of co-stimulatory molecules expressed on the surface of T cells such as CD28 activates PI3K, leading to activation of AKT and mTOR (Acuto and Michel 2003). The co-stimulatory signals mediated by PI3K result in the production of cytokines like IL-2 and upregulation of pro-survival molecules like Bcl-xl (Acuto and Michel 2003). Additionally the metabolic switch to aerobic glycolysis in activated T cells depends upon mTORC1 (Chi 2012). PI3K can also be activated by common γc family cytokines like IL-2 and IL-15 (Fung, Rohwer, and McGuire 2003; Meresse et al. 2004). Changes in gene expression following T-cell activation are mediated in part through the ability of HDAC proteins to remodel chromatin (Akimova et al. 2012), and mitosis in T cells requires the CDK family of cell cycle checkpoint kinases (Wells and Morawski 2014).

The development and function of various innate immune cells also involves many of these pathways. Activation of MEK and ERK promotes the differentiation of myeloid cells from precursor stem cells (Hsu, Kikuchi, and Kondo 2007). Additionally, the survival and differentiation of DCs from monocytes relies on MEK and PI3K signaling (Xie et al. 2005). The ability of DCs and macrophages to upregulate immune-activating genes after toll-like receptor (TLR) recognition of microbial products requires HDAC activity (Roger et al. 2011). Innate immune cells migrate towards sites of inflammation by sensing chemokine gradients, and the receptors for many chemokines (eg fMLP, IL-8, LTB4) signal through PI3K (Hawkins and Stephens 2015). Also, activation of NK cells following ligation of NKG2D is mediated in part through MEK and PI3K signaling (Vivier, Nunès, and Vély 2004). Mention of these examples is not intended as a comprehensive

summary, only to illustrate that pathways targeted in cancer therapy have important functions in immune cells. For this reason, the development of targeted therapies that synergize with antitumor immunity will require dedicated research into how small molecule inhibitors modulate the interaction between the tumor and immune system.

TABLES

Phase	Disease	Clinicaltrials.gov ID
I/II	Relapsed hematologic malignancy (after allogeneic stem cell transplant)	NCT01885897
I/II	Relapsed or refractory multiple myeloma	NCT02099539
I	Melanoma, kidney cancer, non-small cell lung cancer, head and neck cancer	NCT01946789
I/II	Non-muscle invasive bladder cancer	NCT02138734
I/II	Pre-treated, advanced or recurrent Non-small cell lung cancer (+ NivolumaB)	NCT02523469
I/II	Relapse/refractory indolent B cell Non-Hodgkin lymphoma (+ RituximaB)	NCT02384954
I	HIV infection	NCT02191098
I/II	Advanced pancreatic cancer (+ Gemcitabine and Nab-Paclitaxel)	NCT02559674

Table 1.1. Ongoing ALT-803 clinical trials.

FIGURES

Figure 1.1. ALT-803. Illustration showing structure of the IL-15 agonist, ALT-803.

CHAPTER 2

DEVELOPMENT OF GENETICALLY ENGINEERED BREAST CARCINOMA MODELS BY TARGETING OF THE DUCTAL EPITHELIUM WITH ADENOVIRUS-CRE

The following chapter was adapted from a published journal article, and the work was performed in collaboration with Dr. Melanie Rutkowski.

Rutkowski MR*, Allegrezza MJ*, Svoronos N, Tesone AJ, Stephen TL, Perales-Puchalt A, Nguyen J, Zhang PJ, Fiering SN, Tchou J, Conejo-Garcia JR. Initiation of metastatic breast carcinoma by targeting of the ductal epithelium with adenovirus-cre: a novel transgenic mouse model of breast cancer. *J Vis Exp*, doi: 10.3791/51171, 2014.

* These authors contributed equally

ABSTRACT

The development of personalized therapies tailored to the unique molecular features of cancers will require research with preclinical models where the oncogenic driver mutations are precisely known. Breast cancer is a heterogeneous disease involving complex cellular interactions between the developing tumor and immune system, eventually resulting in metastasis to distal tissues and the collapse of anti-tumor immunity. Many useful animal models exist to study breast cancer, but all have their individual limitations. It was my goal to develop a protocol for initiating autochthonous breast tumors in mice that develop after sexual maturity and are driven by consistent, endocrine-independent expression of oncogenes that can be easily interchanged through breeding. In collaboration with Dr. Melanie Rutkowski, I describe here the generation of an inducible transgenic mouse model of ductal carcinoma by delivery of an adenovirus expressing Cre-recombinase into the mammary duct of sexually mature, virgin female mice. Through selective breeding of mice with transgenic alleles flanked by loxP sequences, termed floxed, it is possible to develop different tumor models with a variety of genetic features. For studies in subsequent chapters I use a cell line derived from tumors initiated in LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*Myristoylated-p110α-GFP*$^{+}$ mice, but here I describe the protocol to develop and characterize tumors with LSL-*Kras*$^{G12D/+}$*p53*$^{loxp/loxp}$ LSL-*EYFP*$^{+}$ mice, where delivery of adenovirus-Cre leads to ablation of p53, expression of an oncogenic form of Kras, and activation of YFP. Tumors begin to appear 6 weeks after the initiation of oncogenic events. After tumors become apparent, they progress slowly for approximately two weeks before they begin to grow exponentially. After 7-8 weeks post-adenovirus injection, vasculature is observed connecting the tumor mass to distal lymph nodes, with eventual lymphovascular invasion of YFP+ tumor cells to the distal axillary lymph nodes. Infiltrating leukocyte populations

are similar to those found in human breast carcinomas, including the presence of αβ and γδ T cells, macrophages and MDSCs. This unique model will facilitate the study of cellular and immunological mechanisms involved in tumor formation, metastasis, and dormancy in addition to being useful for designing novel immunotherapeutic interventions to treat invasive breast cancer.

INTRODUCTION

Breast cancer is the most commonly occurring malignancy in women throughout the world and the second leading cause of cancer-related deaths (Youlden et al. 2012; R. Siegel, Naishadham, and Jemal 2016). Complex genetic (Sørlie et al. 2001; Gatza et al. 2010), histological (Bastien et al. 2012) and clinical phenotypes (Montagna et al. 2013) are used to characterize the various subtypes of breast cancer and often are used as a means to predict survival. Analysis of a large cohort of women with breast cancer indicated that most (approximately 80%) of the patients that died had recurred within 10 years post removal of the primary tumor (Karrison, Ferguson, and Meier 1999). For a majority of invasive breast carcinomas, lymphovascular invasion has been shown to be strongly correlated to a poor outcome and more aggressive clinical course of disease (Rakha et al. 2012).

Because of the genetic and phenotypic complexity of breast cancer, there is no animal model that recapitulates the entire course of disease. Human breast tumor cell lines have been frequently used as xenograft or orthotopic (I. S. Kim and Baek 2010) models of invasive and metastatic breast cancer in immune deficient mice. Although informative, these models occur in the absence of immune pressure and because it is a cross species graft, distort the effects of the entire tumor microenvironment. Inducible

genetic mutations driven by mammary specific promoters such as murine mammary tumor virus (MMTV) and whey acidic protein (WAP) have contributed a tremendous amount of knowledge about the genetic nature of breast cancer. However, the tissue specific expression of these promoters are compromised by their responsiveness to the endocrine system (Vargo-Gogola and Rosen 2007; Archer et al. 1995; Cato, Henderson, and Ponta 1987; Schoenenberger et al. 1990; Li et al. 2001; Martelli et al. 2012; Klover et al. 2010), resulting in the variable expression of induced genetic mutations that do not mirror the expression of oncogenes typically overexpressed in human breast cancer. To overcome endocrine control of MMTV driven expression of oncogenes, Moody et al. generated a conditional, doxycycline inducible model overexpressing Neu in the breast epithelium (Moody et al. 2002). This model is useful for turning off Neu expression after tumor formation to study regression and recurrence, but requires constant doxycycline administration for consistent, long-term oncogene expression. A comprehensive discussion of the many relevant breast tumor models available can be found in the review by Vargo-Gogola et al (Vargo-Gogola and Rosen 2007).

My goal was to develop a mouse model of traceable breast cancer on a C57BL/6 background that, after the irreversible induction of mutational events, models the formation of a nascent tumor in the presence of immune pressure. I introduced an adenovirus expressing Cre-recombinase into the mammary ducts of transgenic mice containing floxed alleles of *Tp53,* and an oncogenic form of *Kras,* and YFP. Cre expression ablates *Tp53*, a frequently mutated gene in many breast cancers (Banerji et al. 2012) and induces an oncogenic allele of *Kras* in addition to YFP expression specifically in the mammary ductal epithelium. Although mutations in *Kras* are infrequent in breast cancer, occurring in only 6.5% of breast cancer patients (Miyakis, Sourvinos, and Spandidos 1998; Malaney and Daly 2001), the overexpression of upstream kinases

such as Her2/neu and EGFR result in constitutive activation of the Ras signaling pathway in human breast tumors (Loboda et al. 2010; von Lintig et al. 2000; Downward 2003). Activation of the Ras signaling pathway in many breast tumor cell lines has also been reported (Eckert et al. 2004; Hollestelle et al. 2007). I will describe the initiation of tumor formation and the technique of intraductal injection of an adenovirus expressing Cre-recombinase into sexually mature, virgin female mice. This model of breast cancer develops overt lesions that grow exponentially after about 8 weeks of slow tumor progression, with lymphovascular invasion and metastasis to the axillary lymph node by 7-8 weeks. Because this mouse is on a C57BL/6 background and YFP-expressing tumor cells are traceable in distal lymph nodes, this model provides a relevant tool to study the cellular and immunological mechanisms of latent metastasis and will help to develop novel therapeutic approaches for the treatment of metastatic ductal breast cancer.

PROTOCOL

All animal experiments were approved by the Wistar Institute Animal Care and Use Committee. Materials used for this procedure are listed in **Table 2.1&2.2**.

1. Generation and maintenance of transgenic mice

1.1) Breed LSL-Kras^{tm4Tyj} (Jackson et al. 2001) and Trp53^{tm1Brn} (Jackson et al. 2001) (obtained from NCI mouse models of human cancer consortium on a mixed background) to a full C57BL/6 background (Scarlett et al. 2012) by backcrossing at least 10 generations with C57BL/6 mice. To track tumor metastasis, breed B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J (LSL-*EYFP*, obtained from The Jackson Laboratory on a full C57BL/6 background) with double transgenic LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice.

Transgenic LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice have loxp sites flanking a transcriptionally silenced allele of oncogenic *Kras* and the endogenous *Tp53* locus, so that upon Cre-mediated excision, overexpression of an oncogenic *Kras* mutant and ablation of *Tp53* is achieved.

The LSL-*EYFP* mouse contains a stop codon flanking a gene for enhanced yellow fluorescent protein (YFP) that upon Cre-mediated excision results in the expression of YFP in the tissues where the YFP stop cassette is excised.

1.1.1) Breed transgenic mice to obtain LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice or LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*EYFP* mice for intraductal injections.

Mice are bred as homozygous for *p53*$^{loxp/loxp}$ and heterozygous for LSL-*Kras*^{G12D} because mice with a homozygous deletion of *Kras* die in utero. Use naïve virgin females at least six-weeks old for intraductal injections.

The primers for genotyping homozygous floxed *Tp53* allele are p53-T010-fwd (5′-AAGGGGTATGAGGGACAAGG-3′) and p53-T011-rev (5′-GAAGACAGAAAAGGGGAGGG-3′). They produce a wild type allele at 391 bp and the *Tp53* floxed allele at 461 bp (Vooijs, Jonkers, and Berns 2001; Young, Crowley, and Jacks 2011).

The primers to detect the transgenic allele of mutant *Kras* are oIMR8273 (5'-CGCAGACTGTAGAGCAGCG-3') and oIMR8274 (5'-CCATGGCTTGAGTAAGTCTGC-3'). They produce the mutant band detected at 600bp.

For the YFP reporter triple transgenic mice, the primers to detect the ROSA cassette (5'-AAGACCGCGAAGAGTTTGTC-3'), the wild type allele (5'-GGAGCGGGAGAAATGGATATG-3'), and a shared allele (5'-AAAGTCGCTCTGAGTTGTTAT-3') result in the amplification of bands at 320 bp for floxed allele and 600 bp for the wild type allele.

2. Surgical preparation

2.1) Clean surgical materials with 75% EtOH and autoclave them before and after all injections.

2.2) Perform surgery on a clean uncluttered laboratory bench in a sanitized room within an animal facility. Wipe down all surfaces including the stage and dials of the surgical microscope with a broad-spectrum disinfectant solution followed by 75% EtOH.

2.3) Weigh and anesthetize mice by intraperitoneal injection of a mix of ketamine (80-100 mg/kg) and xylazine (8-10 mg/kg) in sterile saline.

2.4) Gently place mice back into their cages undisturbed for five minutes while they go under anesthesia. During this time generate virus precipitates (see section 3).

2.5) Verify lack of response to pain by toe pinching. Gently cover the eyes of anesthetized mice with veterinary ointment to prevent excessive corneal drying.

2.6) To prevent hypothermia, place anesthetized mice onto a heating pad set to **low heat** during the surgical procedure and until they begin to recover.

2.7) For the management of pain, administer mice meloxicam subcutaneously at 1 mg/kg before the surgery and 24 hours after.

3. Generation of virus precipitates

CAUTION: Adenovirus vectors, although they have been modified and are unable to replicate, pose the risk of infection. Handle adenovirus with caution. All personnel should be appropriately trained according to the institution's guidelines for handling BSL2 agents After intraductal injection, dispose of adenovirus in accordance with BSL2 guidelines.

3.1) Store adenovirus concentrated virus stocks at -80°C frozen in aliquots of 4 x 10^8 pfu each, sufficient for injecting 16 animals with 3 μl of 2.5 x 10^7 pfu of adenovirus particles.

3.2) Store adenovirus aliquots on dry ice until approximately 15-20 minutes before beginning the injections.

Avoid repeated freeze thaw cycles, as virus titer drops significantly between each cycle.

Adenovirus precipitates are formed by modifying a protocol described previously (Dinulescu et al. 2005).

3.3) Reconstitute 504 mg of MEM powder with 50 mL of sterile molecular grade water, supplement with 244 mg of sodium bicarbonate, sterile filter, and store at 4°C.

3.3.1.) Prepare the calcium chloride solution by adding 1.5 g of calcium chloride to 50 ml of molecular grade water and filter in sterile conditions and store at 4°C.

3.3.2) Mix aliquots containing 4 x 10^8 pfu adenovirus-cre with sufficient 3% sucrose in sterile water for a final volume of 10 μl. Add 34 μl of MEM to the virus and gently mix. Then add 4 μl of the $CaCl_2$ solution, gently mix, and incubate at room temperature for 15-20 minutes.

3.3.3) Store adenovirus on dry ice until ready to form precipitates. Avoid thawing of the adenovirus and storing on ice or room temperature for extended times, unless precipitates are formed.

It is also possible to mix the sucrose, MEM and $CaCl_2$ prior to the surgeries if it is not possible to thaw the adenovirus aliquot and begin making precipitates immediately after removal from -80ºC. This aliquot of sucrose, MEM, and calcium can be saved on dry ice until ready to add the adenovirus.

Virus particles are stable for approximately one hour

3.4) Prior to each injection, gently flick the tube to make sure virus particles are mixed. Draw up 3 μl (2.5×10^7 pfu) of virus particles into the 10 μl syringe and prepare the mouse for the intraductal injection.

4. Intraductal injection of virus particles

4.1) Gently place the mouse on its back onto the illuminated stage of a clean dissection microscope. Illuminate the abdominal side with an extra light source and locate the left 4^{th} or right 9^{th} inguinal mammary gland by the small white patches of fur (visible on C57BL/6 females) surrounding each nipple.

4.2) Rub the nipple gently with a sterile ethanol soaked cotton tipped applicator to clear hair away from the nipple and to sterilize the injection site. If they are difficult to locate, gently apply a thick layer of a depilatory cream or use shears to expose the nipples.

4.3) Remove the keratin plug, a layer of dense dead skin cells, which is covering the nipple.

Once the nipple is exposed, the keratin plug should be easily visible under the dissection microscope.

4.4) Secure the nipple with fine surgical forceps and pull up with light force to remove the keratin plug.

4.5) Stabilize the nipple between the forceps.

4.6) Gently insert the needle between the forceps, cannulating the duct canal at a 90-degree angle. Enter the nipple slightly past the bevel of the needle (not more than two millimeters) to prevent penetration through the mammary tissue and into the serous membranes of the ventral body cavity.

4.6.1) Do not insert the needle too deep. To ensure proper depth of injection, gently pull the needle up after inserting it into the lumen of the duct, drawing the nipple up along the edges of the needle as it is pulled up.

Visualization of the injection is difficult, therefore practice for this step is recommended using trypan blue.

4.7) When the needle is appropriately placed into the mammary duct, release the 3 μl of virus precipitates (2.5×10^7 pfu of adenovirus-cre) by gently plunging the syringe with the thumb of the hand holding the syringe. The nipple should slightly inflate as the liquid is added.

5. Recovery of mice

5.1) Place the mouse back onto the heating pad after the injection, until it begins to recover from the anesthesia.

5.2) Once the mouse is recovered, place it back into a clean cage and monitor for full recovery and movement.

5.3) 24 hours after the intraductal injection, subcutaneously administer meloxicam at 1 mg/kg.

6. Monitoring tumor progression

6.1) Palpate the injected mammary gland at day 30 for enlargement and swelling.

6.1.1) Monitor tumor progression every 5-7 days once a swollen and enlarged mammary gland is observed.

6.2) Measure tumor volumes every 3-4 days for tumor growth kinetics once palpable tumors appear (approximately 50-60 days post adenoviral injection).

6.3) Euthanize mice when tumor volumes exceed 10% of the body weight of the mice.

RESULTS

Successful targeting of the mammary ductal tree can be visualized by preparing whole mounts of the mammary gland as previously described (Landua, Visbal, and Lewis 2009) after injection of trypan blue (to verify proper injection technique (**Figure 2.1A**) or an adenovirus expressing mCherry (to verify proper viral preparation and infection of ductal epithelial cells (**Figure 2.1B)**).

When tumors are induced in LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ transgenic mice, tumors will not be apparent until around day 40 when the mammary glands will become enlarged and swollen. It is necessary to begin palpations when this is observed, monitoring tumor growth every 5-7 days. In my hands, hardening of the mammary gland always precedes the onset of tumor development. Tumors will progress slowly for an additional 2 weeks. Beginning around day 56, tumors will begin to grow exponentially (**Figure 2.2B**). At this point, it is critical to measure tumor volumes every 3 days if kinetic studies are desired because there will be slight mouse to mouse variability in tumor progression, which is normal (**Figure 2.2A&2.3A**). Large abdominal masses will be apparent by day 80 (**Figure 2.2A-B&2.3A**), after which mice should be euthanized if tumors exceed more than 10% of their body weight. cDNA analysis of three clones from a tumor-bearing mouse revealed expression of mesothelin, cytokeratin-8, Her2/neu, and estrogen receptor-α (**Figure 2.2C**).

Similar to the cellular microenvironment in human breast cancer, I have observed infiltration of $\alpha\beta$ and $\gamma\delta$ T cells as well as myeloid derived suppressor cells and macrophages into the tumors (**Figure 2.4**). Vasculature draining to the axillary lymph node will begin to engorge before tumors have grown to encompass the entire mammary

tissue where the injection was performed (**Figure 2.3A**). Lymphovascular invasion and metastasis of tumor cells can be tracked by crossing LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice with LSL-*EYFP* mice. After Cre-mediated excision, tumor cells expressing YFP (both high and low) are detected in the tumor and can be traced metastasizing to the draining axillary lymph node (**Figure 2.3B**). Metastasis to the distal axillary lymph node was confirmed by successfully culturing a tumor cell line from this site in a tumor-bearing LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mouse (data not shown).

Due to the anatomy of the mouse and technique of intraductal injections, I find targeting of mammary glands 9 and 4 (**Figure 2.5**) yields the most consistent results and reliable injections. However, any gland can be targeted depending upon the preference of the technician performing the surgery.

DISCUSSION

The success of this procedure hinges on proper technique during intraductal injections, which will be difficult for untrained experimenters. Problems with the injection can result in significantly delayed, variable, or absent tumor development. If the needle is inserted too deep or at an inappropriate angle, the ductal canal may be missed. It is important to enter the nipple slightly past the bevel of the needle (not more than 2 mm), to prevent penetration through the mammary tissue and into the serous membranes of the ventral body cavity. Also, too shallow placement of the needle or the injection of greater than 3 μl of virus precipitates can result in spillage of viral prep outside the mammary gland and the induction of unintentional tumors. One way to overcome these issues is to insert the needle into the nipple slightly deeper than 3 mm, and slowly draw the syringe back up out of the duct until 2 mm from the tip of the bevel. This will ensure that mammary tissue is targeted instead of the muscle surrounding the peritoneal cavity

of mice. This will also stretch the nipple along the edges of the syringe so that when the virus is expelled into the duct, there is no spillage and loss of viral precipitates.

Visualization of the injection is difficult and practice for this step is recommended. I have observed an increase in successful injections following practice, resulting in a higher penetrance of tumor development. Because this technique uses non-lactating virgin females, it is critical to remove the keratin plug covering the nipple to reveal the underlying duct canal. I recommend practicing this step by injecting trypan blue or some other sterile traceable dye inside of the duct and preparing whole mammary mounts to confirm targeting of the ductal tree. Additionally, other protocols describing intraductal injection of reagents have been published (Barham et al. 2012; Murata et al. 2006), which may be useful for developing proper technique. Issues with viral preparation or infection of the ductal lumen can also be investigated by using a mCherry expressing adenovirus. Non-transgenic mice can be used for each of these purposes until the injection technique is optimized.

Although any mammary gland can be used to initiate tumors, I have achieved the most consistent growth rates by targeting mammary gland 4 or 9, which I believe is because it is easier to perform proper injections on these glands, resulting in more efficient targeting of the duct. The proximity of the left 4^{th} or right 9^{th} inguinal mammary glands to the draining inguinal lymph node is also useful to examine antitumor immune responses during different temporal points of tumor progression. To model and track latent metastasis to distal sites, transgenic mice were crossed with LSL-EYFP mice. As the tumor progresses, vasculature connecting the tumor to the axillary lymph node will become slightly engorged at approximately 5 weeks, before the tumor begins to grow exponentially (**Figure 2.3A**). Eventually, after 7-8 weeks, lymphovascular invasion will result in tumor growth within the axillary lymph node (**Figure 2.3A-B**). Using reporter

mice and Cre-LoxP technology, incorporation of YFP creates a platform to track tumor cells metastasizing to distal sites throughout tumor progression. This can facilitate studies aimed at elucidating the cellular and epigenetic mechanisms that promote latent metastasis. In my hands, breast tumor cell lines from LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice expressed cytokeratin-8, mesothelin, estrogen receptor-α, and Her2/neu, confirming targeting of the ductal epithelium. However, depending upon the mutations induced and due to the difficulty and the variability of injections, I recommend histological characterization of tumors once the model is well established in the laboratory.

Because breast cancer is such a deadly and pervasive disease (Youlden et al. 2012; R. Siegel, Naishadham, and Jemal 2016), it is important to use animal models that accurately recapitulate the complex interplay between tumor and host. Here I describe a fully backcrossed C57BL/6 murine model of breast cancer. First, by inducing tumors from native cells, I allow the tumor to evolve naturally in a full immune microenvironment. The immune microenvironment in the advanced mouse breast tumors recapitulates the populations of αβ and γδ T cells, myeloid derived suppressor cells, and macrophages commonly observed in human breast cancer (**Figure 2.4**). Second, endocrine independent expression of oncogenes ensures that tumor cells have persistently high levels of target gene expression. Third, by taking advantage of latent mutations, I can control the timing of tumorigenesis to facilitate precise temporal tracking of tumor evolution. Applications of this model include research on tumor cell biology, studies on factors in the tumor microenvironment, anti-tumor immune responses, and even efficacy evaluation of new therapeutics. Through the availability of the Cre-loxP system, this technique can be used as a platform for investigating a diverse array of additional mutations in the initiation and progression of breast tumors, which I hope will facilitate

the understanding of breast cancer biology and eventually lead to new therapeutics aimed at treating metastatic breast cancer.

TABLES

Name	Company	Catalog Number	Comments
$Trp53^{tm1Brn}$ Transgenic mice K-ras^{tm4Tyj} Transgenic mice	Obtained from NCI mouse models of human cancer consortium		Mice were backcrossed ten times to a full C57BL/6 background
B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J Transgenic mice	Jackson Labs	006148	
Primers $p53^{loxP/loxP}$	Integrated DNA Technologies		5'-AAGGGGTATGAGGGACAAGG-3' 5'-GAAGACAGAAAAGGGGAGGG-3'
Primers LSL-K-$ras^{G12D/+}$	Integrated DNA Technologies		5'-CGCAGACTGTAGAGCAGCG-3' 5'-CCATGGCTTGAGTAAGTCTGC-3'
Primers for LSL-EYFP to detect Rosa promoter	Integrated DNA Technologies		5'-AAGACCGCGAAGAGTTTGTC-3' 5'-GGAGCGGGAGAAATGGATATG-3' 5'-AAAGTCGCTCTGAGTTGTTAT-3'
Primers for detection of Mesothelin expression	Integrated DNA Technologies		5'-TTGGGTGGATACCACGTCTG-3' 5'-CGGAGTGTAATGTTCTTCTGTC-3'
Primers for detection of Progesterone Receptor expression	Integrated DNA Technologies		5'-GCAATGGAAGGGCAGCATAA-3' 5'-TGGCGGGACCAGTTGAATTT-3'
Primers for detection of Cytokeratin 8 expression	Integrated DNA Technologies		5'-ATCAGCTCTTCCAGCTTTTCCC-3' 5'-GAAGCGCACCTTGTCAATGAAGG-3'
Primers for detection of Erbb2 expression	Integrated DNA Technologies		5'-ACCTGCCCCTACAACTACCT-3' 5'-AAATGCCAGGCTCCCAAAGA-3'
Primers for detection of Estrogen Receptor A expression	Integrated DNA Technologies		5'-ATGAAAGGCGGCATACGGAA-3' 5'-GCGGTTCAGCATCCAACAAG-3'
Primers for detection of Estrogen Receptor B expression	Integrated DNA Technologies		5'-ACCCAATGTGCTAGTGAGCC-3' 5'-TGAGGACCTGTCCAGAACGA-3'
Primers for detection of B-Actin expression	Integrated DNA Technologies		5'-GCCTTCCTTCTTGGGTATGG-3' 5'-CAGCTCAGTAACAGTCCGCC-3'

Table 2.1. List of materials.

Name	Company	Catalog Number	Comments
Adenovirus-Cre	Gene Transfer Vector Core from the University of Iowa	Ad5CMV Cre	Store aliquots of virus (4 x 10^8 pfu/aliquot) at -80 °C to avoid repeated freeze thaw cycles.
Adenovirus-mCherry	Gene Transfer Vector Core from the University of Iowa	Ad5CMV mCherry	Store aliquots of virus (4 x 10^8 pfu/aliquot) at -80 °C to avoid repeated freeze thaw cycles.
Hamilton syringe	Hamilton company	701RN	10 µl syringe, RN series. Autoclave before and after each use. Clean with PBS and 75% ethanol.
Custom needle	Hamilton company	7803-05	33 G 0.5 in long RN needle, with a 12° bevel. Autoclave before and after each use. Clean with PBS and 75% ethanol.
Surgical forceps	Dumont	52100-58	Dumostar No. 5 forceps. Clean with 75% ethanol after each use, followed by autoclaving
MEM powder	Cellgro	50 012 PB	Store at 4 °C in powder and reconstituted form
Sodium Bicarbonate	Fisher	S233	Add to MEM and filter sterilize
Calcium Chloride	Sigma	C4901	Minimum 96%, anhydrous

Table 2.2. List of materials continued.

FIGURES

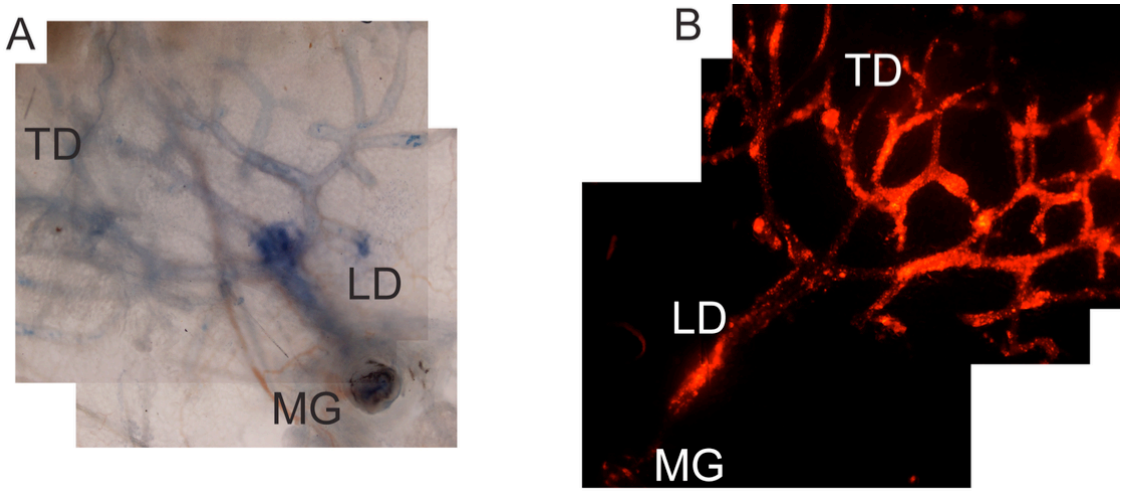

Figure 2.1. Intraductal targeting of mammary glands by injection with trypan blue or adenovirus-mCherry. (**A**) A whole mount, as previously reported (Landua, Visbal, and Lewis 2009), of the mammary gland after injection of mammary gland #4 with trypan blue was prepared 3 hours post injection to visualize/confirm targeting of the entire ductal tree. Images are 4X magnification. (**B**) Infection of the ductal epithelium with adenovirus by injecting 2 x 10^7 pfu of adenovirus expressing mCherry. Mice were injected intraductally, and 4 days post-injection, a whole mount of the mammary gland was prepared to confirm viral infection of the ductal tree. Image is 4x magnification. MG is mammary gland, LD is the lactiferous, or main duct, and TD is the terminal duct.

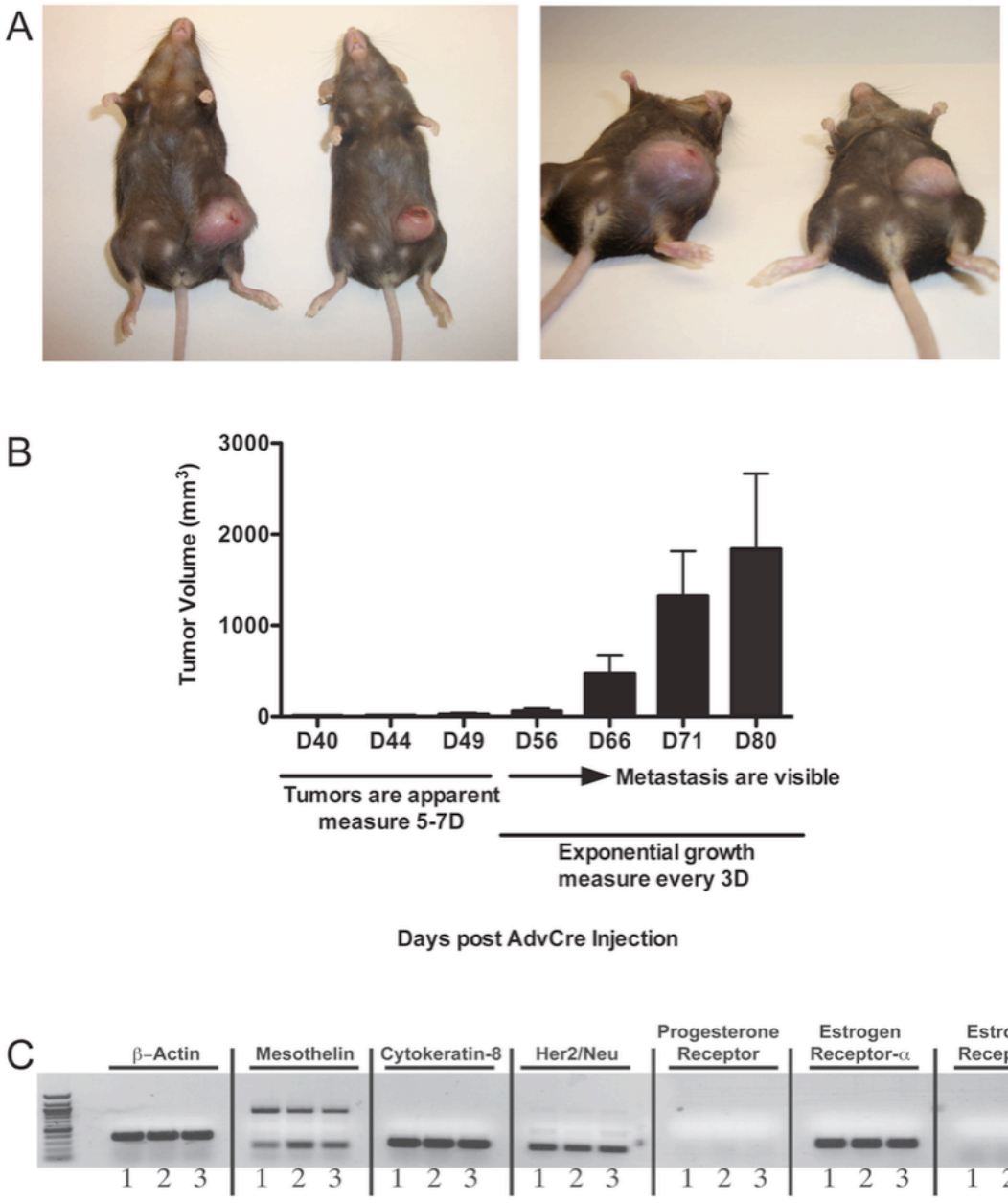

Figure 2.2. Tumor development in LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice injected intraductally with adenovirus-Cre. (**A**) Two examples of tumors 80 days after injection with adenovirus expressing Cre. Mice were given 2.5 x 10^7 PFU of adenovirus expressing Cre and 80 days post tumor-initiation, large palpable masses can be visualized protruding from the abdominal side of the animal. (**B**) Typical tumor kinetics and palpation schedule for tumors induced in LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mice. (**C**) Characterization of three tumor cell clones derived from the same homogenized tumor of a LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$ mouse. RNA was extracted and cDNA synthesized for RT-PCR analysis using primers specific to β-actin, mesothelin, cytokeratin-8, Her2/neu, progesterone receptor, estrogen receptor-α, and estrogen receptor-β.

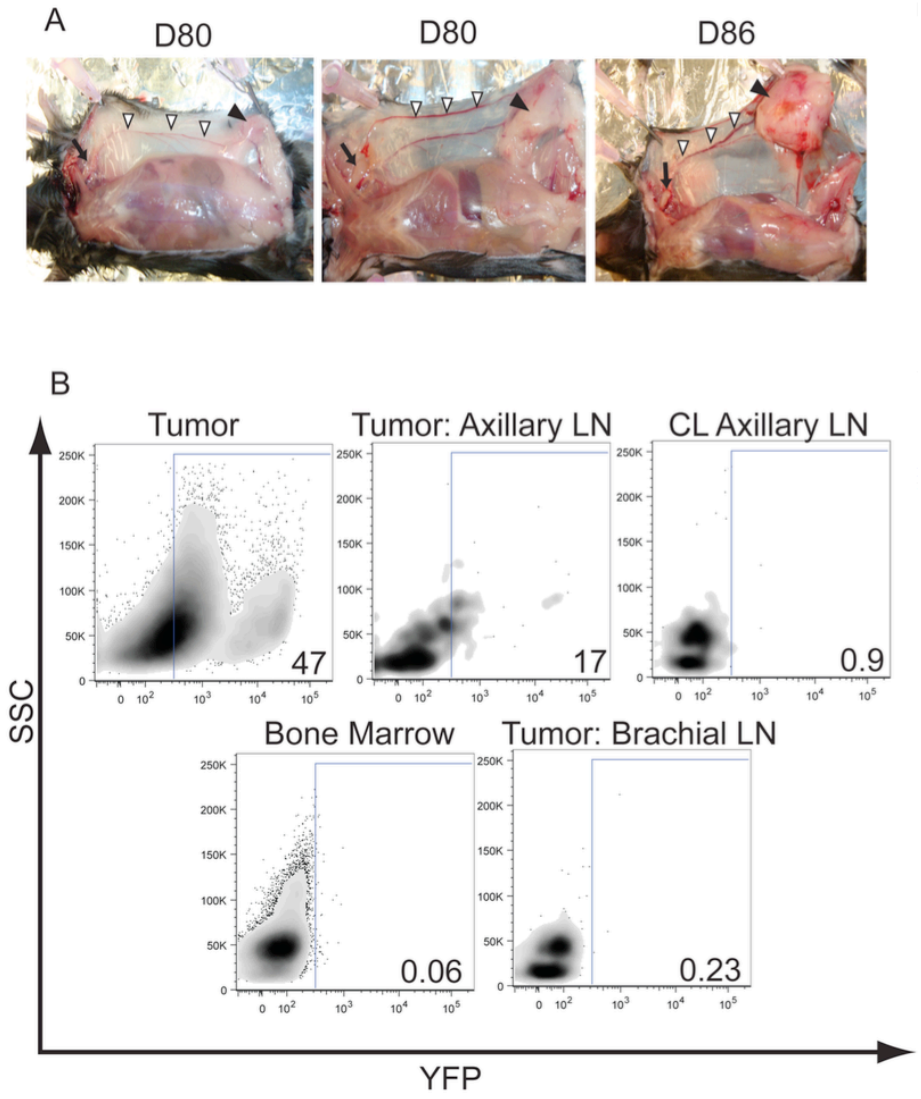

Figure 2.3. Formation of tumors and latent metastasis to the axillary lymph node. (**A**) Example of three advanced breast tumors with different rates of tumor progression. A solid mass, indicated by the arrowhead, forms and eventually grows to the size of the entire abdominal mammary tissue. The tumor stays confined to the mammary tissue and is not observed to invade or attach to the muscle covering the peritoneal cavity. There is evident engorgement of the superficial epigastric vein between the inguinal and axillary lymph nodes, denoted by white arrowheads. After 7-8 weeks, the axillary lymph node begins to become enlarged due to lymphovascular invasion of the tumor cells, indicated by arrow. (**B**) Metastasis of YFP positive tumor cells can be visualized in the axillary lymph node by flow cytometry. LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*EYFP* mice were used to induce tumors and activation of YFP by intraductal delivery of adenovirus-Cre. To verify lymphovascular invasion of tumor cells into the axillary lymph node, 80 days post adenoviral injection, the indicated lymph nodes and organs were harvested and stained for lymphocyte markers and examined for YFP expression. CL represents contralateral non-tumor draining lymph node. Results represent gating on CD45 negative tumor cells, indicating the tumor cells are invading the distal axillary lymph node. Numbers represent percent YFP positive cells from total population.

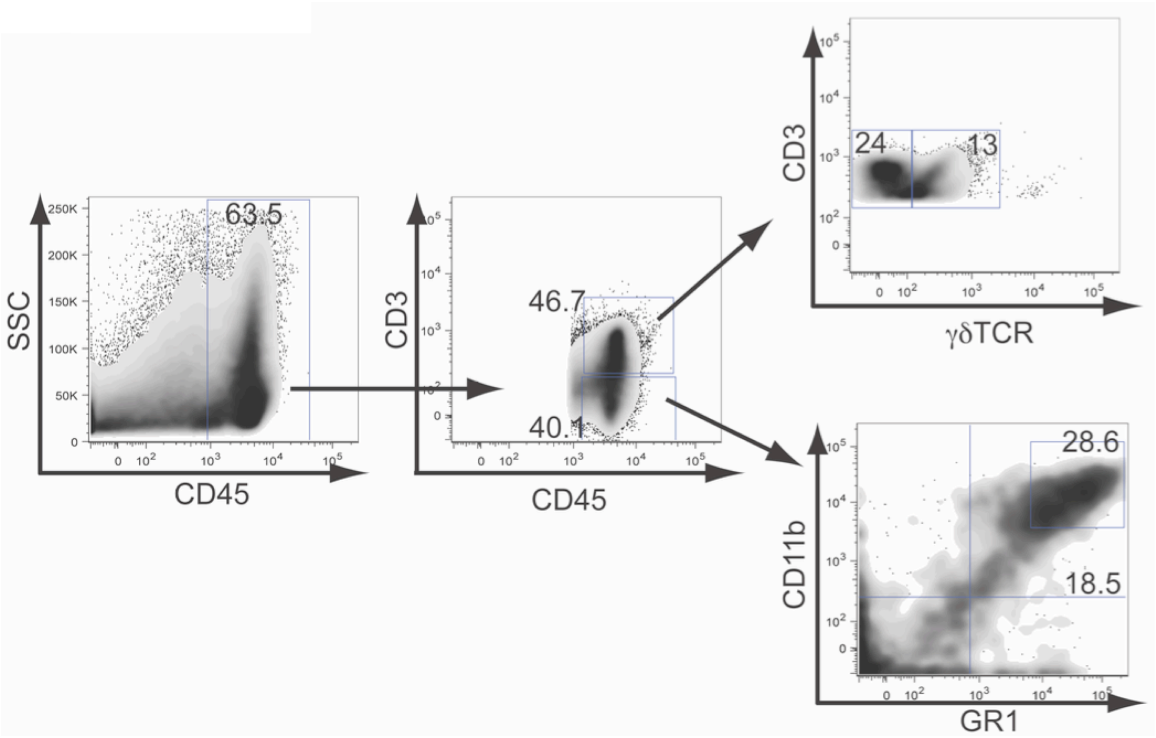

Figure 2.4. Immune infiltrates in mouse transgenic breast tumors. Mouse breast tumors were homogenized and stained for CD45, CD3, γδTCR, CD11b and GR1. Numbers represent percent of positive leukocytes in entire tumor (63.5), total CD3+ (46.7), total CD3 negative (40.1), total CD3+ γδ+ (γδ T cells, 13), CD3+ γδnegative (24), total GR1 high CD11b (MDSC, 28.6) and total CD11b GR1 low (macrophages, 18.5).

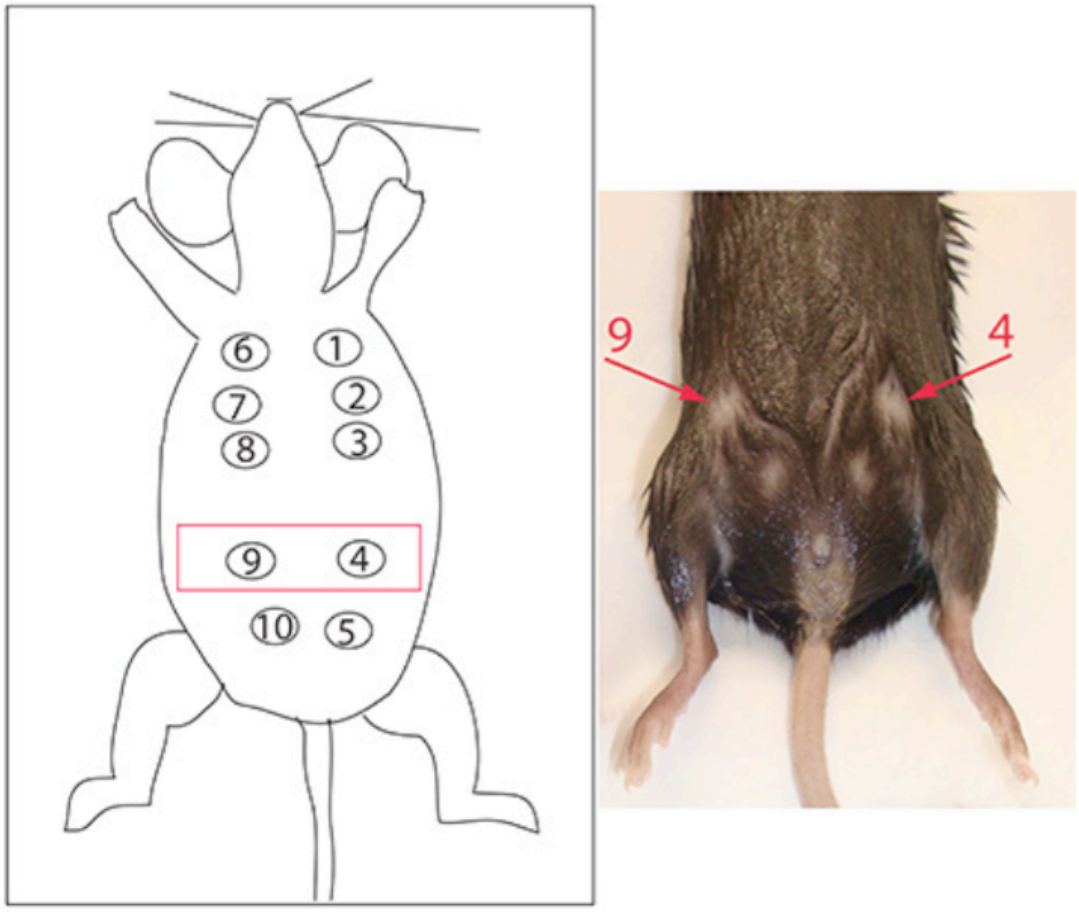

Figure 2.5. Numbering of mammary ducts. Mammary ducts 4 and 9 are highlighted in red on the diagram and indicated with arrows on the mouse. In my hands, we found that injections were easiest to perform on these mammary ducts, however all other mammary tissues that we targeted developed tumors with similar kinetics.

ACKNOWLEDGMENT

This work was supported by NCI Grants RO1CA157664 and RO1CA124515, and a Breast Cancer Alliance award. I would like to thank Jeffrey Faust, David Ambrose, and Scott Weiss from the Wistar Flow Cytometry core facility, James Hayden from the Wistar Imaging facility and the entire staff of the Wistar Institute Animal Facility for their invaluable technical support. I also sincerely thank Dr. Melanie Rutkowski for collaborating with me to perform this work.

CHAPTER 3

IL-15 AGONISTS OVERCOME THE IMMUNOSUPPRESSIVE EFFECTS OF MEK INHIBITORS

The following chapter was adapted from a published journal article.

Allegrezza MJ**,** Rutkowski MR, Stephen TL, Svoronos N, Tesone AJ, Perales-Puchalt A, Nguyen JM, Sarmin F, Sheen MR, Jeng EK, Tchou J, Wong HC, Fiering SN, Conejo-Garcia JR. IL-15 agonists overcome the immunosuppressive effects of MEK inhibitors. *Cancer Res*, Mar 15; pii: canres.28-8.2015, 2016.

Conflict of interest: Hing C. Wong and Emily K. Jeng are employees and stockholders of Altor Bioscience. Data from this chapter was used to file a provisional patent application: Methods and Compositions for Treatment of Metastatic and Refractory Cancers and Tumors. (2015) Allegrezza MJ and Conejo-Garcia JR. Provisional patent WST15-20P1.

ABSTRACT

Small molecule inhibitors are being intensely investigated for specifically targeting the oncogenic signaling that drives the growth of tumors. However, many of these inhibitors target signaling pathways that are also important for the function of anti-tumor lymphocytes. I show with a panel of 41 inhibitors targeting a variety of signaling pathways, that most inhibitors have strong negative effects on T-cell activation at their active doses on cancer cells. FDA-approved MAPK (mitogen-activated protein kinase; MEK) inhibitors were particularly T cell-suppressive *in vitro*. However, these effects are attenuated by multiple cytokines frequently administered to cancer patients. Among them, clinically available IL-15 agonists, through PI3K activation selectively in T lymphocytes, synergize *in vivo* with MEK inhibitors to elicit potent and durable anti-tumor responses that include resistance to tumor re-challenge. My study identifies an actionable approach to overcome the T-cell-suppressive effects of clinical MEK inhibitors and reconciles discrepancies between the immunological effects of targeted anti-cancer small molecule inhibitors *in vitro* versus *in vivo*.

INTRODUCTION

Small molecule inhibitors targeting oncogenic signaling pathways have emerged as a promising new class of drugs in cancer therapy. While these molecules undergo rigorous testing to demonstrate their efficacy in tumor models, their effects on the interplay between leukocytes and tumors have been largely unstudied due to the use of preclinical xenograft models that lack a competent immune system. T cells, crucial for controlling the growth of immunogenic tumors (Vesely et al. 2011), rely upon many of the same signaling pathways targeted by pharmaceutical inhibitors for activation of cytotoxicity against tumor cells. For instance, engagement of the T cell receptor (TCR) and co-stimulatory receptors activates the RAS-MAPK and PI3K-AKT signaling cascades, which are necessary for proliferation and effector function in T cells (Smith-Garvin, Koretzky, and Jordan 2009).

The FDA approved small molecule inhibitor of MEK, trametinib, presents an example of seemingly paradoxical interactions with host anti-tumor immunity. Studies have shown that proper T-cell activation and proliferation is impaired by pharmacological inhibition of MEK signaling with trametinib (Vella et al. 2014; Yamaguchi et al. 2012) and other compounds (Boni et al. 2010). These data seem to imply that trametinib would impair anti-tumor T-cell function in tumor-bearing hosts. However, it was recently found that while trametinib impairs T-cell function *in vitro*, it does not limit the effectiveness of either adoptive cell therapy (Hu-Lieskovan et al. 2015) or checkpoint blockade with antibodies against PD-1, PD-L1, and CTLA-4 (L. Liu et al. 2015) in mouse models. A potential explanation is that trametinib in these studies was co-administered with high doses of IL-2 (Hu-Lieskovan et al. 2015) and checkpoint inhibitors (L. Liu et al. 2015) that enhance the activation of T cells, thus allowing them to overcome small molecule inhibition of MEK. The possibility that cytokines can rescue some deleterious effects

associated with MEK inhibition on T cells has yet to be explored. Additionally, MEK inhibition of tumor cells can lead to increased expression of tumor-specific antigens (Boni et al., 2010; Kono et al., 2006) and the upregulation of surface antigen presentation by preventing internalization of MHC-I (Bradley et al. 2015), which may enhance recognition of tumors by $CD8^+$ effector T cells, offering another explanation for the synergy with immunotherapies. At the moment, the mechanisms explaining the paradoxical effects of MEK inhibitors on T cell-mediated anti-tumor immunity remain elusive.

In a rapidly changing clinical scenario, first-line PD-1 inhibitors, alone or in combination with CTLA4 inhibitors, may become a standard of care in the near future against tumors such as melanoma (Bowyer and Lorigan 2015). However, the combined effects and optimal sequencing of targeted therapies and immunotherapy remains unknown. Clarifying the immunosuppressive effects of targeted therapies *in vivo* is crucial for the design of synergistic combinatorial interventions with emerging immunotherapies. Trametinib was the first MEK inhibitor to be approved for clinical use in 2013, and it improves overall survival in combination with other targeted interventions (Long et al. 2015). To elucidate the effects of multiple targeted therapies on the tumor immunoenvironment and, subsequently, antitumor immunity, I analyzed a panel of molecules for their inhibitory activity on T cells. My results indicate that most small molecule inhibitors, and in particular trametinib, exert direct suppressive effects on human T cells *in vitro* and antitumor mouse T cells *in vivo*. However, the suppressive effects of MEK inhibitors can be overcome by various cytokines. I found that clinically available IL-15 agonists, through a mechanism dependent on the activation of PI3K, were particularly effective at rescuing T-cell function.

MATERIALS AND METHODS

Animals, tissues and cell lines

WT C57BL/6 and congenic Ly5.1 female 6-8 week old mice were procured from the National Cancer Institute or Charles River Laboratory. OT-I C57BL/6-Tg (TcraTcrb)1100Mjb/J transgenic mice were obtained from The Jackson Laboratory. Transgenic *Kras*^{tm4Tyj} and *Trp53*^{tm1Brn} mice (Jackson et al. 2001; Jonkers et al. 2001) were obtained from NCI Mouse Models of Human Cancers Consortium, brought to a full C57BL/6 background (Scarlett et al. 2012; Rutkowski, Stephen, et al. 2014). All animals were maintained in specific pathogen free barrier facilities and used in accordance with the Institutional Animal Care and Use Committee of the Wistar Institute.

The Brpkp110 primary mammary tumor cell line was generated by culturing a mechanically dissociated C57BL/6 LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*Myristoylated-p110α-GFP*$^{+}$ primary breast tumor mass as previously described (Rutkowski, Allegrezza, et al. 2014). Tumor cells were passaged a total of 10 times before deriving the Brpkp110 clonal cell line by limiting dilution. Brpkp110 cells were confirmed to be mycoplasma-free. Tumors were initiated by injecting $5x10^5$ cells in 200 µl PBS into the axillary flanks. Tumor volume was calculated as: 0.5 x (L x W^2), where L is the longer of the two measurements.

Peripheral blood lymphocytes were obtained by leukapheresis/elutriation and Miltenyi bead–purified. A2780 cells were obtained from AddexBio Technologies. ID8 cells (Roby et al. 2000) were provided by K. Roby (Department of Anatomy and Cell Biology, University of Kansas, Kansas City, KS) and retrovirally transduced to express *Defb29* and *Vegf-a* (Conejo-Garcia et al. 2004) or OVA (Cubillos-Ruiz et al. 2009).

T cell stimulation

For human T cell proliferation assays, K562 cells expressing human CD32, termed K32, were generated as described (Maus et al. 2002), γ-irradiated (100 Gy) and loaded with anti-CD3 (500 ng/ml, clone OKT3; eBioscience) plus anti-CD28 (500 ng/ml, clone 15E8; EMD Millipore) antibodies at room temperature for 10 min (aAPCs). PBMCs were labeled with Cell Trace Violet (Invitrogen) according to the manufacturer's instructions and co-cultured with loaded aAPCs at a 10:1 PBMC:aAPC ratio or activated with ConA (2 μg/ml, Sigma). Proliferation of T cells was determined 7 days later by FACS and Division Index was calculated using FlowJo software.

For mouse T-cell proliferation assays, pan-T cells were negatively purified from spleens with antibodies to B220 (RA3), Mac-1 (M170.13), and MHC-II (M5/114) using magnetic beads. T cells were labeled with Cell Trace Violet (Invitrogen) and stimulated with either agonistic CD3/CD28 beads (Dynabeads, Life Technologies) or tumor-pulsed bone marrow dendritic cells (BMDCs) and analyzed for proliferation by FACS either 3 days (CD3/CD28 beads) or 7 days (BMDCs) later. Day 7 BMDCs were generated as previously described (Stephen et al. 2014) and cultured overnight with double-irradiated (γ-irradiated, 100 Gy; and UV, 30 min) ID8-*Defb29/Vegf-a* cells. BMDCs were added to cultures of T cells at a 10:1 (T cell:BMDC) ratio. For recall ELISpot assays, mouse T cells were primed with tumor-pulsed BMDCs plus IL-2 (30 U/ml) and IL-7 (5 ng/ml), and restimulated 7 days later with fresh tumor-pulsed BMDCs at a 10:1 ratio in an IFN-γ ELISpot (eBioscience).

Compounds and cytokines

ALT-803 was generously provided by Altor BioScience Corporation and was diluted in sterile PBS for *in vitro* and *in vivo* studies. Recombinant human IL-15 (Novoprotein), human IL-2, human IL-21, mouse IL-7 (Peprotech), human IL-27

(eBioscience), and Concanavalin A (Type VI, Sigma-Aldrich) were reconstituted in sterile PBS and stored at -20ºC. Trametinib (GSK-1120212) was purchased from LC Laboratories and suspended in vehicle solution of 10% PEG-300 (Sigma Aldrich) and 10% Cremophor EL (EMD Millipore) in sterile dH_20 for *in vivo* oral gavage experiments. For *in vitro* assays, all inhibitors were dissolved in sterile DMSO and diluted in the assays 1:1000, so that the final concentration of DMSO was 0.1%.

Cell proliferation assays

Compound screening on A2780 cells was performed by adding compounds the morning after plating and measuring proliferation 72 hrs later. Screening on human PBMCs was performed by adding compounds simultaneously with ConA stimulation (2 ug/ml) and measuring proliferation 7 days later. Normalized percent inhibition (NPI) was calculated by measuring resazurin fluorescence with respect to values obtained with DMSO negative control and doxorubicin (5 μM) positive control as NPI=100% * (DMSO–compound) / (DMSO–doxorubicin).

Western blotting

Cells were lysed in RIPA buffer (Thermo Scientific) with Complete Protease Inhibitor Cocktail Tablets (Roche) and phosphatase inhibitors (Halt Phosphatase Inhibitor, Thermo Scientific, and Na_3VO_4, 1 mM) and cleared by centrifugation. Proteins were quantified by BCA assay (Thermo Scientific), diluted in reducing Lamelli buffer, denatured at 95°C, run on mini Protean TGX Ready Gels (Bio-Rad Laboratories), transferred to a PVDF membrane, blocked, and incubated with primary antibodies for p-ERK1/2 (D13.14.4E), p-AKT (D9E) and β-tubulin (9F3), all from Cell Signaling; plus β-actin (Sigma; AC-15). Immunoreactive bands were developed using horseradish

peroxidase–conjugated secondary antibodies (Bio-Rad) and ECL substrate (GE Healthcare).

TCR ligation of human CD8 T cells

$CD8^+$ T cells were sorted from PBMCs and rested overnight in R10. T cells ($0.5x10^6$ per condition) were stained with OKT3-biotin (BioLegend, 10 μg/ml) for 15 mins on ice, and washed in cold PBS. TCR ligation was performed by adding streptavidin (Promega, 25 μg/ml) and anti-CD28 antibody (Millipore, clone 15E8, 1 μg/ml) in the presence of indicated inhibitors for 10 min at 37°C.

***In vivo* OT-I proliferation**

Congenic Ly5.1 mice were injected with $1.5x10^6$ ID8-OVA cells i.p. (Huarte et al. 2008). Mice were oral-gavaged with trametinib or vehicle on days 9-13. On day 10, mice were injected i.p. with $1.5x10^6$ Cell-Trace Violet labeled, unstimulated OT-I T cells. Mice were administered ALT-803 (0.2 mg/kg on day 10) or IL-2 (50,000 IU/mouse on days 10-12) i.p. On day 14, peritoneal washes were analyzed for proliferating OT-I T cells.

Statistics

Unless indicated otherwise, all data shown represent means with SEM. All hypothesis testing was two-sided, and unpaired t-tests were performed unless indicated otherwise. A significance threshold of 0.05 for *P* was used (**P*<0.05, ***P*<0.01, ****P*<0.001). Analyses were carried out using GraphPad Prism software. Experiments were repeated at least twice unless otherwise indicated.

RESULTS

Multiple targeted small-molecule inhibitors suppress human T-cell activation *in vitro*

To determine the sensitivity of human T cells to inhibition of signaling pathways commonly targeted by small molecules in cancer therapy (Wargo, Cooper, and Flaherty 2014), I first designed a high-throughput assay to test a diverse panel of 41 inhibitors over a 6 log concentration range on Concanavalin A (ConA)-induced activation and expansion of human T cells from peripheral blood mononuclear cells (PBMCs). At doses equivalent to or below those required to limit proliferation of A2780 ovarian cancer cells – known to be sensitive to PI3K and MEK inhibitors (Gao et al. 2004; Hou et al. 2013) – a variety of inhibitor classes prevented ConA-driven T-cell expansion (**Figure 3.1A)**. Small molecules targeting PI3K, mTOR, MAPK, and CDK signaling, as well as transcriptional regulators (HDACs) and survival molecules (Bcl-2) were deleterious for T-cell expansion. Among these, trametinib, the MEK1/2 inhibitor approved by the FDA for *BRAF*-mutant melanoma, was particularly potent at inhibiting the *in vitro* proliferation of human T cells. Overall, the observed EC50 of every tested molecule with some activity on A2780 cells was lower for human T cells than for A2780 cells (**Table 3.1 and Figure 3.2&3.3**), highlighting the immunosuppressive effects of most small-molecule targeted therapies.

I validated my screening approach by focusing on inhibitors of the PI3K and MEK signaling pathways. Abrogation of T-cell activation elicited by small molecule inhibitors was not restricted to ConA stimulation, because pan-PI3K (BKM120) and MEK (GDC0973) inhibitors also restricted the proliferation of human T cells in response to artificial antigen presenting cells (aAPC) coated with agonistic CD3 and CD28 antibodies (Maus et al. 2002) (**Figure 3.1B**). Importantly, these effects were consistent

among 3 different donors (**Figure 3.1C**). Comparable results were obtained with the MEK inhibitor trametinib (**Figure 3.1D**). As expected, T cells were more sensitive to kinase inhibitors when they were activated in the absence of costimulation (aAPCs lacking anti-CD28), as could occur within the immunosuppressive microenvironment of tumor-bearing hosts (**Figure 3.4A&B**). I noticed only minor and non-significant differences in the repertoire of memory and effector T-cell subsets when cultures were activated without anti-CD28 and with lower amounts of anti-CD3, indicating that differences in sensitivity to the kinase inhibitors cannot be attributed to altered T-cell differentiation (**Figure 3.5A-D**).

In order to determine the effects of MEK and pan-PI3K inhibitors on different T-cell subsets, I FACS purified human CD8 T cells into naïve, memory, and effector populations based upon CD45RA and CD27 expression and activated them with aAPCs. I found that trametinib equally inhibited proliferation of naïve ($CD45RA^{+}CD27^{+}$), memory ($CD45RA^{-}CD27^{+}$), and effector/effector memory ($CD45RA^{-}CD27^{-}$) cells, although one donor showed a trend that memory and effector/effector memory cells were less sensitive than naïve cells to trametinib (**Figure 3.6A&B**). Interestingly, memory and effector/effector memory cells were more sensitive to PI3K inhibition with BKM120 than naïve cells, a result consistent among 3 donors (**Figure 3.6C&D**). Differentiated effector cells ($CD45RA^{+}CD27^{-}$) did not proliferate in response to aAPCs, so I could not conclude their sensitivity to kinase inhibition from this analysis (**Figure 3.7**).

We also explored the result of MEK and PI3K inhibition on physiologic activation of T cells with tumor antigens. MEK and PI3K inhibitors completely abrogated the initial priming response of murine T cells activated with tumor lysate-pulsed dendritic cells (DCs) (Scarlett et al. 2012; Stephen et al. 2014) (**Figure 3.6E&F**). More importantly, the direct suppressive effects of these inhibitors were not restricted to proliferative

responses, because the frequency of tumor-primed T cells secreting IFN-γ in response to re-stimulation with fresh tumor lysate-pulsed DCs was also significantly reduced when either PI3K or MEK were inhibited (**Figure 3.6G&H**).

Overall, these data underscore the T-cell suppressive effects of most small molecule targeted therapies clinically approved or in the pipeline of clinical development, at a time when combinatorial immunotherapeutic interventions are being tested against multiple tumors.

IL-15 rescues the suppressive effects of MEK inhibitors through PI3K activation

Despite the strong T cell inhibitory activity of FDA-approved trametinib on both initial priming and recall responses, recent reports suggest that trametinib does not limit the effectiveness of adoptive cell therapy in preclinical tumor models (Hu-Lieskovan et al. 2015). Interestingly, these studies included the administration of high doses of IL-2. I reasoned that cytokines signaling on immune cells could rescue the deleterious effects of trametinib on T-cell activity. I therefore tested a panel of cytokines known to play a role in T-cell survival and proliferation for their ability to recover T-cell expansion from MEK inhibition. Supporting my hypothesis, IL-2, IL-7, and IL-15 were able to individually rescue the proliferation of human T cells in the presence of trametinib (**Figure 3.8A**). In contrast, IL-21 and IL-27 had no significant rescuing effect (**Figure 3.8A**). Among all cytokines tested, I focused on IL-15 because it provides a strong stimulating signaling to both effector and memory $CD8^+$ T cells without inducing the expansion of Tregs, as compared to IL-2 (Liao, Lin, and Leonard 2013; Waldmann 2006). Indeed, IL-15 was able to dramatically rescue the proliferation of purified CD8 naïve, memory, and effector/effector memory T cells (**Figure 3.8B**).

We found that IL-15 can rescue early (within 10 minutes post-activation) TCR-induced MAPK signaling from MEK inhibition, as shown by ERK1/2 phosphorylation (**Figure 3.8C**). Mechanistically, this effect depends on activation of PI3K by IL-15 (Budagian et al. 2006), because IL-15 was not able to rescue the defect in ERK1/2 phosphorylation in the presence of a pan-PI3K inhibitor (**Figure 3.8C**). Furthermore, activation of protein kinase C (PKC) with phorbol-12-myristate-13-acetate (PMA) completely overcomes the suppressive effect of PI3K inhibition on ERK1/2 phosphorylation without fully restoring PI3K activity as assayed by AKT phosphorylation (**Figure 3.8C**). This is consistent with a mechanism of ERK phosphorylation (Ueda et al. 1996) mediated by the activation of PKC isoforms upon stimulation of the PI3K pathway, which is known to result in the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and, subsequently, activation of PDK1 kinase (Cantley 2002). Together, these results show that IL-15 can augment early signaling events downstream of TCR activation to enhance the amplitude of MAPK signaling to overcome MEK1/2 inhibition by trametinib.

The IL-15 superagonist ALT-803 overcomes the suppressive effects of trametinib *in vivo* in tumor-bearing hosts

To define whether IL-15 signaling can overcome MEK inhibition-induced suppression of T-cell activation in the tumor microenvironment *in vivo*, I investigated the IL-15 agonist ALT-803 for its ability to rescue T cell functions from MEK inhibition. ALT-803 is a new IL-15 agonist complex with IL-15^{N72D} bound to the IL-15RαSu/Fc (Xu et al. 2013). This IL-15^{N72D}:IL-15RαSu/Fc has a significantly longer serum half-life and increased biological activity compared to native IL-15 and is undergoing extensive clinical testing (**Table 1.1**). In this study, I found that ALT-803 was more effective than

IL-7 (**Figure 3.8D**) and IL-15 (**Figure 3.9A&B**) in rescuing human T-cell proliferation *in vitro*. Human T-cell proliferation in the presence of ALT-803 remained unaffected by trametinib, even at the relatively high concentration of 5 μM (**Figure 3.9B**). As was the case for IL-15, I found that ALT-803 activity was also dependent on PI3K signaling because T-cell expansion could not be fully rescued when pan-PI3K and MEK inhibitors were combined (**Figure 3.9B**). I further determined that the reduction in proliferation when pan-PI3K and MEK inhibitors were combined was due to a block in cell division rather than an increase in cell death (**Figure 3.10A-E**).

ALT-803 was also able to rescue the proliferation of purified human $CD8^+$ naïve, memory, and effector/effector memory T cells from trametinib, indicating broad activity on a range of T-cell subsets (**Figure 3.11**). Intriguingly, ALT-803 (and IL-15 for one donor) induced the proliferation of purified, differentiated effector ($CD45RA^+CD27^-$) $CD8^+$ T cells that were otherwise unable to proliferate in response to CD3/CD28 activation (**Figure 3.7**). ALT-803 showed activity on mouse T cells as well, demonstrated by its ability to restore proliferation of bead-activated T cells (**Figure 3.9C**) and IFN-γ recall responses of tumor-primed T cells in the presence of trametinib (**Figure 3.9D**).

To test the activity of ALT-803 in the tumor microenvironment, I transferred Cell Trace Violet-labelled (Ovalbumin (OVA)-specific) OT-I T cells into mice growing OVA-transduced syngeneic ID8 ovarian tumors, a system that allows the recovery of tumor microenvironment lymphocytes through peritoneal wash (Cubillos-Ruiz et al. 2009; Conejo-Garcia et al. 2004; Nesbeth et al. 2010). After 4 days, I found that in mice treated with trametinib, the OT-I T cells proliferated significantly less than in mice gavaged with vehicle (**Figure 3.12A&B**). Importantly, when a single dose of ALT-803 was co-administered with OT-I T cells, proliferation was dramatically enhanced and was not restricted by trametinib (**Figure 3.12A&B**). The observation that ALT-803 induces

proliferation of some CD44^{lo} cells in addition to CD44^{hi} (antigen-experienced) OT-I T cells suggests that ALT-803 may also result in homeostatic proliferation, as has been reported previously (Rhode et al. 2015). I also found that treatment of mice with a high-dose IL-2 regimen was able to rescue OT-I T-cell proliferation (**Figure 3.13B&C**). These results indicate, first, that trametinib impairs antigen-specific T-cell responses *in vivo*, although to a lesser degree than *in vitro*. And secondly, that therapeutic activation of IL-15 or IL-2 signaling can completely overcome trametinib-induced CD8^{+} T-cell suppression in the tumor microenvironment.

Combination of ALT-803 and trametinib induces rejection of *Kras*-mutated tumors

To investigate the therapeutic potential of combining trametinib and ALT-803 against established *Kras*-mutated tumors, I utilized a syngeneic tumor model derived from an autochthonous breast cancer initiated in triple transgenic (LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*Myristoylated-p110α-GFP*$^{+}$) mice with adenovirus-Cre (Jackson et al. 2001; Jonkers et al. 2001; Rutkowski, Allegrezza, et al. 2014), as described in the protocol from Chapter 2. I chose this cell line, termed Breast-p53-Kras-p110α (Brpkp110), to model treatment against tumors that evade single molecule targeting of the MAPK pathway through PI3K activation, as has been commonly reported in human cancer cells (Wee et al. 2009; Mirzoeva et al. 2009; Turke et al. 2012). Brpkp110 cells have detectable signaling through MEK that can be inhibited with trametinib (**Figure 3.14A**) and generate aggressive tumors when grown subcutaneously in mice.

Oral gavage with trametinib significantly reduced the growth of Brpkp110 tumors as a single intervention (**Figure 3.14B**), although all mice eventually progressed to terminal disease (**Figure 3.14B-D**). In contrast, when ALT-803 was combined with trametinib treatment, Brpkp110 tumors progressed even slower, with 19% of mice

remaining tumor free at 50 days, and 15% exhibiting complete regression (**Figure 3.14D**). No mice in either single treatment group remained tumor free after 50 days. Most importantly, mice that recovered from tumor challenge with trametinib/ALT-803 combination treatment developed immunological memory against the tumor because these mice were resistant to subsequent re-challenge with Brpkp110 cells in the opposite flank over 30 days after initial tumor rejection, whereas all naïve control mice developed tumors (**Figure 3.14E**). These results indicate that a chemo-immunotherapy with ALT-803 and trametinib could provide potent anti-tumor activity against some established and aggressive tumors and elicit protective immunity for tumor recurrences.

DISCUSSION

Immunotherapies are revolutionizing cancer treatments (Drake 2015). In addition, the effects of existing chemotherapy on immune cells and the tumor microenvironment have recently become better appreciated (Zitvogel et al. 2008). Here I show that many small molecule inhibitors used as targeted therapies have significant immunosuppressive effects *in vitro* by interfering with signaling pathways that are critical for priming the responses of T cells. Nevertheless, MEK inhibitor-induced suppression can be rescued by signaling from some common γ-chain family cytokines, of which the most effective is the IL-15 agonist, ALT-803.

The FDA-approved MEK inhibitor trametinib demonstrated significant immunosuppressive activity on T cells *in vitro*. These data are in agreement with previous reports (Vella et al. 2014; Yamaguchi et al. 2012); however, recent studies indicate that trametinib does not limit the effectiveness of adoptive cell therapy *in vivo* (Hu-Lieskovan et al. 2015) and synergizes with PD-1 inhibitors (L. Liu et al. 2015). My

study provides a mechanistic rationale to reconcile this paradox by demonstrating that direct T-cell inhibition by trametinib, while still detectable *in vivo* in tumor-bearing hosts, can be effectively overcome by cytokines such as IL-2, IL-7 and IL-15. Therefore, a combination of these cytokines endogenously in tumor-bearing hosts may explain why I observed lower suppressive effects of trametinib *in vivo* as compared to *in vitro*. Additionally, the study showing that trametinib synergized with an adoptive transfer immunotherapy (ACT) in a mouse *BRAF*-driven tumor model included high-dose IL-2 treatment (Hu-Lieskovan et al. 2015), a regimen that was capable of rescuing T cell proliferation from trametinib in my *in vivo* experiments. My results suggest that potential trametinib-driven T-cell inhibition in ACT models may be offset by IL-2 administration. Nevertheless, as MEK inhibitors are being clinically tested against multiple *Kras*-driven malignancies (Infante et al. 2013; Bedard et al. 2015; Blumenschein et al. 2015), my data provide an actionable approach to effectively overcome any direct T cell-inhibitory effects in future combinations of trametinib and emerging immune therapies, through the use of T cell-rescuing agonists.

Our study indicates that ALT-803, an IL-15 agonist complex (Xu et al. 2013), could be the agent of choice for such a combination therapy. ALT-803 induces memory $CD8^+$ T cells to proliferate, upregulate receptors for innate immunity, secrete IFN-γ and acquire the ability to kill tumor cells (Xu et al. 2013; Mathios et al. 2015; H. C. Wong, Jeng, and Rhode 2013). Stimulation of NK cells by ALT-803 can also contribute to enhanced antitumor immunity, thus it will be important for future work to analyze the contribution of NK cells to efficacy in the Brpkp110 model. ALT-803 exhibits more potent anti-tumor activities against tumors than recombinant IL-15 in various animal models (Xu et al. 2013), likely due to its much stronger binding capability to IL2Rβγ displayed on T and NK cells, longer serum half-life, and better biological distribution to and detainment

in lymphoid tissues. Currently, ALT-803 is in multiple clinical trials against solid and hematological tumors either as a single agent or in combination with other FDA-approved immuno-stimulators or therapeutic antibodies (**Table 1.1**).

As most tumor-reactive T cells in cancer patients are unlikely to be naïve T cells, it is important to understand the outcome of kinase inhibition on memory and effector T cells. Sensitivity to MEK inhibition has been reported to correlate negatively with T-cell differentiation, suggesting that naïve T cells require more MEK signaling than memory and effector cells (Shindo et al. 2013). I observed this trend in one of three healthy donors, while two others showed similar sensitivities between memory, effector memory, and naïve cells. IL-15 agonists were remarkably successful at reversing the block in proliferation by trametinib on naïve, memory, and effector memory T cells. Additionally, murine tumor-reactive effector T cells were inhibited by trametinib, but ALT-803 was also able to rescue this activity.

Importantly, I found that IL-15 signaling rescues MEK inhibition-induced T-cell suppression through the activation of the PI3K pathway. The synergy between PI3K and MEK inhibitors on tumor cells (Engelman et al. 2008) therefore also exists for human T cells. These data suggest that combinatorial therapies in patients may compromise anti-tumor immunity, especially considering that combination treatment prevents the rescue by IL-15 agonists *in vitro*. Because treatments combining PI3K and MEK inhibitors are being investigated clinically (Bedard et al. 2015), it is important that future studies determine whether this combination negatively impacts the antitumor activity of T cells in tumor bearing hosts and thus represents a poor option for combination targeted/immunotherapies.

Overall, my finding in this study further illustrates that a greater understanding of targeted small molecules on the host's immune system and tumor microenvironment

could lead to more effective combination therapies against cancer, and provides a novel intervention that should pave the way for combining small molecule inhibitors with immunotherapies.

TABLES

Target	Molecule	EC_{50} T cells (μmol/L)	EC_{50} A2780 (μmol/L)
AKT	GSK2141795	0.0960	2.35
AKT	MK-2206	0.446	8.75
Aurora A	MLN8237 (alisertib)	0.0868	>25
Aurora B	AZD1152 (barasertib)	0.0921	>25
Aurora pan	SNS-314	0.0729	5.83
Autophagy	Spautin-1	>25	>25
Bcl-2	ABT-737	0.0610	N/A
Bcl-2	GX15-070 (obatoclax)	0.0567	1.11
BCR-ABL	AMN107 (nilotinib)	2.48	19.1
BCR-ABL	STI-571 (imatinib)	9.00	20.8
BRAF	GSK436 (dabrafenib)	1.28	11.4
BRAF	PLX4032 (vemurafenib)	5.26	>25
c-MET	AMG-208	>25	>25
CDK	LEE011	2.93	>25
CDK	PD0332991 (palbociclib)	0.110	5.21
CDK	SCH727965 (dinaciclib)	0.00273	2.94
ERK	SCH772984	0.0222	7.64
HDAC	LBH589 (panobinostat)	0.0250	3.61
IGF-1R	AG-1024	9.83	21.3
IGF-1R	GSK1904529A	13.9	>25
IGF-1R	OSI-906 (linsitinib)	>25	>25
MEK	AZD6244 (selumetinib)	24.5	>25
MEK	GDC-0973 (cobimetinib)	0.00435	9.04
MEK	GSK1120212 (trametinib)	0.000337	>25
MEK	U0126	1.92	12.6
mTOR	AZD8055	0.0136	0.0783
mTOR	INK128	0.0184	0.257
mTOR	OSI-027	0.311	5.88
mTOR	Rapamycin	0.00403	N/A
p53	Nutlin-3a	1.13	16.8
PI3K	BKM120 (buparlisib)	0.239	11.5
PI3K	GDC-0941 (pictilisib)	0.0500	1.54
PI3K	GSK2126458 (omipalisib)	0.000237	0.155
PI3K	LY294002	2.69	17.2
PI3K	PX-866	1.20	>25
PI3Kβ	AZD6482	0.962	>25
PI3K/mTOR	BEZ235 (dactolisib)	0.00240	0.0210
PORCN	LGK974	9.03	24.4
RAF/VEGFR2	RAF265	0.128	5.63
Smoothened	LDE225 (sonidegib)	3.57	8.48
Src	AZD0530 (saracatinib)	0.422	>25

NOTE: EC_{50} values were calculated from normalized percent inhibition data using nonlinear curve fitting in PRISM software. N/A indicates failure to fit curve and >25 indicates EC_{50} values greater than the highest concentration tested (25 μmol/L).

Table 3.1. **List of EC50 values of compounds analyzed**. The names, targets, and EC50 values on T cell expansion from PBMCs and A2780 proliferation for each compound tested is listed. EC50 values were calculated from normalized percent inhibition data using non-linear curve fitting in PRISM software. N/A indicates failure to fit curve and >25 indicates EC50 values greater than the highest concentration tested (25 μM).

FIGURES

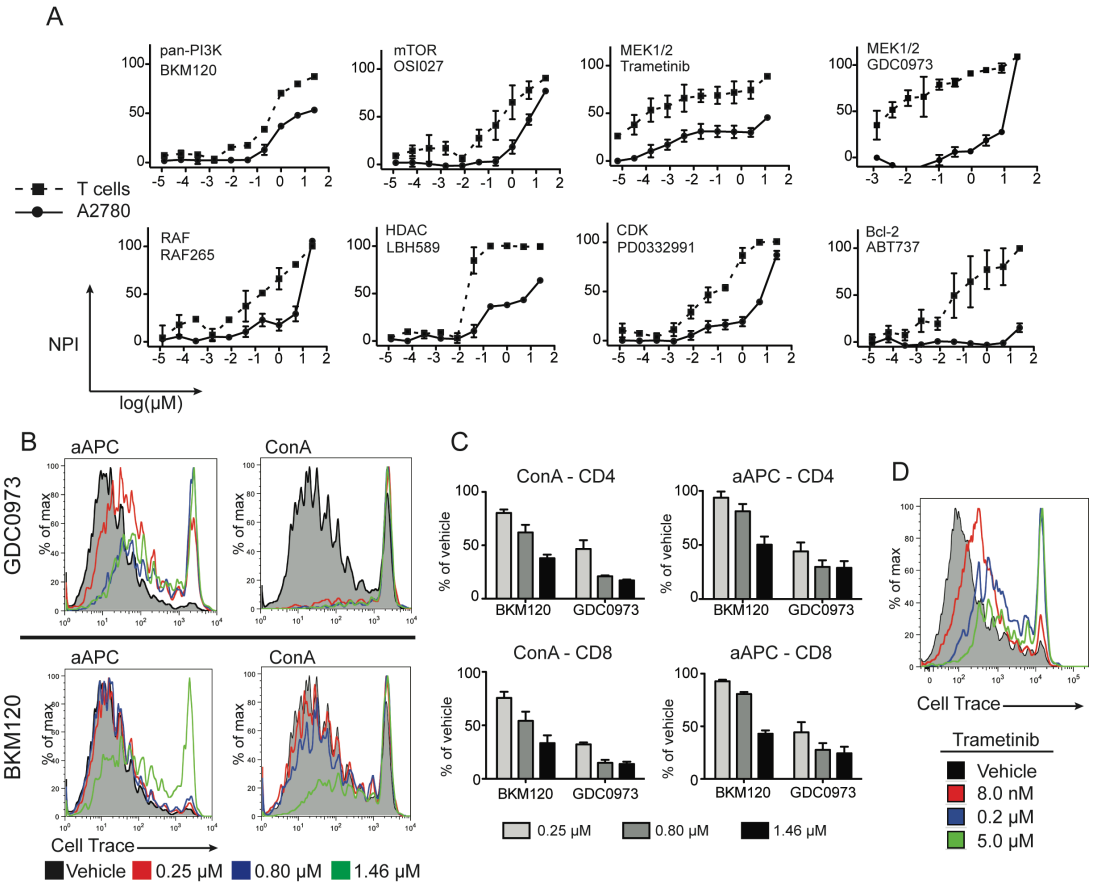

Figure 3.1. **Multiple targeted small molecule inhibitors suppress T-cell responses *in vitro***. (**A**) A2780 cells were cultured for 3 days and human PBMCs were activated with Concanavalin A (ConA) for 7 days in the indicated compounds. Plots show the normalized percent inhibition (NPI) over a range of concentrations. (**B**) Human PBMCs were stained with Cell Trace and activated with either aAPCs or ConA in the presence of inhibitors. Proliferation of live CD8 cells after 7 days is shown. (**C**) Means of the division index normalized to vehicle for CD4 and CD8 cells activated with ConA or aAPCs as in (B) from 3 different donors. All groups are significantly less than 100% (P <0.05, one sample t-test) except ConA-CD8: BKM 0.25 μM and aAPC-CD4: BKM 0.25 μM and 0.80 μM. (**D**) Human PBMCs were stained and activated with aAPCs as in (B) in the presence of trametinib. Shown are live CD8 cells.

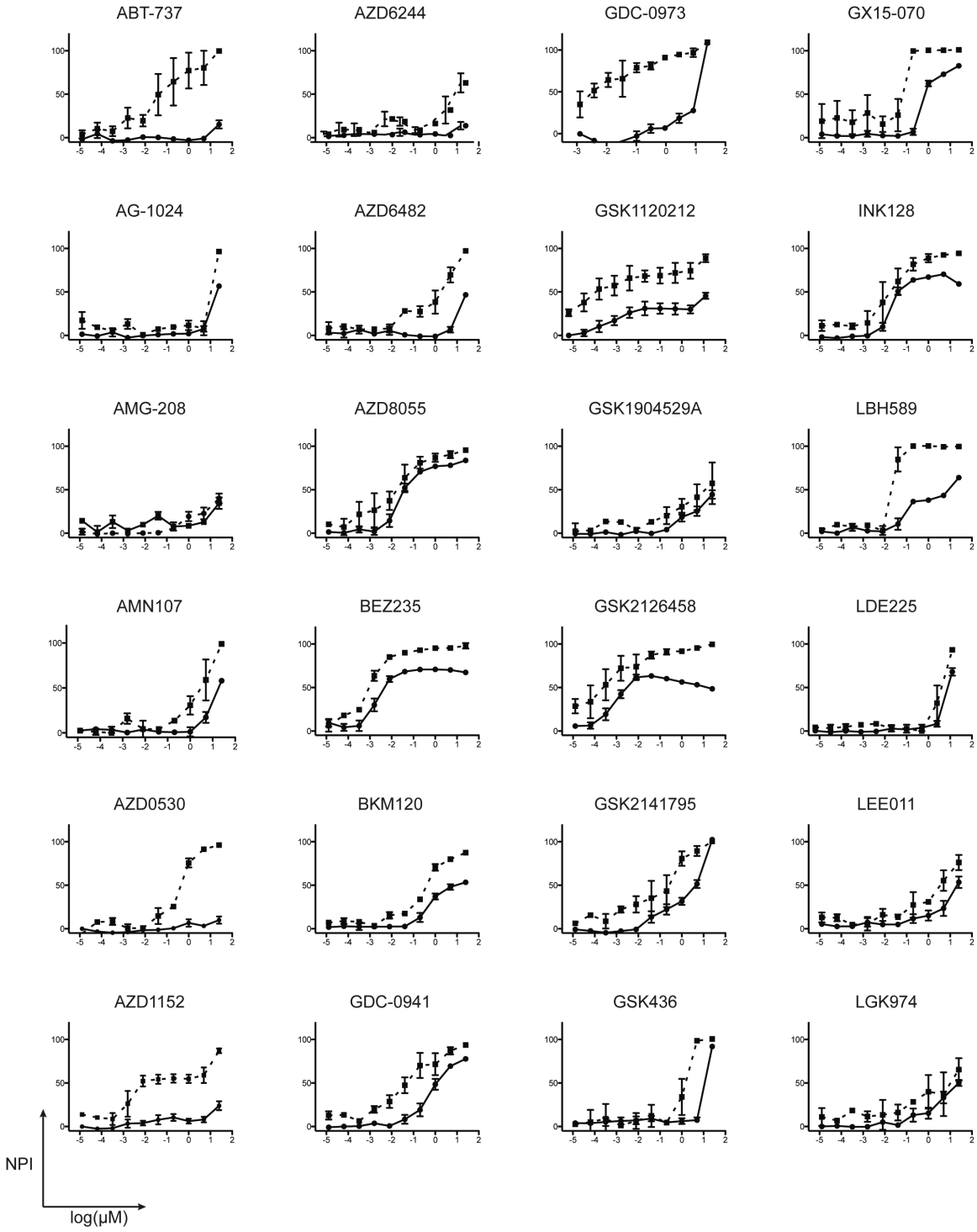

Figure 3.2. Multiple targeted small molecule inhibitors suppress T-cell proliferation. A2780 cells (solid line) were cultured for 3 days and human PBMCs (dotted line) were activated with Concanavalin A for 7 days in the indicated compounds. Plots show the normalized percent inhibition (NPI) over a range of concentrations.

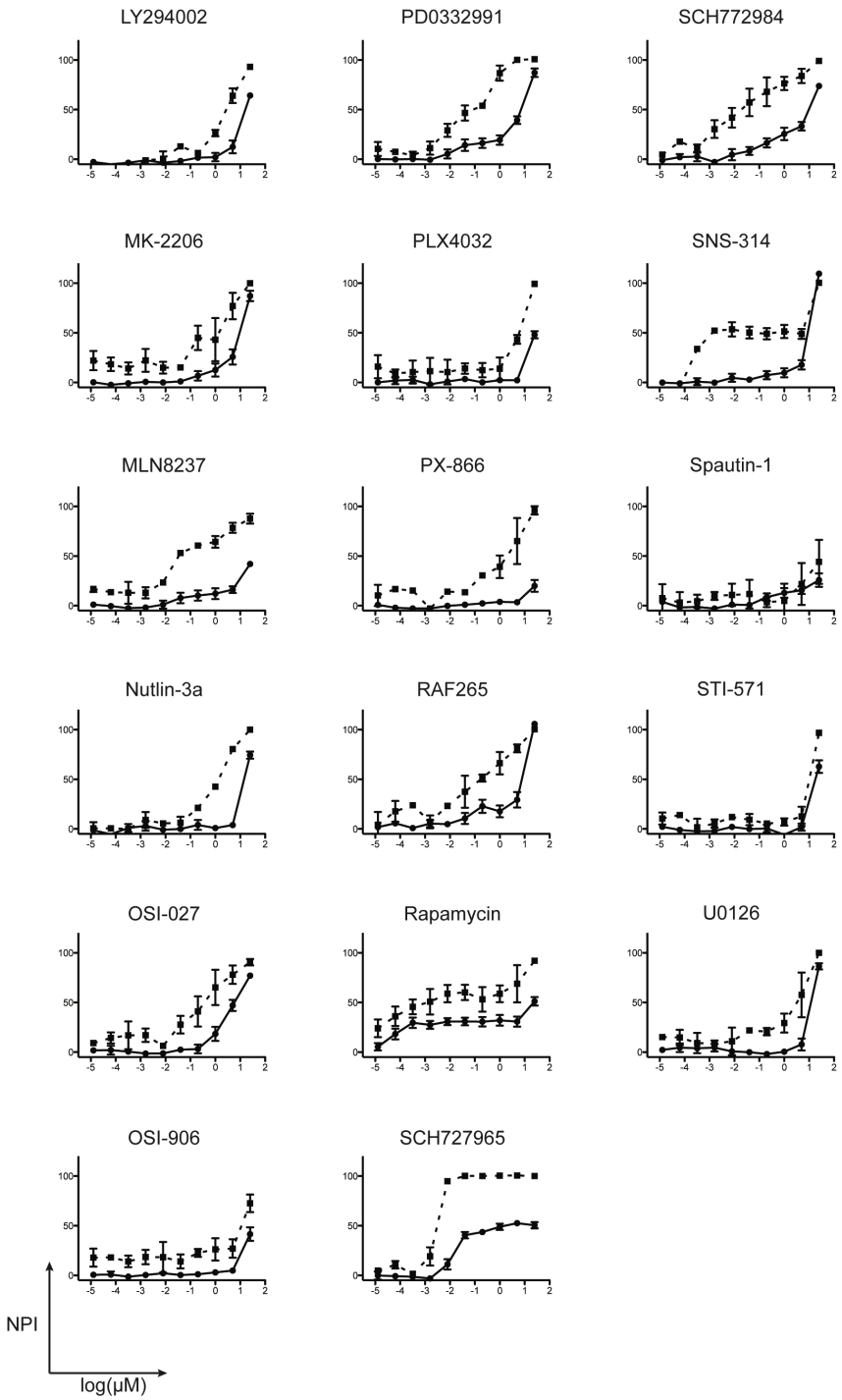

Figure 3.3. Multiple targeted small molecule inhibitors suppress T-cell proliferation continued. A2780 cells (solid line) were cultured for 3 days and human PBMCs (dotted line) were activated with Concanavalin A for 7 days in the indicated compounds. Plots show the normalized percent inhibition (NPI) over a range of concentrations.

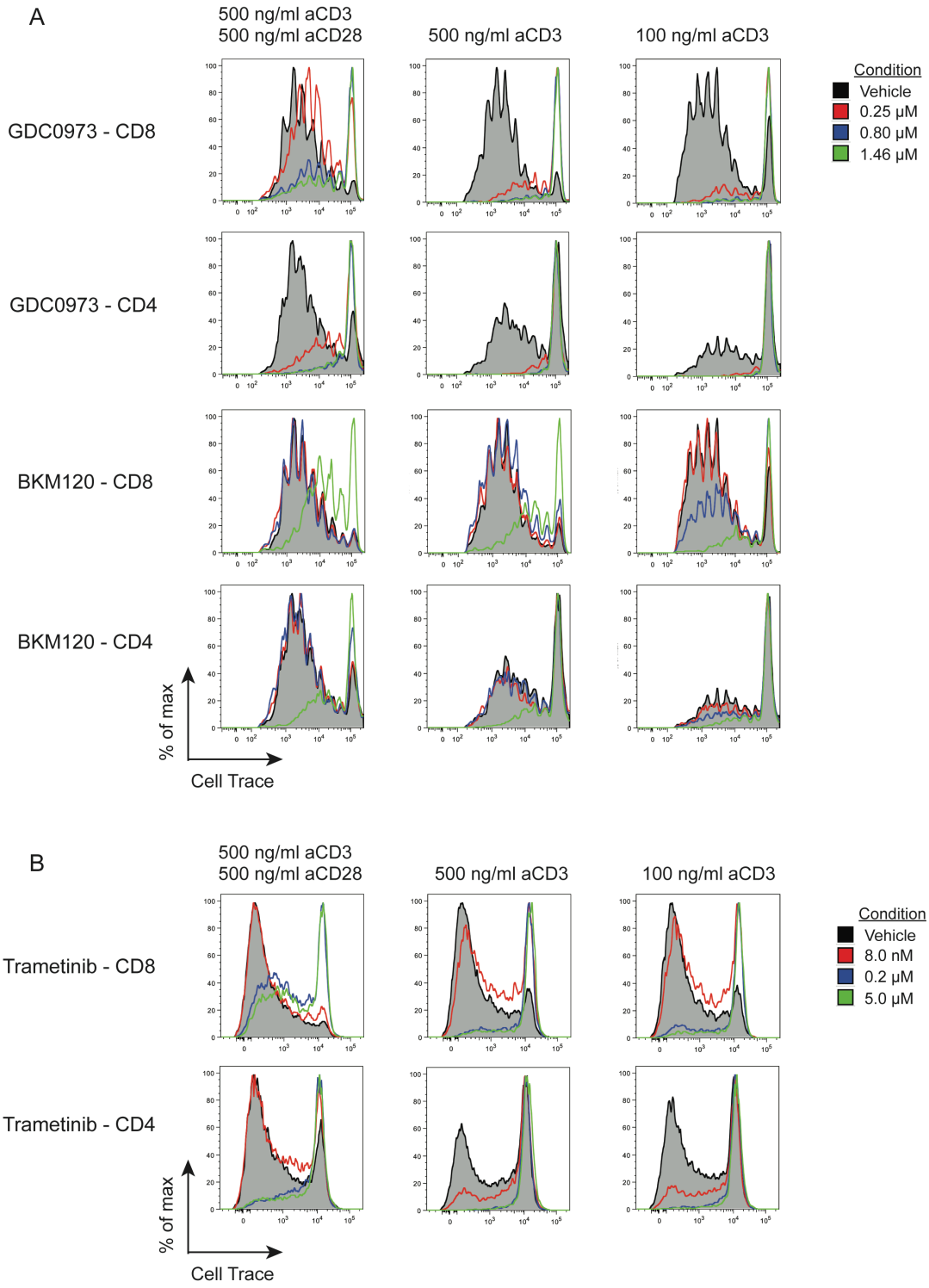

Figure 3.4. Stimulation strength affects T-cell sensitivity to PI3K and MEK inhibitors. (A) and **(B)** Human PBMCs were stained with Cell Trace and activated with either aAPCs loaded with anti-CD3 and anti-CD28 or anti-CD3 alone at various concentrations in the presence of inhibitors. Proliferation of live CD8 and CD4 cells after 7 days is shown for one representative donor of two (**A**) or three (**B**).

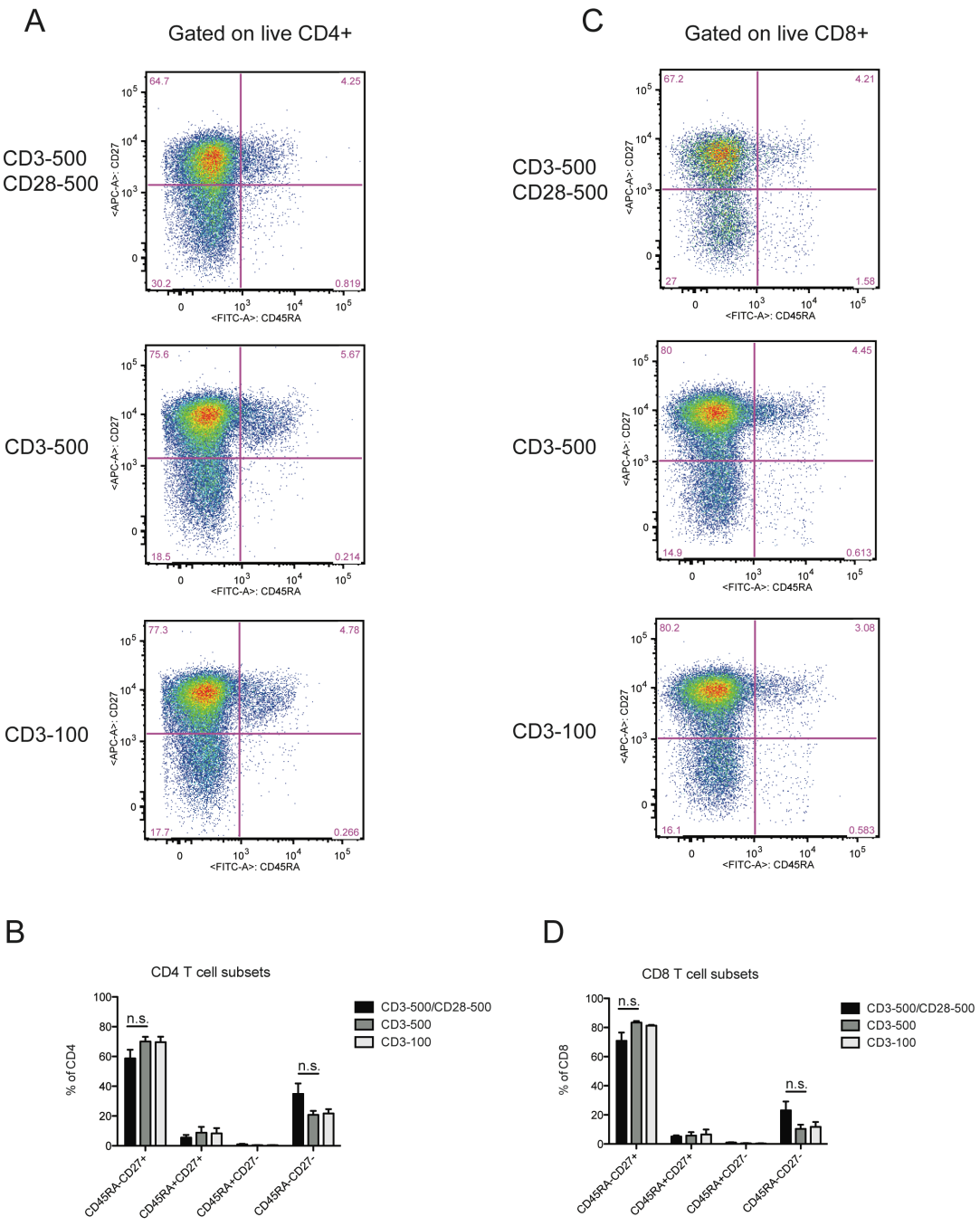

Figure 3.5. Human T-cell differentiation is not affected by lower activation strength with aAPCs. Human PBMCs were stained with Cell Trace and activated with either aAPCs loaded with anti-CD3 and anti-CD28 (500 ng/ml) or anti-CD3 alone at various concentrations (500 or 100 ng/ml) in the presence of inhibitors. After 7 days, cells were stained with ZombieYellow live/dead, CD4, CD8, CD27, and CD45RA. Plots of live CD4 (**A**) and CD8 (**B**) cells for CD27 and CD45RA expression are shown for one representative donor of three. Means of percentages from three donors are shown for the indicated CD4 (**C**) and CD8 (**D**) cell subsets.

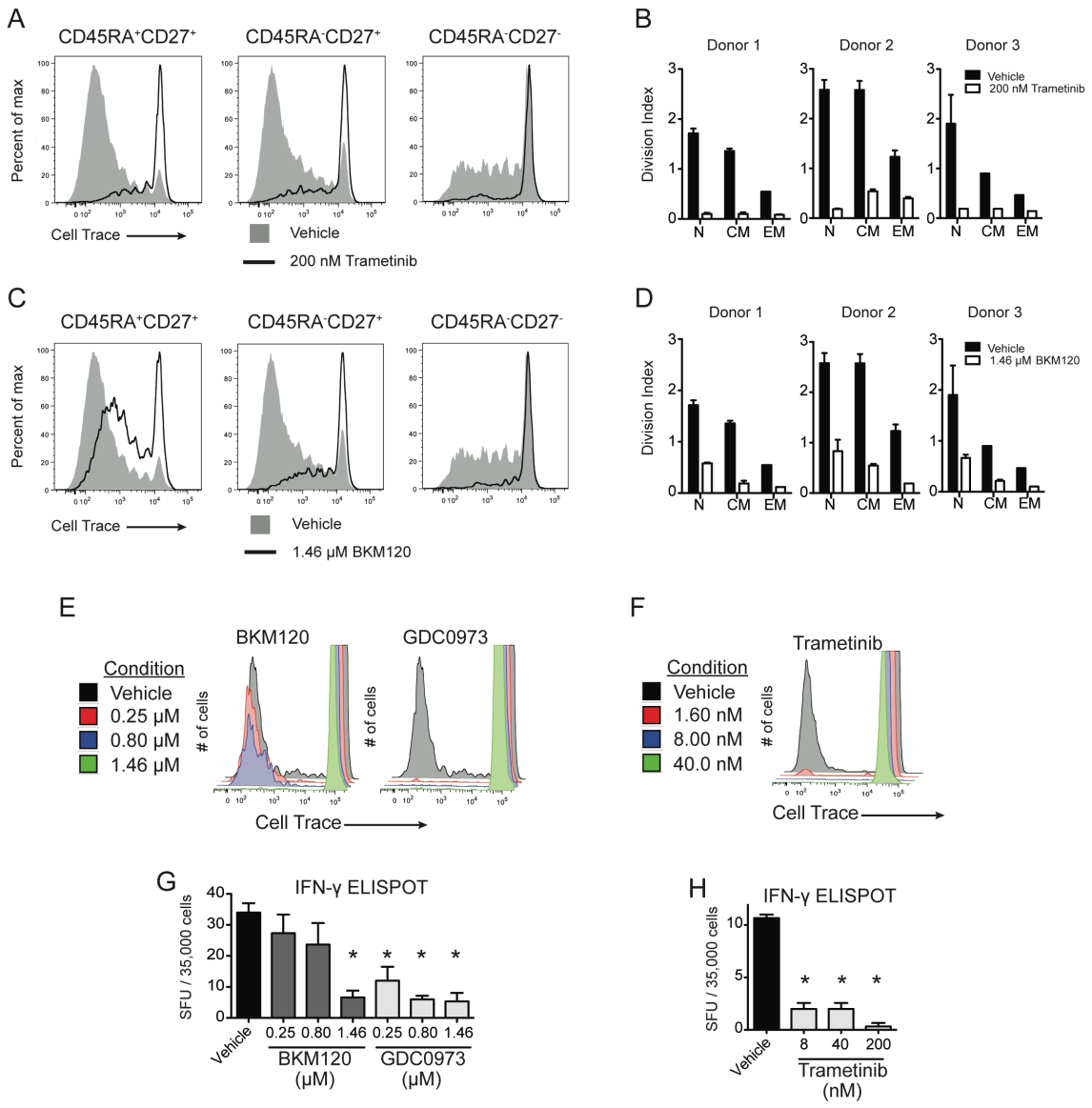

Figure 3.6. Small-molecule PI3K and MEK inhibitors suppress naïve, memory, and effector T-cell responses. Human CD8 T cells were sorted from PBMCs into $CD45RA^{+}CD27^{+}$ (N), $CD45RA^{-}CD27^{+}$ (CM), $CD45RA^{-}CD27^{-}$ (EM) populations, stained with Cell Trace, and activated using aAPCs with or without trametinib (**A**) and (**B**) or BKM120 (**C**) and (**D**). Proliferation was measured 7 days later and quantified from the Division Index. Shown are plots from one representative donor (**A**) and (**C**) and quantifications from all three donors (**B**) and (**D**). (**E**) and (**F**) Mouse splenic T cells were primed with tumor antigen pulsed DCs in the presence of the inhibitors. Proliferation of CD8 cells after 7 days is shown. (**G**) and (**H**) Mouse splenic T cells were primed with tumor antigen-pulsed DCs for 7 days under normal conditions. T cells were then recalled with fresh tumor antigen-pulsed DCs in the presence of inhibitors. The frequency of IFNγ-secreting cells measured by ELISpot is shown.

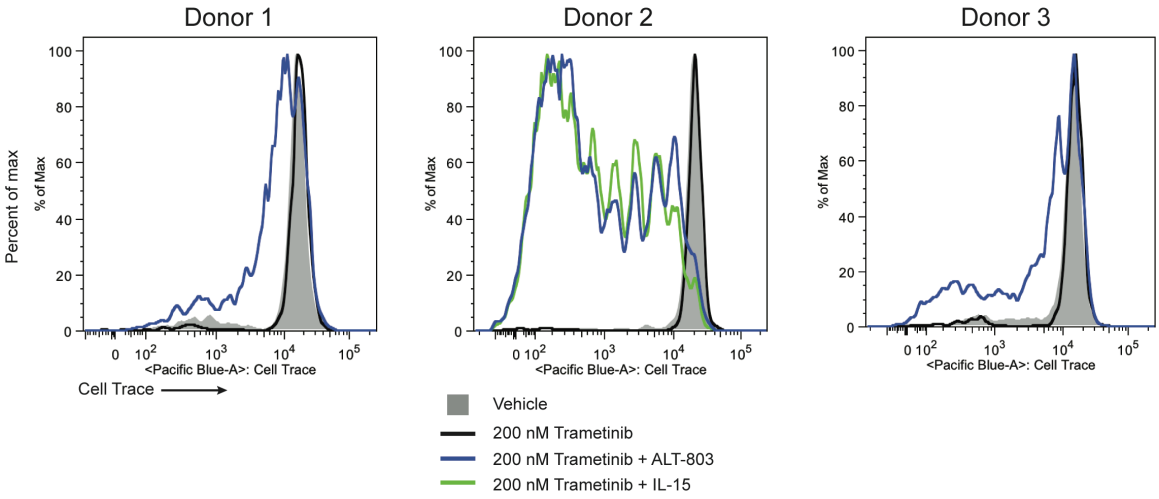

Figure 3.7. Cytokines enhance human effector T-cell proliferation. Human CD8 effector cells ($CD45RA^{+}CD27^{-}$) were FACS sorted from PBMCs from three healthy donors, stained with Cell Trace, and activated with aAPCs in the presence of trametinib and indicated cytokines. Proliferation of live cells after 7 days is shown. IL-15 was not tested for donors 1 and 3 due to insufficient cell numbers.

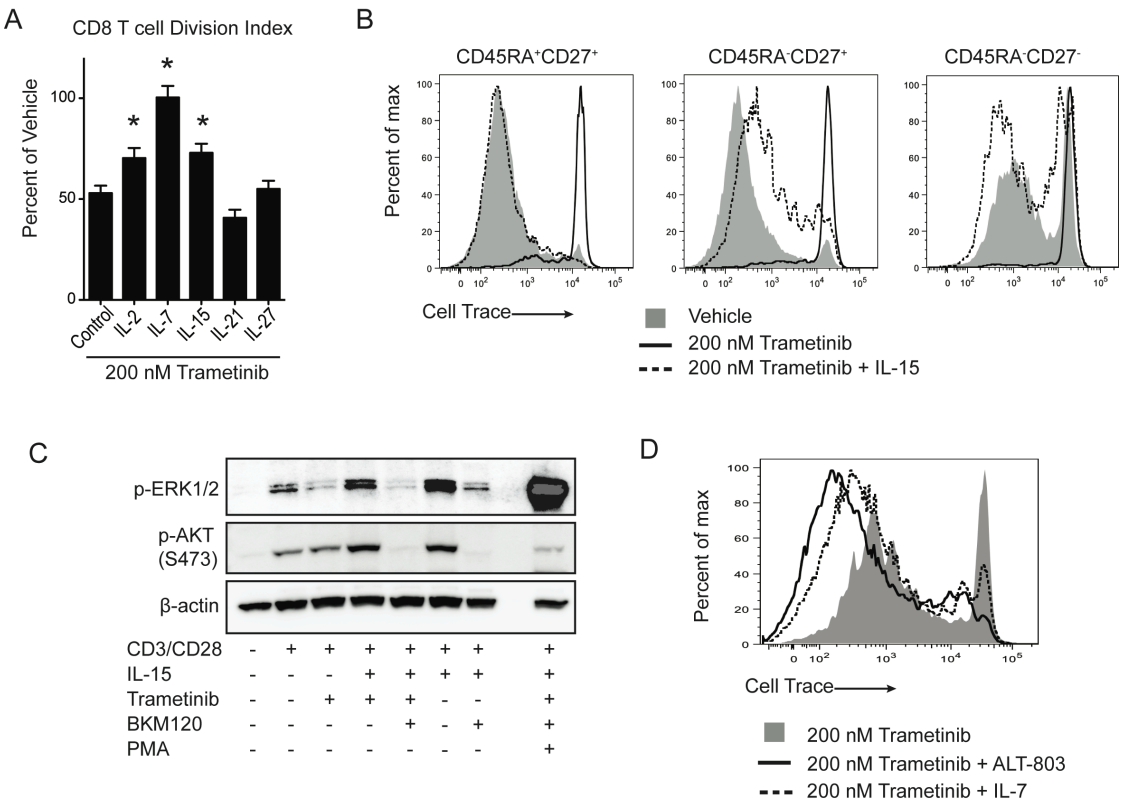

Figure 3.8. Cytokines can rescue T-cell functions from MEK inhibitors. Human PBMCs were stained with Cell Trace and activated with aAPCs with or without indicated cytokines in the presence of vehicle or 200 nM trametinib for 7 days. **(A**) Means of the division index (FlowJo) as a percent of vehicle without cytokines are shown for live CD8 cells from 3 donors. * *P* <0.05 vs control, unpaired t-test. Combined data from two experiments with similar results. (**B**) Human CD8 T cells were sorted from PBMCs into $CD45RA^{+}CD27^{+}$ (N), $CD45RA^{-}CD27^{+}$ (CM), $CD45RA^{-}CD27^{-}$ (EM) populations, stained with Cell Trace, and activated using aAPCs with or without trametinib and IL-15. Shown are proliferation plots acquired 7 days later for one representative donor of three (N, CM) or two (EM). (**C**) Human CD8 T cells were stimulated by staining with biotinylated anti-CD3 followed by crosslinking with streptavidin and soluble anti-CD28 in the presence of the indicated compounds. After 10 min at 37°C, cells were harvested for Western-blotting against the indicated proteins. Representative of 2 independent experiments. (Trametinib=8 nM, BKM120=2 μM, PMA=50 ng/ml). Solid gray indicates oversaturated bands. (**D**) Human PBMCs were activated as in (A) together with indicated cytokines. Proliferation of live CD8 cells from one representative donor of three is shown. (IL-2=20 U/ml, IL-7=2 ng/ml, IL-15=10 ng/ml, IL-21=100 ng/ml, IL-27=50 ng/ml, ALT-803=35.7 ng/ml)

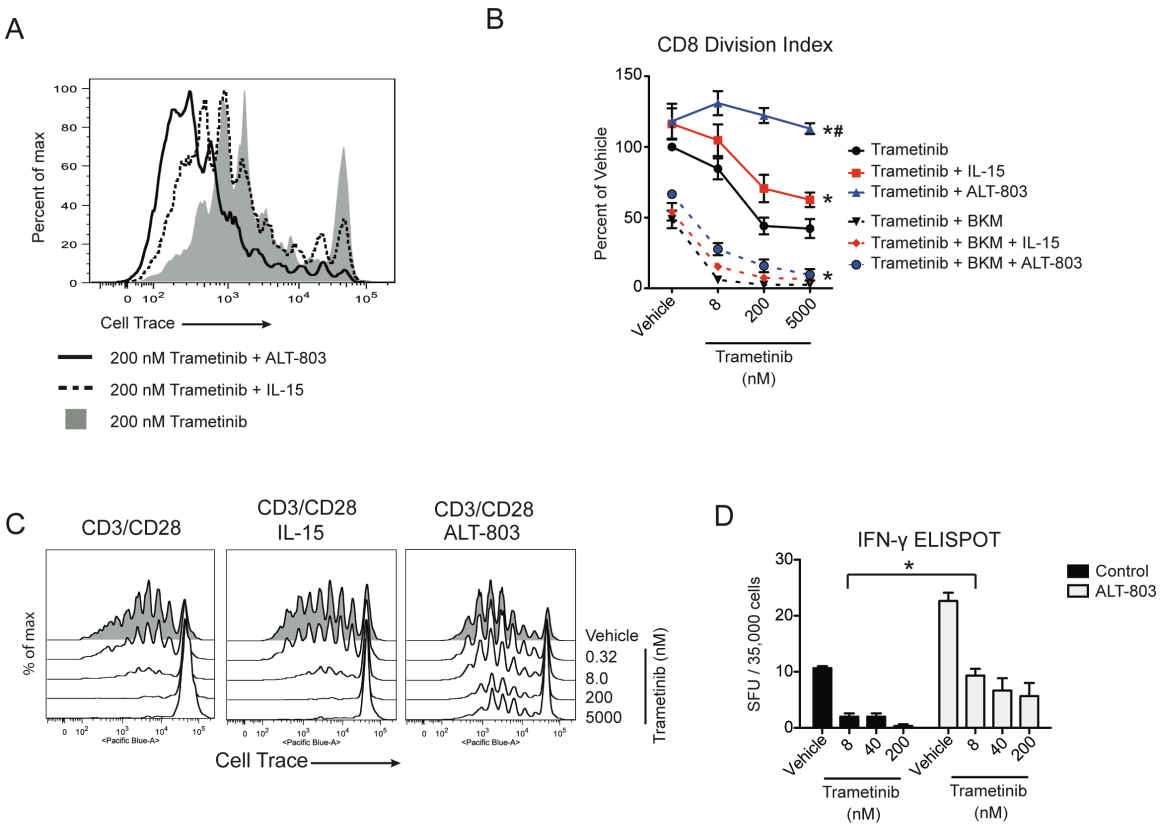

Figure 3.9. IL-15 agonist ALT-803 rescues anti-tumor T-cell activity from MEK inhibition *in vitro*. (**A**) Human PBMCs were stained with Cell Trace and activated with aAPCs with or without IL-15 (10 ng/ml) or equimolar ALT-803 (35.7 ng/ml) in the presence of 200 nM trametinib for 7 days. Histograms of Cell Trace dilution of live CD8 cells are shown for one representative donor of three. (**B**) Human PBMCs were activated as in (A), except with the addition of BKM120 (2 μM) to indicated samples. Division index of live CD8 T cells as percent of vehicle alone is shown as means from three different donors. Representative of two independent experiments. **P* <0.05 compared to trametinib 5000 nM, #*P* <0.05 compared to trametinib 5000 nM+IL-15, Mann-Whitney test. (**C**) Mouse T cells isolated from spleens and Cell Trace labeled were activated with aCD3/aCD28 beads and IL-15 (10 ng/ml) or ALT-803 (35.7 ng/ml) for 3 days with trametinib. Proliferation of live CD8 cells is shown. (**D**) Mouse splenic T cells were primed with tumor antigen-pulsed DCs for 7 days. T cells were then recalled with fresh tumor antigen-pulsed DCs in the presence of trametinib with or without ALT-803 (35.7 ng/ml) in an ELISpot assay. The frequency of IFN-γ secreting cells is shown from one representative experiment of two. **P*<0.05, unpaired t-test.

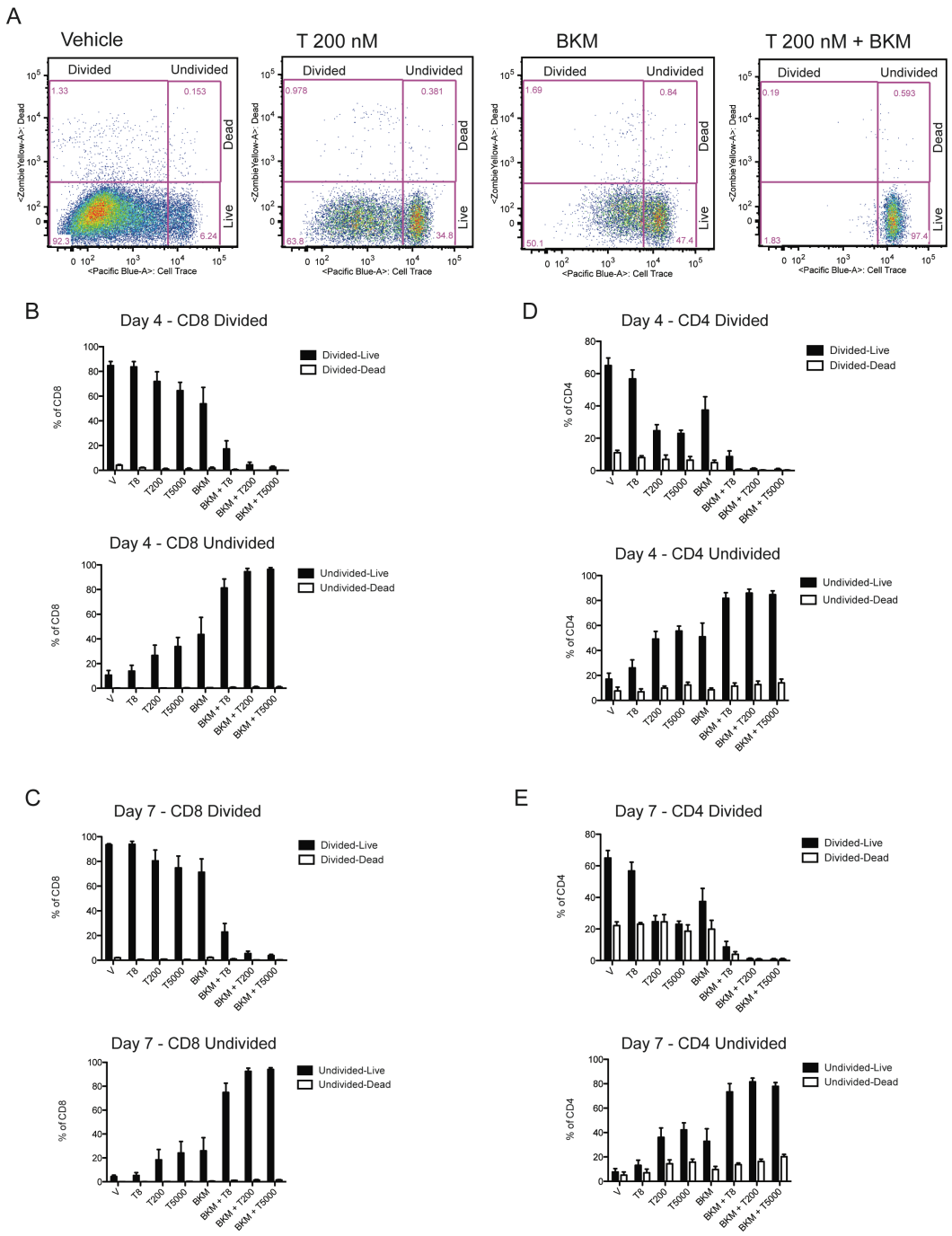

Figure 3.10. MEK and PI3K inhibitors mainly prevent cell division instead of inducing cell death. PBMCs from healthy human donors were stained with Cell Trace and activated with aAPCs in the presence of the indicated inhibitors. On day 4 and day 7, cells were stained for CD4, CD8, and Zombie Yellow (dead cell marker). (**A**) Representative plots of CD8 cells on day 7 showing gating strategy. (**B-E**) Percentages of indicated populations were pooled from 3 donors at day 4 (**B, D**) and day 7 (**C, E**) for CD8 (**B, C**) and CD4 (**D, E**) cells. V = Vehicle, T = Trametinib (8, 200, and 5000 nM), BKM = BKM120 (2 μM)

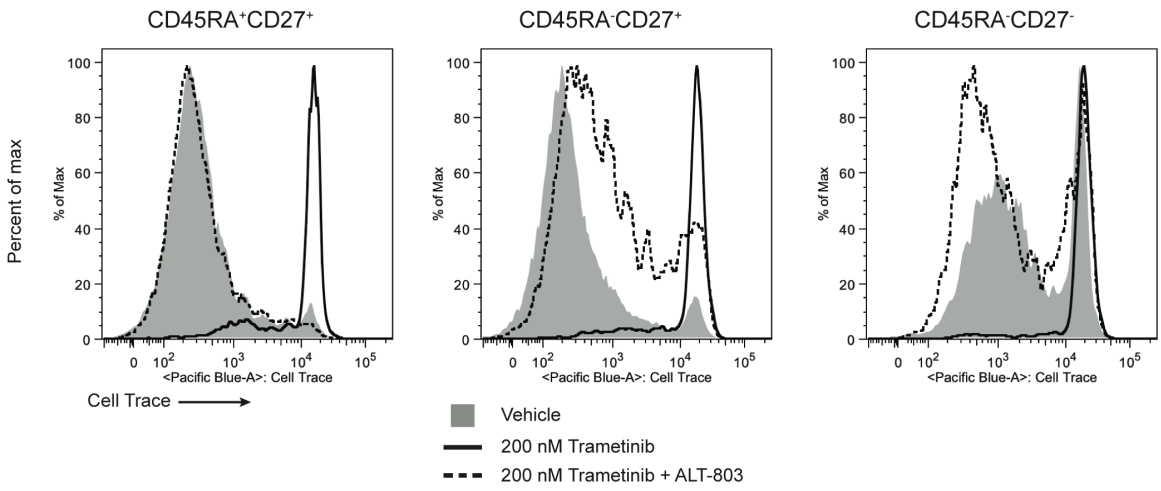

Figure 3.11. ALT-803 can rescue human T-cell proliferation from MEK inhibition. Human CD8 T cells were sorted from PBMCs into CD45RA+CD27+, CD45RA-CD27+, CD45RA-CD27- populations, stained with Cell Trace, and activated using aAPCs with or without trametinib and ALT-803 (35.7 ng/ml). Shown are proliferation plots acquired 7 days later for one representative donor of three.

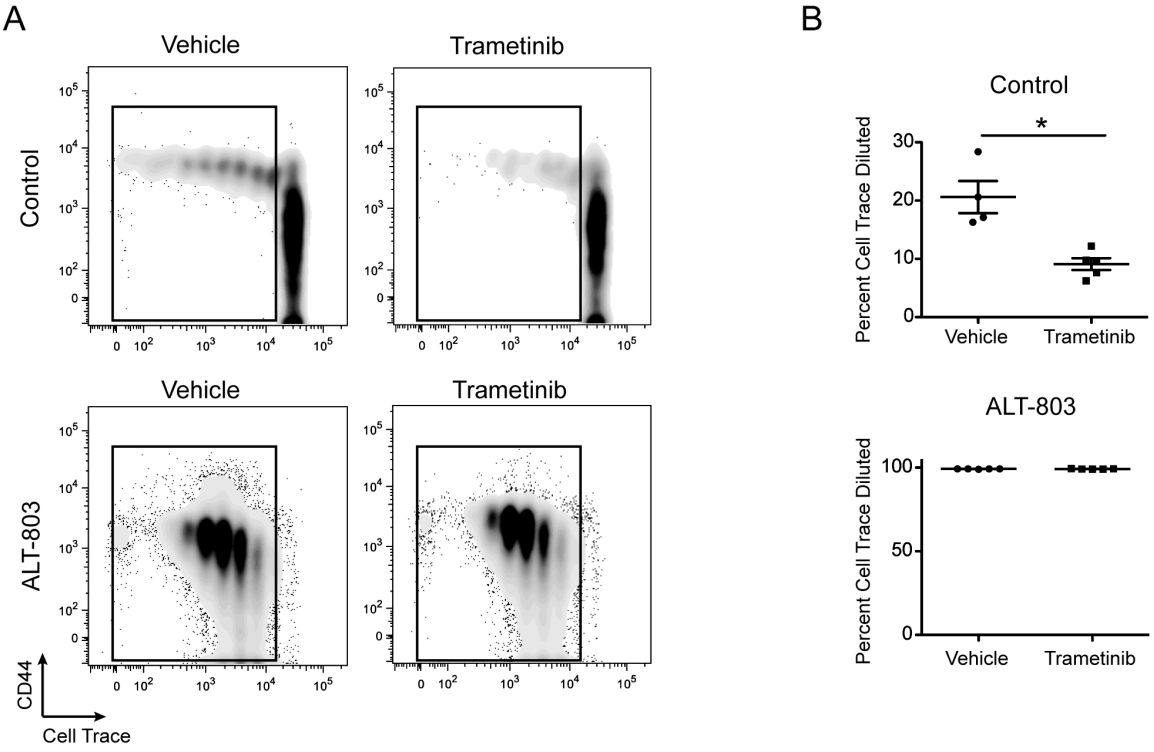

Figure 3.12. IL-15 agonist ALT-803 rescues anti-tumor T-cell activity from MEK inhibition *in vivo*. (**A**) Ly5.1 (CD45.1$^+$) mice were injected with ID8-OVA cells and gavaged once daily with trametinib (1.0 mg/kg) from day 9-13. On day 10, Cell Trace labelled OT-I T cells (CD45.2$^+$) were injected i.p. and mice were treated i.p. with ALT-803 (0.2 mg/kg) or PBS. On day 14 cells were harvested by peritoneal wash. Plots of live CD45.2$^+$CD8b$^+$ OT-I T cells recovered (**A**) and quantification of the percent of OT-I T cells that divided (**B**) are shown for one representative experiment of two. Each data point indicates individual mice. **P* <0.05, unpaired t-test.

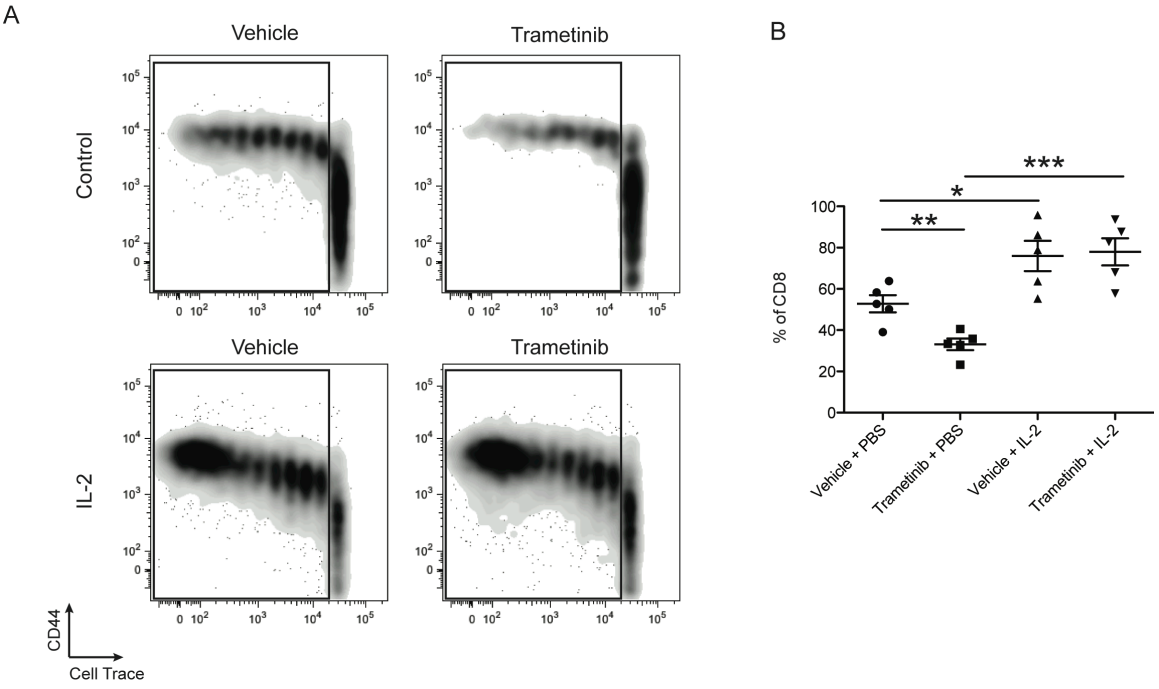

Figure 3.13. IL-2 rescues T-cell proliferation from MEK inhibition *in vivo.* (**A**) Ly5.1 (CD45.1+) mice were injected with ID8-OVA cells and gavaged once daily with trametinib (1.0 mg/kg) from day 9-13. On day 10, Cell Trace labelled OT-I T cells (CD45.2+) were injected i.p. Mice were treated with IL-2 (50,000 IU/mouse) or PBS i.p on days 10, 11, and 12. On day 14 cells were harvested by peritoneal wash. Plots of live CD45.2+CD8b+ OT-I T cells recovered (**A**) and quantification of the percent of OT-I T cells that divided (**B**) are shown for one representative experiment of two. Each data point indicates individual mice. *, **, *** *P* <0.05, 0.01, 0.001, respectively, unpaired t-test.

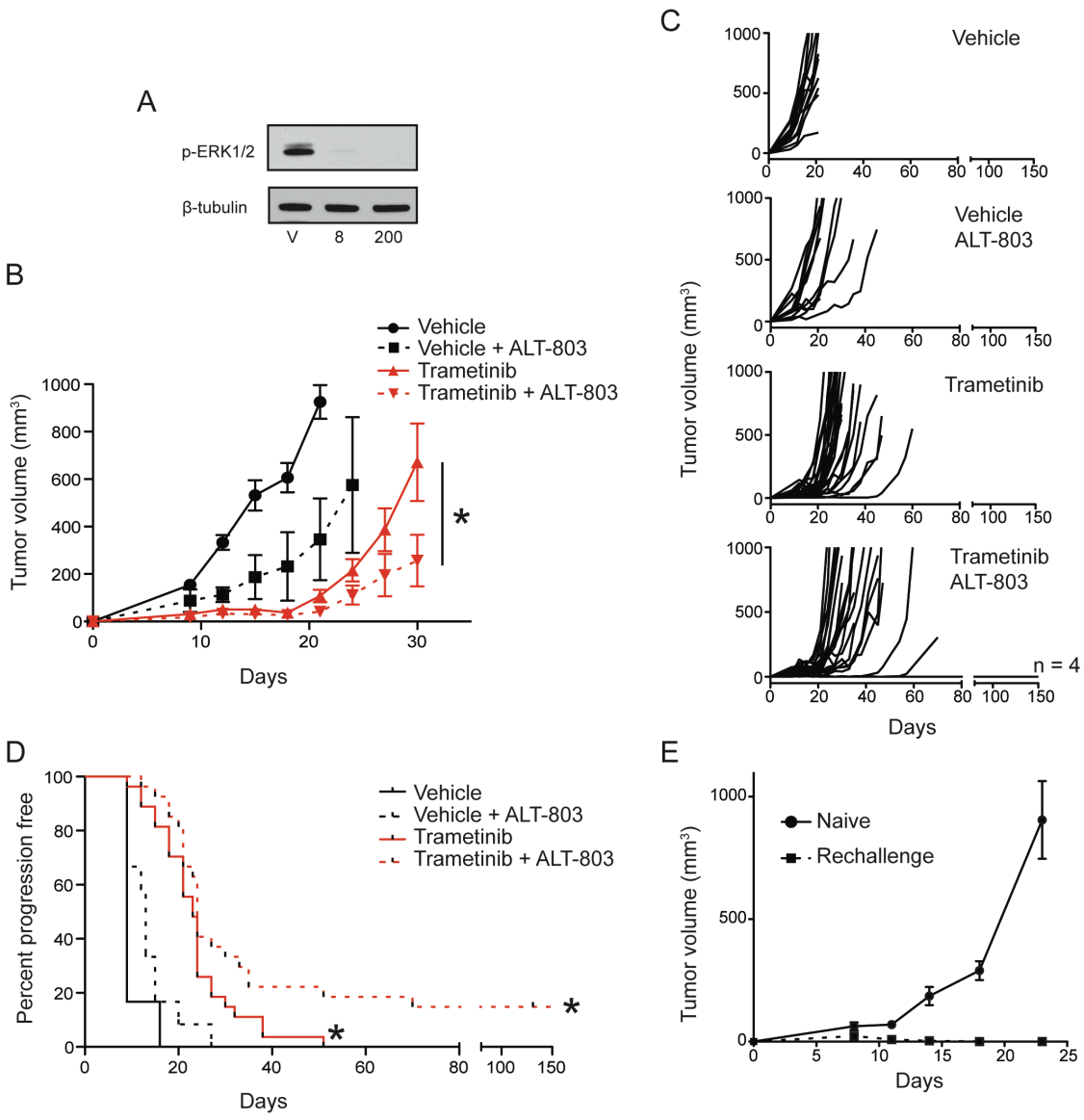

Figure 3.14. **Combination therapy with trametinib and ALT-803 can drive rejection of *Kras*-mutated breast tumors**. (**A**) Brpkp110 cells were treated *in vitro* with vehicle, 8 nM, or 200 nM trametinib for 18 hr and analyzed by Western blot. (**B**) Mice were injected with Brpkp110 cells subcutaneously (day 0) and gavaged once daily with trametinib (1.0 mg/kg) on days 3-13. ALT-803 (0.2 mg/kg) was administered i.p. on days 3, 8, and 13. Tumor growth data from one of three experiments is shown, n=4 for Vehicle and Vehicle+ALT-803 groups; n=12 for Trametinib and Trametinib+ALT-803 groups. *P <0.05, Mann-Whitney test. (**C**) Plots showing growth of individual tumors from all mice in three experiments from (B). n=12 for Vehicle and Vehicle +ALT-803 groups; n = 27 for Trametinib and Trametinib+ALT-803 groups. (**D**) Percentage of mice with tumors <100 mm^3 from (C) is plotted. *Trametinib+ALT-803 is significant from all other curves and Trametinib is significant from Vehicle, P <0.05, log-rank test. (**E**) Mice that rejected tumors from (C) were rechallenged >30 days later with Brpkp110 cells contralaterally and tumor growth was compared to cells injected into naive mice (n=4).

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CHAPTER 4

TRAMETINIB DRIVES T CELL-DEPENDENT CONTROL OF *KRAS*-MUTATED TUMORS BY INHIBITING PATHOLOGICAL MYELOPOIESIS

ABSTRACT

Because small molecule inhibitors can also act on immune cells, their efficacy *in vivo* may involve the modulation of antitumor immunity, effects which are poorly understood for many small molecules, including the MEK inhibitor trametinib. Here I show that trametinib abrogates cytokine-driven expansion of monocytic myeloid-derived suppressor cells (MDSCs) from human and mouse myeloid progenitors. Furthermore, MEK inhibition reduces the production of MDSC-chemotactic osteopontin by tumor cells. Combined, these effects reduce MDSC accumulation in tumor-bearing hosts, resulting in impaired growth of *Kras*-driven breast tumors even though trametinib largely fails to directly inhibit tumor cell proliferation. Accordingly, trametinib impedes tumor progression through a mechanism that, paradoxically, requires $CD8^+$ T cells, despite its reported inhibition of effector lymphocytes. My results demonstrate that the combined anti-inflammatory activity of trametinib on different cell types *in vivo* is at least partially responsible for its effectiveness, irrespective of its activity on tumor cell division. This study unveils elusive mechanistic clues to understand the effect of MEK inhibitors *in vivo* and identifies trametinib as an effective inhibitor of monocytic MDSC expansion.

INTRODUCTION

The main purpose of targeted kinase inhibitor development for cancer therapy has been to precisely block oncogenic signaling in tumor cells. However, many other cells in the tumor microenvironment, including immune cells, rely upon the same signaling pathways for normal activity. The attractiveness of targeting these pathways in tumor cells has led to concerns that an on-target side effect would disrupt beneficial anti-tumor immunity (Vella et al. 2014; Yamaguchi et al. 2012).

For instance, T cells require the Ras-MAPK signaling cascade following antigen activation for proliferation and effector function (Smith-Garvin, Koretzky, and Jordan 2009). Indeed, proper T-cell activation and proliferation is impaired by pharmacological inhibition of MEK signaling by the FDA-approved drug trametinib (Vella et al. 2014; Yamaguchi et al. 2012). However, while trametinib impairs T-cell function *in vitro*, it does not prevent the effectiveness of adoptive cell therapy (Hu-Lieskovan et al. 2015) or checkpoint blockade (L. Liu et al. 2015) in mouse models and can actually synergize with these immunotherapies. While signaling from common γ-chain cytokines reduces sensitivity of T cells to trametinib *in vivo* (Allegrezza et al. 2016), another potential explanation is that trametinib acts on tumor and stromal cells in the tumor microenvironment that, overall, ameliorate its immune-modulatory effects.

The occurrence of neoplasia results in a chronic inflammatory response that promotes the pathologic expansion and recruitment of myeloid-derived suppressor cells (MDSCs) (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012; Rutkowski et al. 2015; Scarlett et al. 2012; Tesone et al. 2016). Immune suppression by MDSCs is a critical factor in the ability of tumor cells to avoid adaptive immune responses (Dmitry I Gabrilovich, Ostrand-Rosenberg, and Bronte 2012; Rutkowski, Stephen, et al. 2014;

Kumar et al. 2016; Munn and Bronte 2016; Hossain et al. 2015). Although the impact of trametinib on MDSC mobilization has not been studied, MEK signaling is known to promote the lineage commitment of myeloid cells from hematopoietic stem cells and multipotent-progenitor cells (Hsu, Kikuchi, and Kondo 2007). Similar findings have also implicated MEK's target proteins, ERK1/2, in the development of myeloid cells (Staser et al. 2013). However, the requirement for MEK-ERK signaling in tumor-driven MDSC expansion and the susceptibility of this pathological axis to small-molecule MEK inhibition have yet to be studied.

A possible outcome of therapeutic MEK-signaling inhibition is alterations in the secretion of inflammatory cytokines by *Kras*-mutated tumor cells. Among these, osteopontin has been implicated in the recruitment of macrophages into tumors (Giachelli et al. 1998) and its expression is positively correlated with $CD204^+$ M2-like macrophages (Lin et al. 2015). Osteopontin secreted by tumor cells also drives the expansion of MDSCs in the spleens of tumor-bearing mice through activation of the MAPK pathway in myeloid progenitors (E.-K. Kim et al. 2014).

To examine the effects of trametinib on the tumor immunoenvironment, I dissected the role of trametinib in restricting the growth of a *Kras*-driven breast tumor cell line in immune-competent mice. I find that treatment of tumor-bearing mice with trametinib results in a reduction in monocytic MDSCs (M-MDSCs) that allows $CD8^+$ T cells to control tumor growth. The impairment of MDSC mobilization is attributable to a direct blockade of MDSC expansion from bone marrow precursors and a reduced secretion of chemotactic molecules by tumor cells. This study enhances understanding of trametinib's antitumor efficacy by demonstrating a mechanism of enhanced immunity through a reduction in pathological MDSC mobilization.

MATERIALS AND METHODS

Mice

WT C57BL/6 female 6-8 week old mice were procured from the National Cancer Institute or Charles River Laboratory. OT1 C57BL/6-Tg (TcraTcrb)1100Mjb/J transgenic mice were obtained from Jackson Labs. All mice were randomized into treatment groups.

Cell lines and media

The Brpkp110 primary mammary tumor cell line was generated by culturing a mechanically dissociated C57BL/6 LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*Myristoylated-p110α-GFP*$^{+}$ primary breast tumor mass (Rutkowski, Allegrezza, et al. 2014). Tumor cells were passaged a total of 10 times before deriving the Brpkp110 clonal cell line by limiting dilution. Brpkp110 cells were confirmed to be mycoplasma-free. Cell lines and lymphocytes were cultured in R10 (RPMI-1640 (CellGro, with L-glutamine), 10% FBS, penicillin (100 I.U./ml), streptomycin (100 µg/ml), L-glutamine (2 mM), sodium pyruvate (0.5 mM), b-mercaptoethanol (50 µM)).

Cell proliferation assays

Cells were plated in 96-well plates and the next morning, trametinib (dissolved in DMSO) was diluted into the wells 1:1000, so that the final concentration of DMSO was 0.1%. Cell proliferation was measured 48 hrs later with the CellTiter 96 MTS assay (Promega) according to the manufacturer's instructions.

Tumor inoculation and treatments

Brpkp110 tumors were initiated by injecting $5x10^5$ cells in 200 µl PBS into the subcutaneous axillary region. Mice were treated by oral gavage once daily with a dose of 1.0 mg/kg trametinib (GSK-1120212, LC laboratories) suspended in a vehicle solution of 10% PEG-300 (Sigma Aldrich) and 10% Cremophor EL (EMD Millipore) in sterile dH_2O. For CD8 depletion, mice were injected with anti-CD8α (BioXcell, clone YTS 169.4) on day 3 (500 µg/mouse) and day 10 (250 µg/mouse) post tumor inoculation. All antibodies, including isotype control (BioXcell, clone LTF-2), were injected i.p. in sterile PBS. Tumor volume was calculated as: $0.5 \times (L \times W^2)$, where L is the larger dimension.

Tumor dissociation

For flow cytometry analysis, tumors were dissected and mechanically dissociated through a 70 µm nylon cell strainer. For cell sorting, tumors were dissected, minced with a scalpel, digested in RPMI-1640 containing 1 mg/ml collagenase (Type V, Sigma-Aldrich) for 1 hr at 37° C. Cells were dissociated through a 70 µm nylon cell strainer followed by passage through a 23G needle.

***In vitro* MDSC differentiation, suppression, and chemotaxis**

Bone marrow from naïve mice was cultured for 4 days with IL-6 (40 ng/ml, Peprotech) and GM-CSF (40 ng/ml Peprotech) or media containing 50% tumor-conditioned media, prepared by filtering supernatant from a confluent flask of tumor cells through a 0.45 µm membrane. For suppression assays, MDSCs were either added to 2×10^5 CellTrace-labelled, WT splenocytes simultaneously activated with anti-CD3 (500 ng/ml, clone 2C11, Tonbo) and anti-CD28 (1 µg/ml, clone 37.51, Tonbo) or 2×10^5 CellTrace-labelled, OT-I splenocytes simultaneously activated with $OVA_{257-264}$ peptide (1 µM, GenScript) in 96-well plates. Proliferation of T cells was measured 3 days later. For

chemotaxis assays, MDSCs were separated into $Ly6G^+$ and $Ly6G^-$ fractions with anti-Ly6G MicroBeads (Miltenyi) according to the manufacturer's protocol. Chemotaxis was measured toward recombinant carrier-free osteopontin (R&D Systems) after 1 hr on 3 μm filter plates ($Ly6G^+$ cells) or 4 hrs on 5 μm filter plates (Ly6G- cells) (NeuroProbe).

Human bone marrow

All patients with Stage I-II lung cancer, who were scheduled for surgical resection, were consented for tissue collection of a portion of their tumor and/or blood for research purposes at the Hospital of the University of Pennsylvania and The Philadelphia Veterans Affairs Medical Center after obtaining consents that had been approved by their respective Institutional Review Boards. All patients selected for entry into the study met the following criteria: (i) histologically confirmed pulmonary squamous cell carcinoma (SCC) or adenocarcinoma (AC), (ii) no prior chemotherapy or radiation therapy within two years, and (iii) no other active malignancy. Bone marrow cell suspension was obtained from the rib fragments that were removed from patients as part of their lung cancer surgery. The single cell suspension was obtained by vigorous pipetting of cells flushed from bone marrow and passing the disaggregated cells through a 70 μm nylon cell strainer. Total bone marrow cells were cultured in 6 well plates (2 x 10^6 leukocytes/well) in 3 ml of complete-IMDM (IMDM (CellGro, with l-glutamine and 25 mM HEPES), 15% FBS, penicillin (100 I.U./ml), streptomycin (100 μg/ml), l-glutamine (2 mM), β-mercaptoethanol (50 μM)) supplemented with 40 ng/ml human IL-6 (Peprotech) and 40 ng/ml human GM-CSF (Peprotech) for 4 days. Cells were stained for surface marker expression, fixed in 1% para-formaldehyde, and analyzed by flow cytometry.

Quantitative PCR

RNA from FACS-purified cell populations was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA RT Kit (Applied Biosystems) and analyzed by qPCR with SYBR Green (Applied Biosystems) on a 7500 Fast machine (Applied Biosystems). Primers used were: Arg1-fwd, 5'-GGAATCTGCATGGGCAACCTGTGT-3'; Arg1-rev, 5'-AGGGTCTACGTCTCGCAAGCCA-3'; Nos2-fwd, 5'-GTTCTCAGCCCAACAATACAAGA-3'; Nos2-rev, 5'- GTGGACGGGTCGATGTCAC-3'; TBP-fwd, 5'-CACCCCCTTGTACCCTTCAC-3'; TBP-rev, 5'-CAGTTGTCCGTGGCTCTCTT-3'.

Western blotting

Cells were lysed in RIPA buffer (Thermo Scientific) with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (Halt Phosphatase Inhibitor, Thermo Scientific, and Na_3VO_4, 1 mM) and cleared by centrifugation. Proteins were quantified by BCA assay (Thermo Scientific), diluted in reducing Laemmli buffer, denatured by incubation at 95° C, run on mini Protean TGX Ready Gels (Bio-Rad Laboratories), transferred to a PVDF membrane, blocked, and incubated with primary antibodies for p-ERK1/2 (Cell Signaling, clone D13.14.4E) and β-actin (Sigma, clone AC-15). Immunoreactive bands were developed using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories) and ECL substrate (GE Healthcare).

Immunohistochemistry

Tissues were embedded in Tissue-Tek OCT and frozen. Endogenous peroxidases were quenched from acetone-fixed sections (8 μm) by incubating in 0.3% H_2O_2 for 10

minutes at room temperature. Following quenching, sections were blocked using 3% goat serum followed by staining with antibodies against Ki-67 (clone D3B5, Cell Signaling Technology). Immunohistochemistry using the ABC Kit (Vector labs) was performed according to the manufacturer's instructions, and sections were counter stained with hematoxylin. Slides were then imaged at 10X objective magnifications on a Nikon E600 Upright microscope with a Nikon DS-Ri1 Digital camera. Nikon NIS-Elements software was used for image acquisition and image stitching of the entire tumor. Image Pro Plus 7 analysis software was used to measure the percentage of Ki67 stained nuclei within each sample. Percentage of stained nuclei was calculated as total area of brown stained nuclei divided by total area of sample.

LC-MS/MS

Brpkp110 cells were cultured in serum-free RPMI with DMSO or trametinib for 40hrs. Supernatants were collected, centrifuged, passed through a 0.22 μm filter, and concentrated by centrifugation in Amicon 3000 MWCO tubes (EMD Millipore). Concentrated supernatants were run 0.5 cm on a NuPage 12% Gel with MES buffer, extracted, digested with trypsin, and subjected to LC-MS/MS analysis by the Wistar Proteomics Facility.

Flow cytometry

Zombie Yellow (BioLegend) was used for all live/dead staining. For mouse experiments, antibodies from the following companies were used: Tonbo Biosciences: CD45.2-PerCP/Cy5.5 (104), CD11b-APC (M1/70), F480-PerCP/Cy5.5 (BM8.1), CD45-PE/Cy7 (30-F11) CD3-FITC (145-2C11); BioLegend: F480-PE/Cy7 (BM8), Ly6G-FITC (1A8), Ly6C-APC/Cy7 (HK1.4), I-A/I-E-PacBlue (M5/114.15.2), CD4-APC (RM4-5),

CD25-APC/Cy7 (PC61), Foxp3-PacBlue (MF-14), CD8b-PerCP/Cy5.5 (YTS156.7.7), H-2Kb-PE (AF6-88.5). For human experiments, antibodies from the following companies were used: Tonbo Biosciences: CD45-PE/Cy7 (HI30); BioLegend: CD14-APC (HCD14), CD15-FITC (HI98), HLA-DR-APC/Cy7 (L243), CD33-PerCP/Cy5.5 (WM53), CD11b-PacBlue (ICRF44). Samples were run on a BD LSRII cytometer and analyzed by FlowJo.

ELISA

Osteopontin concentrations were measured using ELISA kits (RayBiotech) according to the manufacturer's instructions. Plasma was isolated from peripheral blood of mice by centrifugation in lithium heparin tubes (Becton Dickinson) and stored at -80° C. Intratumoral fluid was isolated from advanced Brpkp110 tumors after careful excision and blotting on gauze tissue (to remove excess fluid) by squeezing the tumor through a 10 ml syringe (BD, Luer-Lok^{TM} Tip) into a microcentrifuge tube followed by two centrifugation steps to obtain debris-free liquid, which was stored at -80° C.

Statistics

Unless indicated otherwise, all data shown represent means with SEM. All hypothesis testing was two-sided, and unpaired t-tests were performed unless indicated otherwise. A significance threshold of 0.05 for *P* was used (*P<0.05, **P<0.01, ***P<0.001). Analyses were carried out using GraphPad Prism software. Experiments were repeated at least twice unless otherwise indicated.

Study Approval

All animals were maintained in specific pathogen free barrier facilities and used in accordance with the Institutional Animal Care and Use Committee of the Wistar Institute.

RESULTS

Trametinib reduces M-MDSC accumulation in tumor bearing mice.

I previously demonstrated that MEK inhibitors, including trametinib, inhibit T-cell responses *in vitro* and *in vivo*, which can be rescued with some common γ-chain cytokines (Allegrezza et al. 2016). To dissect the effects of trametinib treatment on other compartments of the immune-environment of solid tumors, I analyzed the effect of trametinib against the progression of transplantable Brpkp110 breast tumors, derived from a syngeneic autochthonous LSL-*Kras*^{G12D}*p53*$^{loxp/loxp}$LSL-*Myristoylated-p110α-GFP*$^{+}$ primary breast tumor (Allegrezza et al. 2016; Rutkowski, Allegrezza, et al. 2014). Trametinib alone dramatically delayed breast cancer growth in multiple independent experiments (**Figure 4.1A**). This was slightly unexpected because, in addition to the direct T cell-suppressive effects of trametinib, Brpkp110 cells were only weakly sensitive to trametinib (70% growth compared to vehicle) *in vitro* (**Figure 4.1B**). Accordingly, trametinib did not significantly reduce Ki-67 staining in Brpkp110 tumors (**Figure 4.1C&D**), indicating that *in vivo* proliferation of tumor cells was largely unaffected by direct MEK inhibition. I therefore reasoned that trametinib-induced changes in the microenvironment might be responsible for the decrease in Brpkp110 tumor growth.

To elucidate possible changes in the immuno-environment, I treated mice with established tumors for 3 days (to minimize differences in tumor burden) and analyzed populations of tumor-infiltrating leukocytes. Despite the sensitivity of T cells to MEK inhibition (Allegrezza et al. 2016), I did not observe a decrease in $CD8^{+}$ or $CD4^{+}$ T cells

at tumor beds in trametinib treated tumor-bearing mice (**Figure 4.2A**). The proportion of $Foxp3^+$ Tregs and differentiated ($MHC\text{-}II^+$) macrophages also did not significantly change after trametinib treatment (**Figure 4.2B**). In contrast, trametinib induced a dramatic reduction in the accumulation of specifically $CD11b^+MHC\text{-}II^-Ly6C^{hi}Ly6G^-$ monocytic MDSCs (M-MDSCs), while $CD11b^+MHC\text{-}II^-Ly6C^{low}Ly6G^+$ polymorphonuclear MDSCs (PMN-MDSCs) remained unaffected, whether analyzed as a proportion of total leukocytes (**Figure 4.2C)**, or as a proportion of $CD11b^+MHC\text{-}II^-$ myeloid cells (**Figure 4.2D&E**). The apparent increase in PMN-MDSCs as a percentage of $CD11b^+MHC\text{-}II^-$ cells was entirely due to a reduction in M-MDSCs because overall percentages of PMN-MDSCs among leukocytes remained constant while the respective percentages of M-MDSCs were reduced. Confirming their identity as immunosuppressive cells, these M-MDSCs expressed high levels of Arginase and Nos2 (**Figure 4.2F**) and were capable of suppressing the proliferation of activated T cells *ex vivo* (**Figure 4.2G**). The selective reduction in M-MDSCs was also observed in the spleens of trametinib treated tumor-bearing mice (**Figure 4.2H-I**), suggesting a systemic effect on M-MDSC mobilization. These data demonstrate that trametinib decreases the accumulation of immunosuppressive M-MDSCs in Brpkp110 tumor-bearing mice, resulting in significant delays in tumor growth independent of proliferative sensitivity of tumor cells to the drug.

Trametinib abrogates M-MDSC expansion by inhibiting the Ras-MAPK pathway in myeloid precursors.

To dissect the mechanism whereby trametinib treatment inhibits the mobilization of M-MDSCs, I stimulated myelopoiesis in bone marrow cells with a combination of GM-CSF and IL-6 (Marigo et al. 2010). Recapitulating my *in vivo* observations, trametinib significantly impaired the expansion of M-MDSCs in a dose-dependent manner (**Figure**

4.3A&B). A reduction in the expansion of PMN-MDSCs was also observed with trametinib treatment, although to a much smaller degree than for M-MDSCs, further supporting the preferential inhibition of trametinib on the mobilization of myeloid cells of the monocytic lineage. Similar inhibitory effects were observed when MDSCs were differentiated from bone marrow using Brpkp110-conditioned media (**Figure 4.3C&D**), a system that effectively promotes expansion of MDSCs capable of suppressing T-cell proliferation (**Figure 4.3E**). I confirmed that trametinib abrogated MEK signaling at both early and late stages in bone marrow cultures incubated with Brpkp110-conditioned media (**Figure 4.3F**).

To support the clinical relevance of MDSC inhibition by trametinib, I next obtained human bone marrow isolates from four different lung cancer patients undergoing partial rib resection, and induced MDSC expansion by culturing in GM-CSF and IL-6 (Marigo et al. 2010). Although a more diverse population of myeloid cells was expanded in these cultures in comparison to murine bone marrow, a $CD14^{+}CD15^{int}HLA\text{-}DR^{-/low}CD11b^{+}CD33^{+}$ population of immature myeloid cells (corresponding to M-MDSCs (Condamine et al. 2015)) did reproducibly expand (**Figure 4.4A&B**). As in my mouse bone marrow and *in vivo* experiments, trametinib induced a dose-dependent reduction preferentially in $CD14^{+}CD15^{int}$ cells across all patient samples (**Figure 4.4A&C**). Taken together, these results indicate that trametinib, by inhibiting the Ras-MAPK pathway in myeloid precursors, disproportionately decreases the mobilization of immature M-MDSCs in mice and humans. This potentially explains the decreased accumulations of M-MDSCs in the periphery and in the tumor beds *in vivo*, and it suggests a novel mechanism that contributes to the antitumor activity of trametinib in human patients.

$CD8^{+}$ T cells are required for optimal anti-tumor activity of trametinib.

My results above suggest that trametinib may be effective in the Brpkp110 tumor model by decreasing the expansion of immunosuppressive MDSCs, thus allowing anti-tumor T-cell responses to control tumor growth. This mechanism however requires a functional effector response by tumor-infiltrating T cells in the presence of trametinib treatment. I found that treatment of tumor-bearing mice with trametinib did not decrease the frequencies of $CD8^+$ or $CD4^+$ tumor-infiltrating T cells capable of producing IFN-γ when re-stimulated *ex vivo* (**Figure 4.5A).** Additionally, trametinib did not impair the ability of OVA-vaccinated mice to lyse adoptively transferred $OVA_{257-264}$-loaded targets during an *in vivo* cytotoxicity assay, indicating that cytotoxic effectors function during trametinib treatment (**Figure 4.5B&C**). Most importantly, I examined whether T-cell immunity contributes to the efficacy of trametinib by depleting $CD8\alpha^+$ T cells during trametinib treatment of Brpkp110-bearing mice. Tumors grew significantly faster in trametinib treated mice depleted of $CD8\alpha^+$ T cells compared to trametinib treated mice injected with an irrelevant IgG (**Figure 4.5D**). These data demonstrate that tumor-infiltrating effector T cells are not restricted by trametinib *in vivo* and are actually necessary for the full efficacy of trametinib.

Trametinib abrogates the production of osteopontin by *Kras*-mutated tumor cells.

I finally reasoned that, by modulating the *Kras*-dependent secretion of inflammatory factors by tumor cells, trametinib could also alter the non-cellular composition of the tumor microenvironment. Supporting this proposition, LC-MS/MS analysis of culture supernatants from Brpkp110 cells revealed that several cytokines were dramatically altered in response to trametinib treatment. A number of factors known to be involved in recruitment of myeloid cells such as CSF1, CCL2, and CX3CL1 (**Figure 4.6A**) were decreased after trametinib treatment. Strikingly, osteopontin (SPP1)

was decreased >18-fold upon trametinib treatment. I confirmed the trametinib-driven decrease in osteopontin secretion in separate ELISA experiments (**Figure 4.6B**). I focused on osteopontin because it has been reported to induce MDSCs expansion (E.-K. Kim et al. 2014) and tumor recruitment of macrophages (Lin et al. 2015). Supporting the relevance of my proteomic analysis, the tumor-driven increase of osteopontin in plasma was abrogated by short-term (3 day) treatment with trametinib in Brpkp110-bearing mice (**Figure 4.6C**). Importantly, when *in vitro*-derived $Ly6G^+$ and $Ly6G^-$ MDSCs were isolated with Ly6G-MACS separation (**Figure 4.6D**), they were able to migrate towards a gradient of recombinant osteopontin (**Figure 4.6E&F**). This trafficking was genuine chemotaxis and not chemokinesis because the migration was greatly diminished when osteopontin was supplied on the same side of the transwell chamber as the cells. Although the osteopontin concentration required for chemotaxis (100 μg/ml) is much higher than the concentration observed in the plasma (<1 μg/ml), we found that the concentration of osteopontin in the intratumoral fluid obtained from Brpkp110 tumor samples exceeded 200 μg/ml (**Figure 4.6G**). These data indicate that the physiological range of osteopontin concentrations in tumor bearing mice should induce migration of MDSCs from peripheral blood into tumor tissue. Together, these results suggest that trametinib may also decrease MDSCs in the tumor by reducing the production of chemotactic cytokines by tumor cells through a direct anti-inflammatory effect that is independent of changes in the proliferation of tumor cells.

DISCUSSION

Here I show that the *in vivo* growth of a mouse breast tumor driven by KRAS and PI3K signaling is restricted by trametinib, despite tumor cell proliferation being relatively

resistant to MEK inhibition. Full growth inhibition by trametinib requires $CD8^+$ T cells, indicating that trametinib is acting to enhance control of tumor growth by cytotoxic T cells. This mechanism is likely achieved through a reduction in MDSC mobilization into tumors, a result of trametinib directly impairing MDSC differentiation from bone marrow precursors and reducing tumor-secreted chemotactic molecules.

A limitation of this study is that the reduction in M-MDSCs in trametinib treated mice was correlative and not directly linked to enhanced antitumor immunity and decreased tumor growth. Two experiments would support a causal link for trametinib induced MDSC reduction in tumor growth. First, non-pharmacologic depletion of M-MDSCs during tumor progression would establish that a reduction in M-MDSCs independent of trametinib restricts tumor growth. Second, the adoptive transfer of M-MDSCs to trametinib treated mice to restore M-MDSC levels would establish the relative importance of these cells to the net effect of trametinib *in vivo*. I have tried variations of both experiments several times, but in each case the results were inconsistent. These problems may arise from the challenges in depleting MDSCs in general, specifically depleting M-MDSCs, and adoptively transferring enough M-MDSCs to restore their numbers in tumors. Unfortunately, I will not have time to troubleshoot these experiments, so it will be important for future studies to establish these protocols.

Nonetheless, my data offer mechanistic insight into the apparent inconsistency between *in vitro* and *in vivo* effects of trametinib on antitumor adaptive immunity. Previous reports showed that trametinib has a direct inhibitory effect on proliferation and effector function in both naïve and memory T cells *in vitro* (Vella et al. 2014; Yamaguchi et al. 2012; Allegrezza et al. 2016). In contrast, recent studies indicate that trametinib does not limit the effectiveness of adoptive T-cell therapy (Hu-Lieskovan et al. 2015) or checkpoint blockade with antibodies against PD-1, PD-L1, and CTLA-4 (L. Liu et al.

2015) in other mouse models. I previously showed that the ability of common γ-chain cytokines, which were administered exogenously or possibly up-regulated in response to immunotherapy, to rescue the immunosuppressive activity of trametinib could explain some of these discrepancies (Allegrezza et al. 2016). My current work offers a more comprehensive picture of how trametinib impacts multiple cell types in tumor-bearing hosts.

I demonstrate that trametinib reduces the accumulation of a major immunosuppressive cell compartment in this tumor model; namely, monocytic MDSCs. This occurs at two levels: On the one hand, inflammation-induced MDSC expansion clearly depends on the MAPK pathway, which is abrogated upon MEK inhibition, in both human and mouse bone marrow precursors. On the other hand, trametininb reduces tumor-derived osteopontin secretion, which correlates with a decrease in M-MDSCs. In addition, my study identifies for the first time that osteopontin can chemoattract MDSCs. Although relatively high levels of osteopontin are required for chemoattraction of MDSCs *in vitro*, these levels are physiologically achieved in tumor beds. Together, these combined effects, in addition to the rescuing activity of endogenous cytokines, likely compensate for the direct inhibitory effects of trametinib on T cells. This ultimately results in $CD8^+$ T cells being paradoxically required for the full efficacy of trametinib.

In their seminal study, Hu-Lieskovan *et al.* (Hu-Lieskovan et al. 2015) also looked at the proportions of MDSCs in tumors and spleens of mice treated with trametinib. However, they did not observe a decrease in M-MDSCs and instead found a decrease in PMN-MDSCs in tumors of mice treated with trametinib and dabrafenib, while my study shows a preferential suppressive effect on the mobilization of M-MDSCs. This difference might be due to different treatment schemes. In their experiments trametinib was combined with either dabrafenib or adoptive cell therapy, and it was not tested as a

single agent as it was in my study. In addition, their study used a B-Raf-driven melanoma tumor, which is likely more sensitive to trametinib than my KRAS and PI3K-driven breast tumor and may respond differently in the secretion of inflammatory mediators under MEK inhibition. My *in vitro* studies clearly demonstrate that MEK inhibition abrogates cytokine-induced MDSC expansion, with a preferential direct effect on M-MDSCs. In support of my findings, a murine study of allograft transplantation found that rapamycin treatment induced the expansion of M-MDSCs (defined as $CD11b^{+}GR1^{int}$), but this effect was prevented by trametinib administration (Nakamura et al. 2015). These combined findings show that in some systems M-MDSC expansion can be blunted by MEK inhibition. To understand why trametinib seems to selectively impair M-MDSCs, it will be important for future work to dissect the molecular activity of trametinib on myeloid precursors and determine whether these populations (e.g. common myeloid progenitors and granulocyte-monocyte progenitors) are altered during trametinib treatment.

My results also highlight the importance of tumor microenvironmental and systemic responses to trametinib. Because the activity of trametinib appeared to be independent of the effects on Brpkp110 proliferation, it is likely that trametinib's effectiveness depends in some tumors on whether they mobilize MDSCs to suppress antitumor T cells, rather than the direct cytotoxic or cytostatic effects of trametinib on cancer cells.

Overall, my study offers novel mechanistic understanding to reconcile inconsistent effects of trametinib *in vitro* and *in vivo*, and explain how, by influencing multiple cells types in tumor-bearing hosts, MEK inhibition could have overall permissive effects on protective anti-tumor immunity. Subsequent analyses of trametinib's

effectiveness as a function of MDSC burden and activity in cancer patients will further substantiate whether its therapeutic activity is immune-dependent.

FIGURES

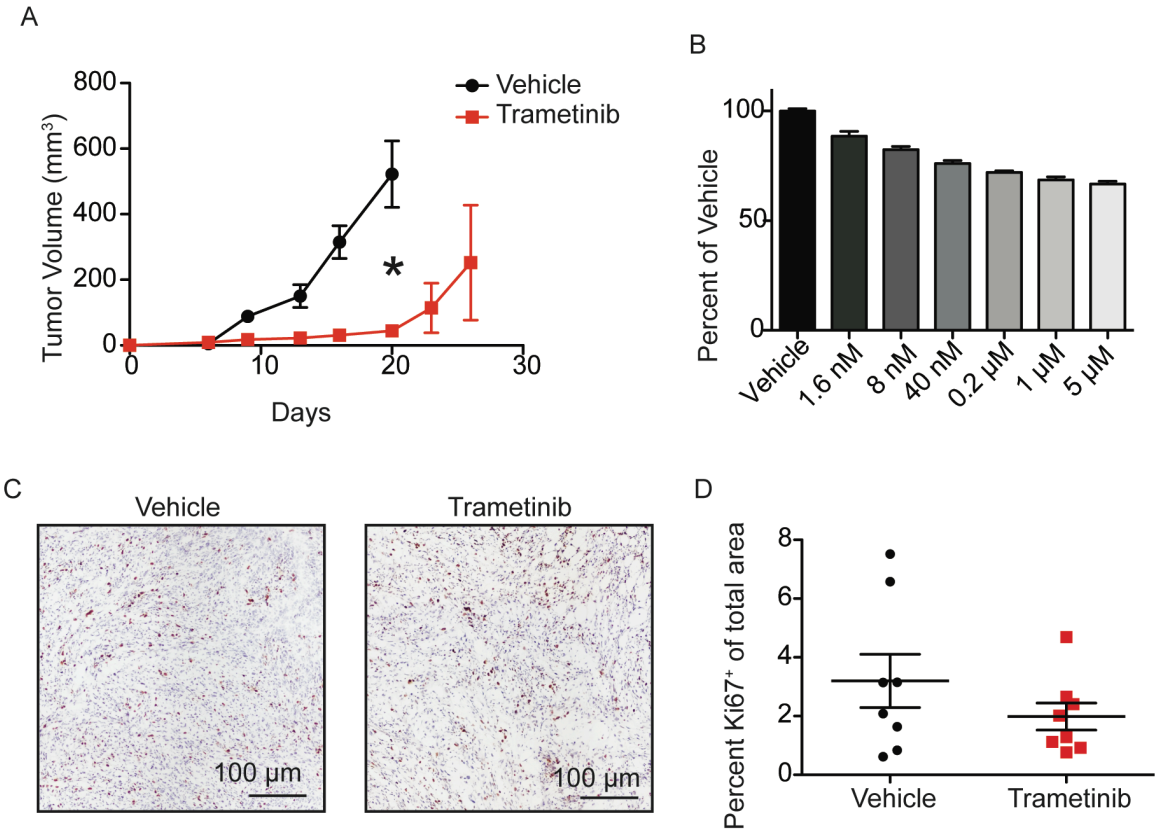

Figure 4.1. **Trametinib impairs the growth of Brpkp110 tumors independent of tumor cell proliferation.** (**A**) Mice with Brpkp110 subcutaneous tumors were gavaged daily with trametinib or vehicle on days 3-13. One representative experiment of three, *P*<0.05, unpaired t test. (**B**) Brpkp110 cells were cultured for 2 days with trametinib and proliferation was quantified by MTS assay. (**C**) Mice with Brpkp110 tumors were treated daily with trametinib or vehicle on days 7-9 and tumors were excised on day 10 and stained for Ki-67. Scale bars = 100 mM. (**D**) Positive Ki-67 as % of total tumor area is shown from two experiments.

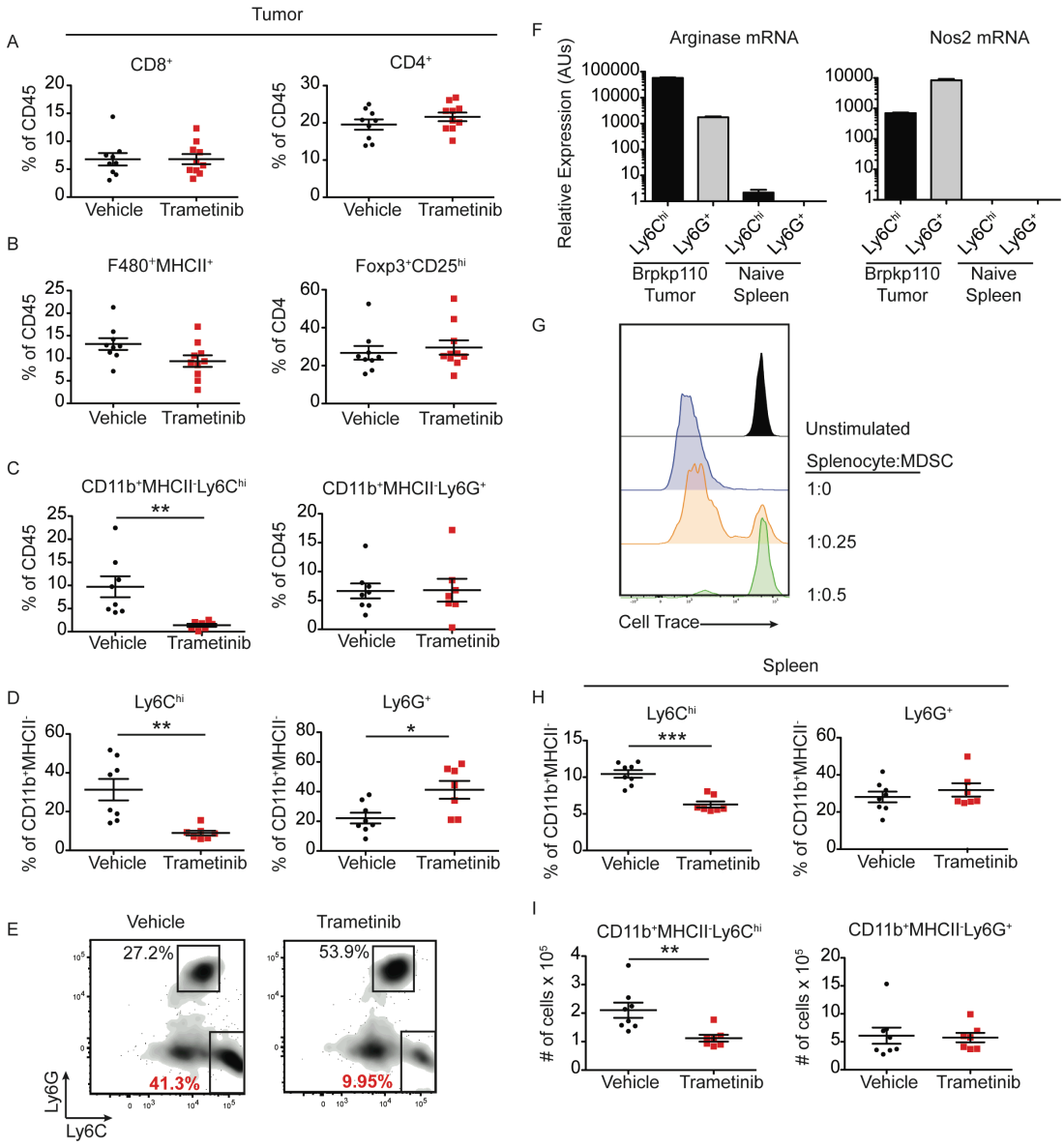

Figure 4.2. **Trametinib reduces the accumulation of Ly6C^{hi} M-MDSCs in tumors**. (**A-E**) Mice with Brpkp110 subcutaneous tumors were gavaged daily with trametinib or vehicle on days 7-9, harvested on day 10, and analyzed by flow cytometry. (**A-D**) Percentages of cell populations found in dissociated tumors from two independent experiments. (**E**) Representative gating for Ly6C^{hi} and Ly6G^{+} from CD11b^{+}MHCII^{-} cells in tumors. (**F**) CD11b^{+}MHCII^{-}Ly6C^{hi} or CD11b^{+}MHCII^{-}Ly6G^{+} were FACS sorted from advanced Brpkp110 tumor-bearing or naive mice and analyzed by qPCR. Expression normalized to TATA binding protein is shown. (**G**) CD11b^{+}MHCII^{-}Ly6C^{hi} cells were sorted from advanced Brpkp110 tumor-bearing mice and mixed at the indicated ratios with OVA$_{257-264}$-peptide-loaded OT-I splenocytes. Proliferation was measured by CellTrace dilution of CD8^{+} cells 3 days later. Representative of two experiments. (**H-I**). Percentages or total numbers of cell populations from spleens of Brpkp110 tumor-bearing mice from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, unpaired t test.

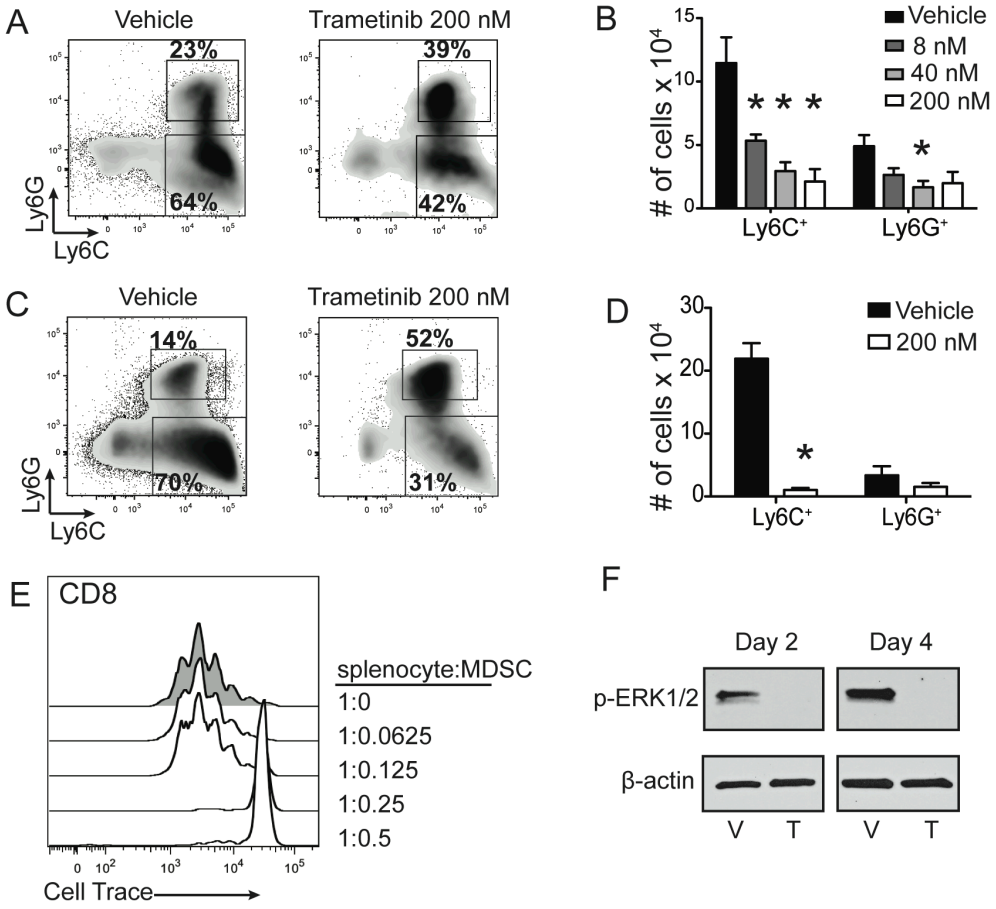

Figure 4.3. Trametinib selectively reduces the differentiation of Ly6C$^+$ MDSCs from bone marrow. (**A**-**B**) MDSCs were differentiated from mouse bone marrow with IL-6 and GM-CSF in the presence of vehicle or trametinib for 4 days. Shown are representative gating from all live cells (**A**) and total number of cells (**B**) from 4 experiments. *P<0.05, Mann-Whitney test. (**C**-**D**) MDSCs were differentiated from mouse bone marrow with Brpkp110 conditioned medium (50%) in the presence of vehicle or trametinib for 4 days. Shown are representative plots (**C**) and total number of cells (**D**) from 4 experiments. *P<0.05, Mann-Whitney test. (**E**) MDSCs differentiated with Brpkp110 conditioned medium were added at the indicated ratios to mouse splenocytes activated with anti-CD3 and anti-CD28 and cultured for 3 days. (**F**) MDSCs were differentiated as in (**C**) and analyzed by Western blot on days 2 and 4 of culture. V=vehicle, T=trametinib 200 nM.

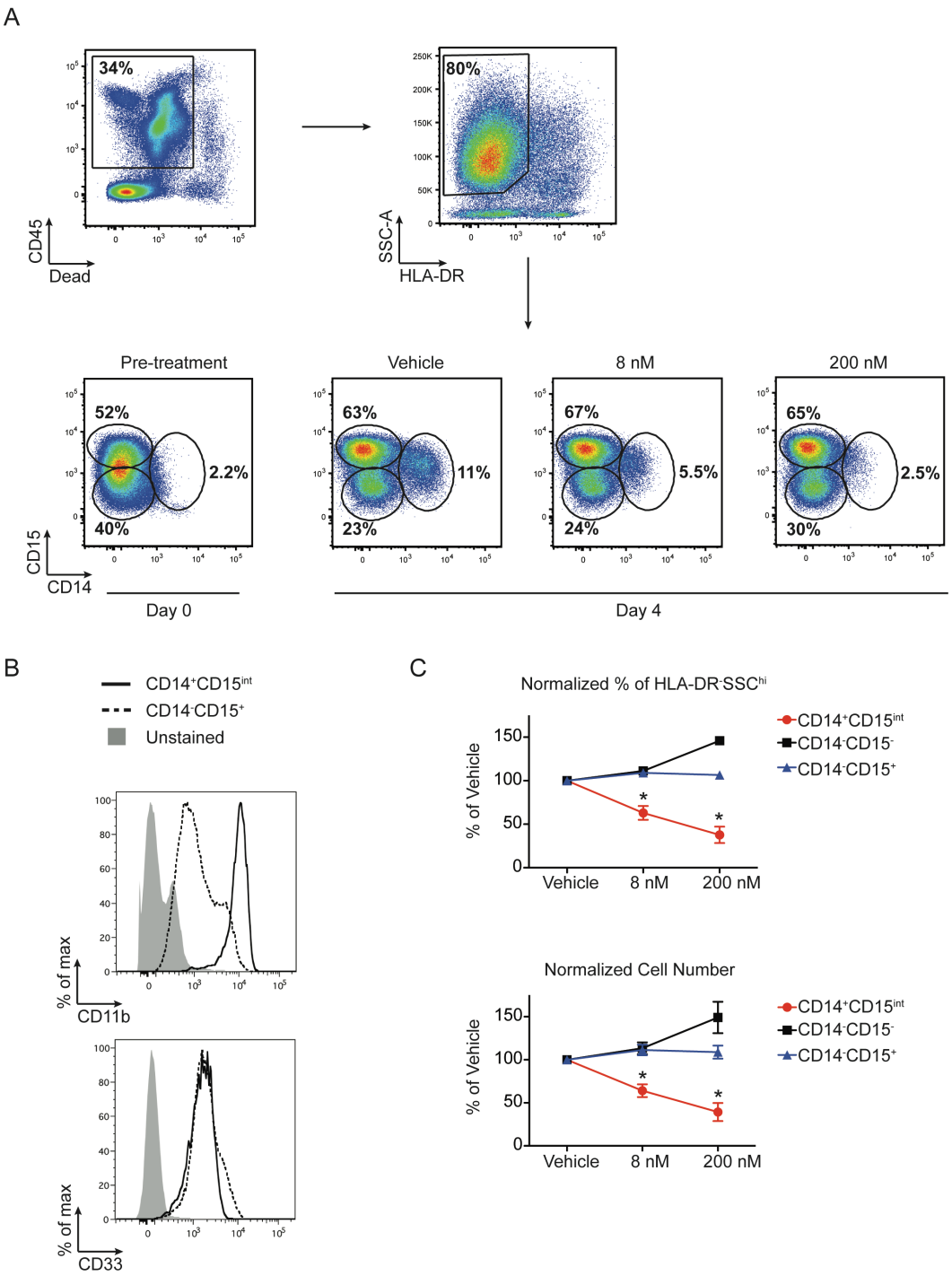

Figure 4.4. Trametinib reduces the expansion of CD14^{+} MDSCs from human bone marrow. Dissociated human bone marrow was cultured in GM-CSF and IL-6 with vehicle or trametinib for four days and analyzed by flow cytometry. (**A**) Gating analysis for one representative donor out of seven is shown. (**B**) Expression of CD11b and CD33 is shown for indicated cell populations. (**C**) Proportions within HLA-DR^{-}SSC^{hi} cells and total numbers for indicated cell populations normalized to vehicle are shown. Data combined from seven individual donors. P<0.05, one sample t-test with respect to 100%.

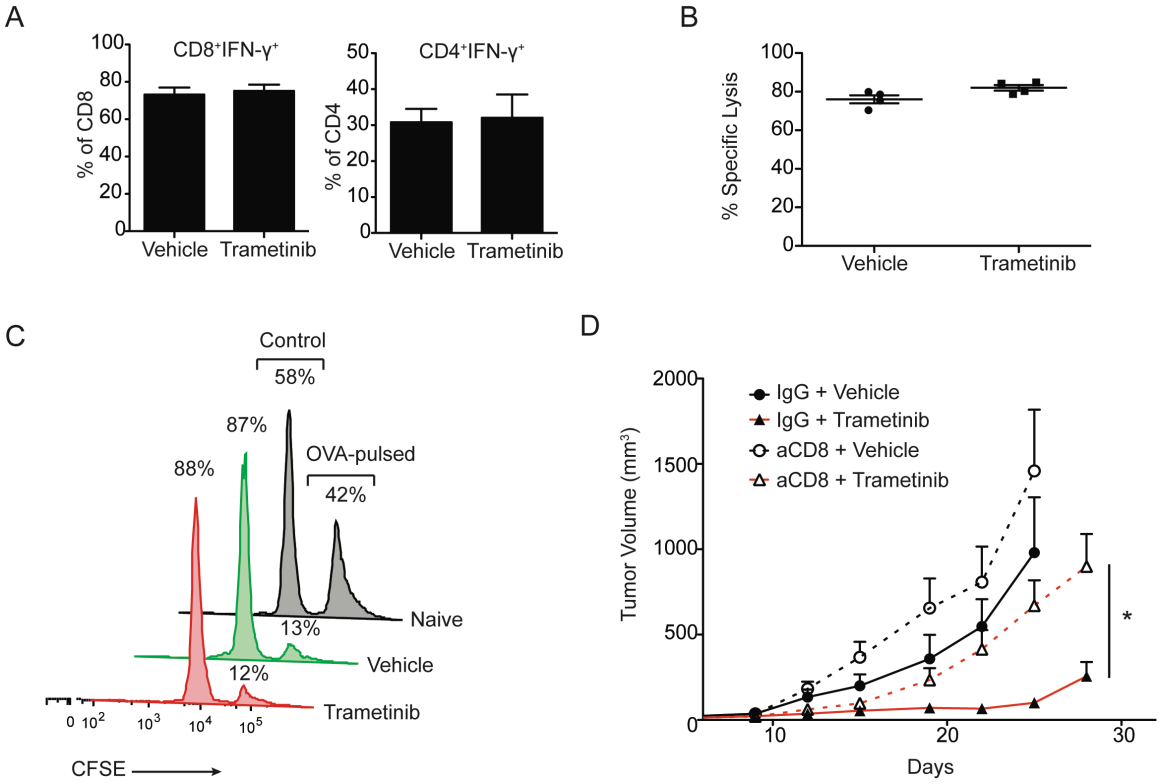

Figure 4.5. Full efficacy of trametinib requires CD8$^+$ T cells. (**A**) Tumors from Brpkp110 tumor-bearing mice treated daily with vehicle or trametinib on days 7-9 were dissociated on day 10, stimulated with PMA/Ionomycin for 5 hrs, and stained for intracellular IFN-γ. (**B**) Mice were vaccinated with OVA-pulsed BMDCs 12 and 5 days before adoptive i.v. transfer of a 1:1 mixture of $OVA_{257-264}$-pulsed ($CFSE^{hi}$) and unpulsed ($CFSE^{low}$) target splenocytes. Mice were gavaged the day before and the day of adoptive transfer (immediately preceding i.v. injections) with vehicle or trametinib and spleens were harvested 5 hrs after transfer for flow cytometry analysis (n = 4 mice/group). Specific lysis (**B**) and representative histograms (**C**) from one experiment of two are shown. *Naïve* indicates unvaccinated mouse. (**D**) Mice with Brpkp110 subcutaneous tumors were gavaged daily with trametinib or vehicle on days 3-13. Anti-CD8α or control anti-LTF was also administered. *Tumor volume different from corresponding non-CD8α depleted mice, $P<0.05$, unpaired t test.

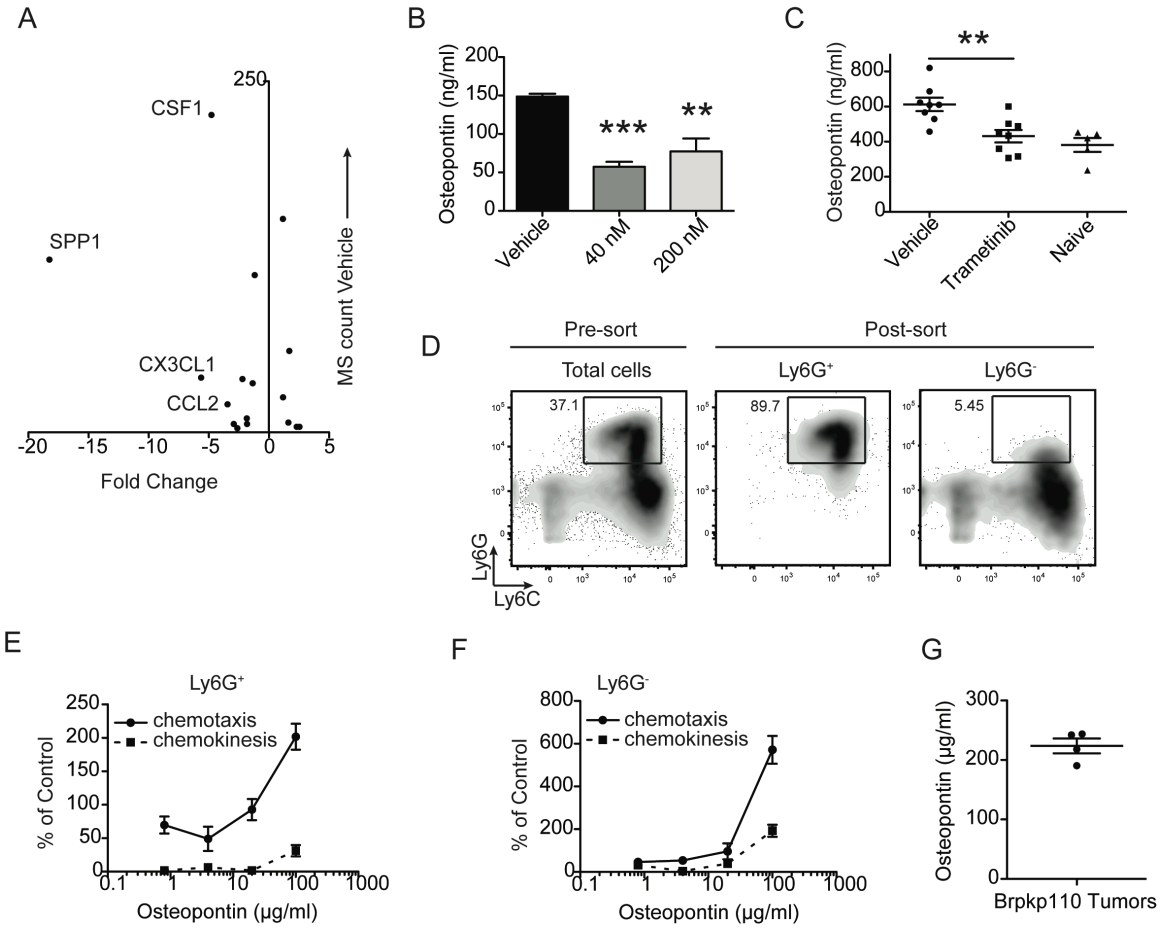

Figure 4.6. **Osteopontin chemoattracts MDSCs and is reduced by trametinib treatment of tumor cells.** (**A**) LC-MS/MS data of cytokines found in supernatants of Brpkp110 cells cultured for 40 hrs in vehicle or 200 nM trametinib. Y axis=MS count (abundance) in vehicle supernatants. X axis=fold change. Positive values=(trametinib/vehicle), negative values = -(vehicle/trametinib). (**B**) Osteopontin concentration measured from supernatants of Brpkp110 cells cultured overnight in the indicated conditions. (**C**) Osteopontin concentration from plasma samples collected from Brpkp110-bearing mice (or naïve tumor-free mice) gavaged daily with trametinib on days 7-9, and harvested on day 10. (**D**) GM-CSF and IL-6 *in vitro* derived MDSCs were separated with Ly6G-MACS microbeads into $Ly6G^+$ and $Ly6G^-$ populations. Pre- and post-sort cell populations were analyzed for Ly6G and Ly6C expression by flow cytometry. (**E-F**) GM-CSF and IL-6 *in vitro* derived MDSCs were separated with Ly6G-MACS microbeads into $Ly6G^+$ and $Ly6G^-$ populations and assayed for their ability to migrate in a transwell assay towards osteopontin (chemotaxis) or within the presence of osteopontin (chemokinesis). (**G**) Osteopontin concentration measured from intratumoral fluid collected from four separate Brpkp110 tumors. **P*<0.05, ***P*<0.01, ****P*<0.001, unpaired t test.

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CHAPTER 5

DISCUSSION AND FUTURE RESEARCH

Cancers are systemic diseases that profoundly affect hematopoiesis and the host's immune response. Here I show that many small molecule inhibitors actively inhibit human T cells, and that although the MEK inhibitor trametinib impairs T-cell functions *in vitro*, it actually facilitates control of tumor growth by $CD8^+$ T cells likely because it also reduces immunosuppressive MDSCs. Considering that a similar dependence upon $CD8^+$ T cells was observed in the efficacy of the $B\text{-}Raf^{V600E}$ inhibitor vemurafenib in murine melanoma (Knight et al. 2013), the contribution of immunity to therapeutic mechanisms should not be overlooked. Additionally, a current obstacle in the treatment of cancer with targeted therapies is the strong selective pressure for tumors to evolve resistance, which has been documented clinically for many small molecules (Sierra, Cepero, and Giordano 2010).

Because T cells can to respond to new antigens and develop memory, they have the potential to eradicate every last tumor cell and maintain surveillance against the future emergence of micrometastases. Combining small molecule inhibitors with immunotherapies could therefore synergistically combat the development of resistance to targeted therapy, although it will first be necessary to understand how immune cells are impacted by small molecule inhibitors. In the following sections, I examine how my work in this thesis contributes to the progress in understanding immune mechanisms in targeted small molecule cancer therapy and the potential for synergy with immunotherapies. I also highlight outstanding questions and avenues of promising research that will help advance the field toward better personalized cancer therapy.

DIRECT EFFECTS OF SMALL MOLECULE INHIBITORS ON T-CELL ACTIVITY

Research into this topic has lagged significantly behind that into the effects of small molecule inhibitors on tumor cells, as evidenced by the fact that most preclinical studies are performed with xenografts in immune-deficient mice. Additionally, because the presence of T cells infiltrating tumors correlates positively with clinical outcome in many cancers (L. Zhang et al. 2003; Galon et al. 2006; van Houdt et al. 2008), caution should be taken to avoid the use of small molecules that impair the functional activity of tumor-reactive T cells. Developing targeted therapies that synergize with antitumor immunity will require dedicated research into how small molecule inhibitors modulate the interaction between the tumor and immune system.

Many leukocytes use the same signaling pathways targeted by small molecule inhibitors in cancer cells. For instance, T cells use the RAS/MAPK, PI3K/mTOR, HDAC, CDK, pathways for activation, proliferation, and cytotoxic activity (Smith-Garvin, Koretzky, and Jordan 2009; Wells and Morawski 2014; Krangel 2007). Accordingly, when I tested a diverse panel of over 40 small molecule inhibitors on activated human T cells, I found that most of them impaired T-cell proliferation at concentrations where they were active against tumor cells (Allegrezza et al. 2016). Molecules restricting T-cell proliferation included those targeting members of the MEK, PI3K, AKT, mTOR, Aurora, CDK, HDAC, and Bcl-2 protein families. Other groups have also found that pharmaceutical inhibition of the MEK (Boni et al. 2010; Vella et al. 2014), PI3K (So et al. 2013; Blanco et al. 2015), HDAC (D. J. L. Wong et al. 2014; Schmudde et al. 2010), and CDK (Nellore et al. 2014) pathways limits the *in vitro* activity of human T cells.

Studies of small molecule inhibitors *in vivo* are critical, though, where the complexity of environmental factors and pharmacokinetics can alter drug activity. Initial research with the MEK inhibitor trametinib (GSK1120212, named JTP-74057 at the time) demonstrated that it was effective at reducing rheumatoid arthritis in mice and rats,

activity the authors attributed to its ability to prevent the activation of auto-reactive T cells when assayed *ex vivo* (Yamaguchi et al. 2012). Although Yamaguchi et al did not determine whether trametinib prevented *in vivo* activity of T cells, I found that once daily oral gavage with trametinib reduced the proliferation of T cells responding to tumor antigen (Allegrezza et al. 2016). The combined interpretation of these results suggests that MEK inhibition *in vivo* limits the generation of T-cell driven immune responses in at least some disease contexts.

Intriguingly, recent studies indicate that trametinib does not restrict the effectiveness of adoptive T-cell therapy (ACT) (Hu-Lieskovan et al. 2014) and in fact synergizes with PD-1 inhibitors in mice (L. Liu et al. 2015). A possible mechanistic explanation is that direct T-cell inhibition by trametinib can be effectively overcome by cytokines such as IL-2, IL-7, and IL-15 through PI3K activation (Allegrezza et al. 2016). The former study included high-dose IL-2 treatment (Hu-Lieskovan et al. 2014), a regimen that I found capable of rescuing T-cell proliferation from trametinib *in vivo* (Allegrezza et al. 2016), and PD-1 is known to inhibit proximal TCR signaling (Riley 2009) and PI3K activity (Patsoukis et al. 2013). It is possible that enhanced activation of the PI3K and MAPK pathways through cytokine signaling and/or decreased inhibitory signaling from PD-1 renders T cells more resistant to MEK inhibitors in the tumor microenvironment. Notably, the PI3K inhibitor BKM120 has been shown to prevent the expansion of tumor-specific T cells in mice (Peng et al. 2016). Because the combination of PI3K and MEK inhibitors is being clinically investigated (Bedard et al. 2015), it will be important to determine if PI3K inhibition prevents the ability of cytokines and immunotherapies to ameliorate MEK inhibition of T-cell function *in vivo*.

Not all small molecule inhibitors are detrimental to T cells, however, particularly when they target signaling pathways irrelevant for lymphocyte function. For instance,

Axitinib, an inhibitor of the VEGFR proteins, does not affect T cell activity (Du Four et al. 2015). I observed that an inhibitor of IGF-1R, OSI-906, had negligible activity on human T cell activation and proliferation at concentrations relevant to limit proliferation of IGF-1R dependent cells (Allegrezza et al. 2016). The rational use of isoform specific PI3K inhibitors might also avoid T-cell inhibition. The class I PI3K family has four separate isoforms of the catalytic p110 subunit (α, β, γ, δ) with variable expression among different cell types. The p110γ and p110δ isoforms are required for effector and memory T-cell responses (So and Fruman 2012; A. L. Martin et al. 2008; Okkenhaug 2013), while activity of the p110α and p110β isoforms often contributes to tumor growth. Therefore, use of isoform selective inhibitors instead of pan-PI3K inhibitors (like BKM120) could theoretically target tumor cells while preserving immune cells. Indeed, although pan-PI3K inhibitors impair T cells, one study found that p110α specific inhibitors such as MLN1117 and A66 did not impair T-cell proliferation or cytokine production at concentrations able to limit the proliferation of tumor cells (So et al. 2013). The researchers also found that the detrimental effect of pan-PI3K inhibition on immunity *in vivo* was avoided in mice treated with MLN1117.

In addition to molecules targeting signaling pathways not utilized by immune cells, molecules that target mutation-specific proteins would be expected to spare the activity of T cells. Indeed, B-Raf^{V600E} inhibitors do not impair T-cell function (Knight et al. 2013; Boni et al. 2010; Comin-Anduix et al. 2010), and in fact there is evidence that B-Raf^{V600E} inhibitors promiscuous for wild-type B-Raf can actually enhance T-cell activation via increased MAPK signaling (Callahan et al. 2014). Small molecules can even be designed to boost T-cell activation, as shown with Avasimibe, an inhibitor of the ACAT1 cholesterol esterification enzyme (Yang et al. 2016).

Certain small molecules can also influence the differentiation of various T cell subsets. For instance, although mTOR inhibition by rapamycin can limit the proliferation of activated T cells (Allegrezza et al. 2016; Dumont et al. 1990; Kay et al. 1991), it can also enhance the differentiation of memory T cells (Araki et al. 2009). Because memory T cells are ultimately more beneficial for tumor control than effector cells (Klebanoff et al. 2005), rapamycin improves the therapeutic efficacy of tumor antigen vaccination in a mouse B16-OVA model when specifically administered during the effector-to-memory transition phase (day 10-30 post vaccination) (Diken et al. 2013).

Several studies have demonstrated enhanced T-cell responses in tumor bearing mice treated with HDAC inhibitors (Vo et al. 2009; Woan et al. 2015), although it is difficult to conclude whether these findings were direct results of HDAC inhibition in T cells or from the effects of HDACs on tumor cells or APCs. Nonetheless, HDAC inhibition has been reported to enhance the expression of the effector molecules granzyme B (Agarwal et al. 2009) and IFN-γ (Agarwal et al. 2009), the expression of chemokines CCL5 and CXCL10 (Zheng et al. 2016) and the differentiation of $CD8^+$ T cells into functional memory cells that can provide protective immunity (Northrop, Wells, and Shen 2008). In mice, HDAC inhibition can either accelerate or ameliorate Th1-driven GVHD depending on the individual inhibitor used (D. Wang et al. 2012), demonstrating that effects of HDAC inhibition may depend on the specificity of each inhibitor.

Because Treg cells can suppress anti-tumor immunity, therapies that modulate the numbers and function of these cells can have important consequences for tumor growth. For that reason, several groups have characterized the effects of kinase inhibition on Tregs, with conflicting results depending on the specific inhibitors or experimental systems used. One group found that inhibitors of PI3K and AKT impaired human and mouse Treg proliferation while having negligible activity on conventional

$CD4^+$ T-cell proliferation at the same concentrations (Abu-Eid et al. 2014). Administration of these molecules prior to vaccination in mice was able to reduce Tregs and enhance antitumor $CD8^+$ T-cell responses. In contrast, other researchers found that PI3K inhibition suppressed the proliferation of human conventional $CD4^+$ T cells more than the proliferation of Tregs (Zwang et al. 2016). Among the several different variables between these studies was the use of high concentrations of IL-2 in the former study, which is known to activate PI3K and enhance the activity of Tregs (Yates et al. 2007). Various murine studies have demonstrated that PI3K inhibition can either inhibit or promote Tregs, depending on which functions and subsets of Tregs are analyzed, but the general conclusion suggests that the net effect of PI3K inhibition is a reduced and less suppressive Treg compartment (Soond et al. 2012). The precise timing of PI3K inhibition could also be important, demonstrated by the ability of naïve $CD4^+$ T cells to turn into Tregs when PI3K is inhibited 18hr after TCR activation, while earlier and later timepoints are less efficient at producing Tregs (Sauer et al. 2008). Tregs may be less susceptible to mTOR inhibition, however, as it was found that rapamycin, everolimus, or BEZ235 favored the expansion of Tregs compared to conventional T cells, while PI3K inhibition with BKM120 showed similar inhibition of both subsets (Huijts et al. 2016).

Fewer studies have analyzed how MEK inhibition directly influences Tregs, but results suggest that it may encourage their expansion. One study found that MEK inhibition with MEK-162 inhibited the proliferation and activation of human conventional T cells more than Tregs, possibly explained by the relatively lower expression of MEK (and thus reliance upon) in Tregs (Zwang et al. 2016). The Treg phenotype is actively maintained because Tregs can lose Foxp3 expression to transition into a Th1 phenotype. One group found that Foxp3 downregulation was signaled through the MEK-ERK pathway and that a MEK inhibitor was able to prevent Foxp3 loss, thus maintaining

the Treg phenotype (Guo et al. 2014). Despite these data, when I treated tumor-bearing mice with trametinib, I did not observe a difference in the percentages of Tregs. I did not assess the functional activity of these cells, however, so it remains possible that Tregs from trametinib treated mice have higher suppressive ability. Experiments to test this idea, along with experiments analyzing the relative activation and expansion of human Tregs during *in vitro* culture with trametinib, would be useful for a more complete characterization of trametinib's activity on the tumor microenvironment.

THE MYRIAD EFFECTS OF SMALL MOLECULE INHIBITORS ON THE IMMUNOENVIRONMENT OF CANCER

Chronic inflammation in cancer promotes the expansion and recruitment of a variety of immunosuppressive myeloid cells that contribute to tumor growth. The activity of small molecule inhibitors on these cells may contribute to their pharmacological mechanisms of action and may also be therapeutically advantageous. Cytokines such as IL-6, GM-CSF, and VEGF are upregulated as a result of chronic inflammation in many cancers and lead to the expansion of MDSCs. These cells suppress antitumor T cells and their numbers are correlated with disease progression in many cancers (Solito et al. 2014). Although little is known about how small molecule inhibitors impact MDSCs, several findings have shown the relevance of targeting MDSCs. Continuous activation of STAT3 in MDSCs contributes to their suppressive activity and impaired differentiation into mature APCs (Kumar et al. 2016). Inhibition of JAK2/STAT3 signaling with JSI-124 was able to reduce the numbers of immature myeloid cells by promoting their differentiation into macrophages and DCs, which enhanced the effectiveness of immunotherapy in preclinical models (Nefedova et al. 2005; Kumar et al. 2016). Several studies have also shown that MDSC accumulation in tumor bearing mice can be

attenuated by small molecule inhibition of VEGFR with Axitinib (Du Four et al. 2015; Yuan et al. 2014) or SAR131675 (Espagnolle et al. 2014). Additionally, inhibition of phosphodiesterase-5 with sildenafil or tadalafil prevents MDSCs from suppressing T cells (Serafini et al. 2006).

MAPK signaling is also important for the differentiation and function of myeloid cells (Hsu, Kikuchi, and Kondo 2007). Accordingly, I demonstrate that MEK inhibition reduces the mobilization of monocytic MDSCs ($Ly6C^{hi}Ly6G^{-}$) in *Kras*-driven tumors, which likely facilitates control of tumor growth by $CD8^{+}$ T cells. Trametinib also impairs cytokine-induced expansion of their $CD14^{+}MHC\text{-}II^{-}$ counterparts from human bone marrow, supporting the promise of MEK inhibition against MDSC-dependent tumors in patients. Therefore, the immunosuppressive *vs.* immunostimulatory activity of MEK inhibitors appears to depend on their collective effects on a variety of immune and non-immune cells, a balance that may have distinct outcomes in different malignancies.

Epigenetic modifiers could also be used to modulate MDSC differentiation. For instance, Youn and colleagues found that around 40% of monocytic MDSCs differentiate into granulocytic PMN-MDSCs in tumor bearing mice, controlled by HDAC2 silencing of the *Rb1* gene. Treatment of M-MDSCs *ex vivo* with an HDAC inhibitor upregulated *Rb1* expression and promoted their differentiation into macrophages and DCs, the usual terminal differentiation products of immature monocytes under non-inflammatory conditions (Youn et al. 2013).

In addition to influencing the activity of suppressive immature myeloid cells, small molecules can also alter the function of mature APCs. MEK inhibition can impair cross-presentation by human monocyte-derived DCs (Vella et al. 2014), leading to ineffective priming of $CD8^{+}$ T cells. Conversely, pan-PI3K inhibition was able to skew TLR agonist-activated DCs towards priming polyfunctional antitumor T cells by reducing DC

production of TGF-β and IL-10 while maintaining favorable IL-12 and IL-1β secretion (Marshall et al. 2012). The HDAC inhibitor LAQ824 was found to reduce IL-10 secretion from macrophages and make them better at priming antigen specific T cells (H. Wang et al. 2011).

Finally, small molecules may modulate antitumor immunity indirectly through their effects on tumor cells. Signaling pathway inhibition of tumor cells has been reported to alter their expression of antigens and costimulatory molecules (Boni et al. 2010), secretion of chemokines (Knight et al. 2013; Zheng et al. 2016), and sensitivity to killing by NK and T cells (Skov et al. 2005; Armeanu et al. 2005; Schumacher et al. 2006; Shanker et al. 2015). Small molecules can also induce autophagy to sensitize tumor cells to killing by CTLs, such as through PI3Kβ inhibition in PTEN deficient melanoma cells (Peng et al. 2016). These various mechanisms can result in drug activity superior to that when only direct cytoxic/cytostatic activity on tumor cells is considered in immune-deficient mice.

Clearly, this broad array of mechanisms can lead to *in vivo* activities very different from that when only direct cytotoxic/cytostatic activity on tumor cells is considered in immune-deficient mice. While it may not be feasible to study the interaction of every small molecule in clinical development with all the various leukocyte populations, I feel that it is essential to consider the function of immune cells when investigating the mechanistic actions of these new therapies.

STRATEGIES TO PROMOTE ANTITUMOR IMMUNITY WITH SMALL MOLECULE INHIBITORS

In order to avoid detrimental effects on antitumor immunity, better therapies can be contrived by designing more specific inhibitors that target protein isoforms predominantly expressed in cancer cells (as with PI3Kα) and mutation specific inhibitors with little off-target activity on wild type proteins, such as mutant IDH1/2 inhibitors (F. Wang et al. 2013; Rohle et al. 2013). Yet in cases where certain molecules have unavoidable activity on leukocytes, careful dosing titration and timing may maintain beneficial antitumor immunity. Proteosome inhibition by bortezomib provides a compelling example, where it was found that high doses led to immune suppression, while lower doses given less frequently were not immunosuppressive and instead sensitized tumors to FasL lysis by T cells (Shanker et al. 2015). PI3K inhibitors may also be candidates for specifically timed dosing, for instance in order to boost the activity of DCs during vaccination (Marshall et al. 2012) without limiting the proliferation of antigen specific T cells by continuous administration. Additionally, certain immunotherapies may be able to neutralize the negative effects of small molecule inhibitors on immune cells, as I show here with the ability of IL-2 and IL-15 agonists to rescue T cells from MEK inhibition.

In cases where cross-reactivity of small molecules on T-cell signaling pathways cannot be avoided, I envision several strategies to protect T cells that should be investigated. Exciting progress has been made utilizing nanoparticles to target chemotherapeutics to tumors, a process which increases the maximum concentration achievable in tumor cells by reducing drug release throughout the rest of the body (Goodall, Jones, and Mahler 2015). It is conceivable that nanoparticle encapsulation of small molecule inhibitors could also be used to specifically target tumor cells. Indeed, this concept was very recently demonstrated with the aurora B kinase inhibitor, AZD2811 (Ashton et al. 2016), although it was only tested in immune-deficient

xenografts models. More precise targeting may be achieved by covalently conjugating small molecule inhibitors to tumor-targeted antibodies, thus facilitating their direct entry into tumor cells by endocytosis.

T cells could also be protected through genetic engineering, for instance through adoptive transfer of T cells with enhanced expression of ATP-binding cassette (ABC) transporters. ABC transporters actively pump out xenobiotics and their activity has been demonstrated to increase the resistance of cancer cells to small molecule inhibitors (Özvegy-Laczka et al. 2005). Endogenous expression of ABC transporters is known to protect memory T cells from cytotoxic chemotherapy (Turtle et al. 2009), so it is likely that their engineered expression in T cells could limit the activity of other small molecules. Lastly, signaling networks could be restored (or enhanced) in T cells through genetically expression of protein mutants resistant to the activity of small molecules, such as the MEK2-Q60 mutant for trametinib resistance (Villanueva et al. 2013). Experiments would need to be performed to determine that these T cells function normally and do not pose an unacceptable risk of malignant transformation.

However, it may not be optimal to simply avoid signaling inhibition in immune cells for all small molecules. At least several targets exist where it seems possible to enhance the activation of APCs or T cells, which could result in therapeutic synergy and the possibility to completely eradicate metastatic disease. Small molecules could also be useful to prevent the function of regulatory immune cells in tumors, particularly the accumulation of suppressive myeloid cells. While inhibitors of JAK2/STAT3 and MEK/ERK may selectively impair MDSCs, a targeted delivery approach could also be utilized. Regulatory myeloid cells with phagocytic ability, such as tumor-associated DCs, are very efficiently targeted by nanoparticles (Cubillos-Ruiz et al. 2012), and the incorporation of small molecules may be achievable. Antibody-drug conjugates (ADC)

could be employed to target small molecules to non-phagocytic regulatory cells. For instance, an ADC has been developed to selectively deliver an Lck inhibitor to T cells (R. E. Wang et al. 2015), thus modifications could allow delivery of other inhibitors to Tregs or MDSCs.

INTEGRATING TARGETED AND IMMUNOTHERAPY COMBINATIONS INTO PERSONALIZED MEDICINE

Due to the unique nature of each cancer patient's disease, better therapeutic results will be achieved with more personalized therapies. It is an ongoing effort to more precisely classify which patients will respond to different treatments. For instance, patients with high numbers of tumor-infiltrating leukocytes that express PD-L1 respond better to an antibody targeting PD-L1 (Herbst et al. 2014), reflecting that those are the patients where the PD-1/PD-L1 axis is relevant to immune suppression.

The most important variable for determining the applicability of immunotherapy is the quality of tumor antigens. In mouse models, the induction of potent neoantigens by chemical carcinogens or the genetic introduction of foreign antigens allows the adaptive immune system to control tumor growth (Koebel et al. 2007; DuPage et al. 2012). One important feature of my studies is that the Brpkp110 tumor cells express EGFP, a protein not present in the mouse genome. It is possible that EGFP functions as a tumor antigen in this model. In BALB/c mice, which express the H-2Kd MHC-I allele, it is well established that EGFP is a potent antigen because EGFP expressing tumors are rejected and EGFP-specific effector T cells can be detected from tumor-challenged mice (Stripecke et al. 1999).

However, it appears that EGFP is a much weaker antigen in C57BL/6 mice, which express the H-2Kb MHC-I allele. Although one lab found that EL-4/EGFP+

lymphoma cells had an impaired ability relative to the parental EL-4 cells to form tumors in C57BL6 mice, 4 of 5 mice did eventually develop EL-4/EGFP+ tumors and the authors were unable to detect any T cell responses against EGFP (Stripecke et al. 1999; Skelton, Satake, and Kohn 2001). Follow up work by a separate group actually found no detectable delay in EL-4/EGFP+ tumor development compared to control EL-4 cells in C57BL/6 mice (Denaro et al. 2001). C57BL/6 mice are capable of developing adaptive immune responses to EGFP, though, as was demonstrated by the appearance of EGFP-specific CD8+ T cells and antibodies leading to the eventual clearance of transduced EGFP+ cells in mice given a lentiviral vector (Annoni et al. 2007).

Thus, EGFP seems to be a weak antigen in C57BL/6 mice, such that in the context of tumor-driven immunosuppression it may not elicit significant T-cell responses, while in adjuvant settings (such as with viral vectors), both cellular and humoral responses can be generated. Because it is a weak antigen, EGFP may function in a similar manner as natural tumor antigens in advanced cancers, a context where multiple suppressive mechanisms prevent effector T-cell activity against tumor cells, but perturbation by immune stimulation can tip the balance to favor tumor clearance by cytotoxic effector T cells. These insights should be considered when interpreting results from Brpkp110 tumors because the translational relevance of my findings may only apply to patients with tumor antigens capable of eliciting T-cell responses. As such, it will be important for future work to analyze the efficacy of trametinib and IL-15 agonists in tumor models without foreign proteins.

In addition to the potency of tumor antigens, it also seems that the clonality of antigens in tumors is important. Tumors with neoantigens present in all cells (clonal) tend to respond better to PD-1 and CTLA-4 blockade than those with subclonal neoantigens (McGranahan et al. 2016). Studies using clonal tumor cells, such as

presented here, may only be useful for extrapolating results to patients with clonal mutations. It may be possible for certain small molecules to enhance immunotherapeutic efficacy in subclonal tumors by activating antigen spreading, where dying tumor cells that contain previously unrecognized antigens elicit the expansion of naïve T cells into cytotoxic effectors. Small molecules that facilitate tumor cell death, enhance tumor cell antigen presentation, or promote the activation of APCs could potentially induce antigen spreading for synergy with immunotherapy.

Novel insights into the host microbiota have led to increased appreciation for its influence on cancer growth and therapeutic outcome (Rutkowski, Stephen, et al. 2014; Viaud et al. 2013). In fact, the presence of certain bacterial species is required for the efficacy of CTLA-4 and PD-L1 blockade in mice (Vétizou et al. 2015; Sivan et al. 2015). Small molecule inhibitors may modulate the interaction between commensals and the immune system. Prokaryotes also signal through kinases that have homology in their catalytic domains to eukaryotic kinases (Pereira, Goss, and Dworkin 2011), so it would be interesting to know if any small molecule inhibitors demonstrate activity on commensal bacteria. Small molecules could also act on gut epithelial and immune cells to modulate the sensing of bacterial products by those cells. Because the microbiota is extremely important in regulating immunity, it is possible that important drug mechanisms will be found in the interactions between commensals and host. These mechanisms and their ability to determine the optimal categorization of patients for immunotherapy and targeted therapy combinations should be investigated.

Developing better-quality personalized therapies also depends on the use of more relevant animal models for preclinical cancer studies. Although xenografts have the advantage of using human tumor cells, it is now evident that these models lack a critical biological component relevant to disease progression in many cancers. In this

regard, syngeneic mouse tumor cell lines are preferable because they allow the analysis of tumor growth in the presence of host adaptive immunity. Because of their ease of use and rapid, consistent tumor growth, transplantable syngeneic tumor cell lines are valuable in developing proof-of-concept studies. However, they fail to recapitulate the natural progression from transformation to malignancy and the heterogeneous nature of many tumors. Also, the injection of tumor cells in a bolus, which includes dead/dying cells, may induce an inflammatory reaction, thus priming the immune system in an artificial manner. For these reasons, studies should also be performed in more representative cancer models.

Genetically inducible autochthonous models allow tumor initiation and development with knowledge of the precise molecular features driving tumorigenesis. In this thesis I describe a method for generating inducible, autochthonous breast cancer models in which the use of endocrine agonists is avoided and the precise timing of tumor initiation is known. The beauty of the Cre-Lox system is that any mutational combination can be developed, given that it is feasible to develop the desired genetic background through breeding. Another advantage is that adenovirus-Cre can be delivered in many locations to develop different cancers. Here I describe initiation of breast cancer by infection of mammary duct cells, but other researchers have used adenovirus-Cre to generate ovarian (Scarlett et al. 2012) and lung carcinomas (Jackson et al. 2001) and sarcomas (Rutkowski, Stephen, et al. 2014). This method of tumor initiation can be used to generate a variety of genetically distinct cancers to model the diversity observed clinically, and it will be important to determine if the mechanism of trametinib activity and the synergy with IL-15 agonists I observed here is similar in other tumor models.

Most patients being treated with targeted and immunotherapies have metastatic disease. Thus, murine models of metastasis are more clinically relevant than primary

tumor models. Unfortunately, few genetically engineered mouse models recapitulate metastasis well (Saxena and Christofori 2013). Although the breast cancer model I describe here did not develop macroscopic metastases by the time that mice needed to be euthanized from the large primary tumor, I did observe dissemination of YFP+ tumor cells to the distal LN. It is possible that surgical resection of the primary tumor would allow time for the metastases to form tumors. This method might also allow the study of cancer dormancy, where tumor cells remain quiescent and undetected for long periods of time (sometimes many years in patients) before they switch into active tumor formation later. Cancer dormancy is an important aspect of cancer biology that has been difficult to study for obvious reasons. If surgical resection of this breast tumor model produced a period of dormancy, it would be fascinating to determine if trametinib and/or IL-15 agonists affected the length of dormancy or elimination of dormant cancer cells. Considering the many options provided by Cre-Lox engineered tumor models, future studies should be conducted to determine if the combination of trametinib and IL-15 agonists is appropriate for clinical use, and if so, which patients are most likely to benefit from such therapy.

CONCLUDING REMARKS

In this thesis, I demonstrate that the immune system can contribute in important ways to the mechanisms of small molecule inhibitors. Although cancer biology and immunology operated as separate disciplines for many years, it is now clear from this work and the research of many others that the knowledge of immune mechanisms in cancer will be critical for designing optimal therapeutic strategies. And while tumor cells may still be the primary targets for inhibition by small molecules, modulation of signaling

pathways in leukocytes must be examined, including more comprehensive monitoring of immune readouts in clinical trials. In fact, it appears that there may only be a few classes of inhibitors that do not impact the immune system. Most inhibitors have been reported to affect at least some leukocytes, and several classes of inhibitors have broad activity on a wide range of immune cell compartments. Considering that the success of checkpoint inhibitors has kindled dreams of immunotherapies as first line interventions, research is urgently needed to identify drugs that perform well in not only immunocompromised mice, but also in the context of antitumor immunity. Looking forward, I see an optimistic future for the combination of small molecules and immunotherapies to usher in a new era of personalized cancer treatment.

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