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Tumor Antigens Revealed By Exome Sequencing Drive Editing of Tumor

Immunogenicity

by

Matthew David Vesely

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2013

St. Louis, Missouri

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LIST OF ABBREVIATIONS

AOM	azoxymethane
APC	antigen presenting cell
ATM	ataxia-telangiectasia mutated
B2m	beta-2 microglobulin
CCL11	chemokine (C-C motif) ligand 11
CD	cluster of differentiation
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated protein-4
DC	dendritic cell
DEN	diethyl-nitrosamine
DMBA	7,12-di-methylbenz[a]-anthracene
DNA	deoxyribonucleic acid
DNAM1	DNAX accessory molecule-1
DSS	dextran sodium sulfate
FACS	fluorescence-activated cell sorting
FASL	Fas ligand
FR4	folate receptor 4
gDNA	genomic DNA
<i>Gld</i>	generalized disease mutant mice
GM1	ganglioside

GM-CSF	granulocyte-macrophage colony stimulating factor
HTLV	human T cell leukemia virus
IFN	interferon
IFNAR1, 2	interferon alpha receptor chain 1, 2
IFNGR1	interferon gamma receptor chain 1
Ig	immunoglobulin
IL	interleukin
JAK	Janus activated kinase
KRAS	v-Ki ras2 Kristen rat sarcoma viral oncogene homolog
LMP	low-molecular-mass protein
mAb	monoclonal antibody
MCA	3'-methylcholanthrene
MHC I	major histocompatibility complex class I
MDSC	myeloid derived suppressor cell
MNU	N-methyl-N-nitrosurea
MyD88	myeloid differentiation primary response gene 88
NK	natural killer
NK1.1	NK-cell-associated antigen 1.1
NKG2D	NK group 2, member D
NKT	natural killer T
PCR	polymerase chain reaction
<i>Pfp</i>	perforin
PD-1	programmed cell death 1

PD-L1	programmed cell death 1 ligand 1
RAE	retinoic acid early transcript
RAG	recombination activating gene
RNA	ribonucleic acid
SCID	severe combined immunodeficient
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
STAT	signal transducer and activation of transcription
TAA	tumor associated antigen
TIL	tumor-infiltrating lymphocyte
TCR	T cell receptor
TNF	tumor necrosis factor- α
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TRAMP	transgenic adenocarcinoma of the mouse prostate
TRP53	transformation related protein 53
UV	ultraviolet
WT	wild type

ACKNOWLEDGEMENTS

The efforts of many exceptional people have contributed not only to the work presented in this thesis, but also to my education and development as a scientist. First and foremost, I would like to thank my advisor, Dr. Robert Schreiber for his unwavering support, mentorship and teaching throughout my years in the laboratory. His infectious enthusiasm and ability to tackle difficult scientific questions with inventive approaches created a collegial training environment and has provided me with a framework to aspire to in future scientific endeavors. Secondly, I would like to thank the other members of my thesis committee—Drs. Marco Colonna, Ken Murphy, Barry Sleckman, Andrey Shaw, Emil Unanue, and Wayne Yokoyama—for their advice, encouragement and scientific acumen.

This thesis would not have been possible without the efforts of Dr. Hirokazu Matsushita, a post-doctoral fellow in the Schreiber laboratory who contributed immensely to the collaborative effort of identifying tumor antigens. Not only are his dedication to science, tireless work ethic, and elegant experimentation inspiring, but also his kind and gentle character are greatly admired. I would also like to acknowledge the efforts of the Genome Institute at Washington University, especially Dr. Elaine Mardis, Dan Koboldt, Vince Magrini, Ryan Demeter, Todd Wylie, and Jasreet Hundal for their technical assistance and expertise as well as their eager willingness to collaborate. I am deeply grateful to past and present members of the Schreiber laboratory for their invaluable assistance and advice as well as their generous friendship. Although it is

impossible to list the many contributions of my colleagues to this work, I would especially like to acknowledge the substantial efforts of Chuck Rickert, Dr. Ravi Uppaluri, Sang-hun Lee, Cora Arthur, Mike White, and Jessica Archambault. In addition, Dr. Ruby Chan, Dr. Kathy Sheehan, Dr. Mark Diamond, Dr. Brian Weaver and Dr. Jack Bui have been vital resources throughout my time in the Schreiber laboratory. I would also like to thank Dr. Gavin Dunn, Dr. Catherine Koebel, Samuel Siu-kit Lam, Andy Chen and Lauren Shea for helpful instruction, assistance, and critical thinking. In addition, I am grateful to Jeanne Silverstrini and Sharon Smith who provided support and assistance throughout my years in the Schreiber laboratory. This work has benefited from a stellar immunology community at Washington University, and I would like to particularly thank Drs. Thaddeus Stappenbeck, Ted Hansen, Daved Fremont, Paul Allen and Steve Horvath for collaboration, reagents, and advice.

Scientific research is an international enterprise and I have been very fortunate to collaborate with many esteemed immunologists both in and out of the United States. In particular, I would like to acknowledge Dr. Mark Smyth from Melbourne University for his collaborative spirit, scientific enthusiasm, and critical insight he has generously offered over the past few years.

Finally, I am deeply thankful for the encouragement, love and support of friends and family during graduate school. My father, Dr. David Vesely and my late mother, Cloatta Farrell Vesely, have been and continue to be sources of inspiration. I am also fortunate to have many loving and supportive siblings, including my sisters, Susanna Vesely Renzy and Catherine Vesely Backus, brothers, Brian and Jonathan Vesely, brothers-in-law, Ryan Renzy and Brett Backus, and sister-in-law, Tiffany Crawford who

have all provided encouragement. Lastly, I would like express tremendous gratitude to my classmate, best friend, and fiancée, Shuba Srivatsan, for unfaltering love, encouragement, and dedication throughout graduate school.

Throughout my graduate education I have been supported by a Medical Scientist Training Program (MSTP) training grant, an Immunology Program training grant from the National Institute of Health (NIH), and a pre-doctoral fellowship from the Cancer Research Institute.

ABSTRACT OF THE DISSERTATION

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Professor Robert D. Schreiber, Chairperson

Accumulated data from animal models and human cancer patients strongly support the concept that immunity cannot only function as an extrinsic tumor suppressor, but also shape tumor immunogenicity. These observations led to the development of the cancer immunoediting hypothesis that stresses the dual host-protective and tumor-sculpting actions of immunity on developing cancers. We previously demonstrated important roles for lymphocytes and type I (IFN- α/β) and type II (IFN- γ) interferons in cancer immunoediting. In the present work, we confirmed the role of IFN- γ in sculpting tumor immunogenicity and provide evidence that antigens expressed by tumors drive the destructive or sculpting actions of immunity on cancers.

Initial studies confirmed the finding that IFN- γ is a critical mediator of cancer immunoediting. Wild type mice treated with antibodies that neutralize IFN- γ developed more sarcomas than control mice. Furthermore, a subset of sarcomas generated in IFN- γ neutralized mice spontaneously reject when transplanted into wild type mice. Finally,

these unedited tumors had differential requirements for IFN- γ responsiveness at the level of the host and the tumor to mediate tumor rejection.

Although many immune components that participate in cancer immunoediting are known (e.g. IFN- γ), its underlying mechanisms remain poorly defined. We used massively parallel sequencing to characterize the expressed mutations in a highly immunogenic sarcoma, d42m1, and identified mutant spectrin- β 2 as the major rejection antigen. Moreover, we demonstrate that editing of d42m1 tumor cells occurs via a T cell-dependent immunoselection process that promotes outgrowth of variants lacking mutant spectrin- β 2. Thus, the strongly immunogenic characteristic of an unedited tumor can be ascribed to expression of a highly antigenic mutant protein.

Subsequent studies established that antigen loss variants of d42m1 and edited sarcomas from wild type mice exhibit residual immunogenicity and respond to checkpoint blockade immunotherapy (anti-CTLA-4). Exome sequencing of these tumors has laid the groundwork for the eventual identification of the antigens targeted for destruction by this form of cancer immunotherapy. Taken together, these studies demonstrate that antigens drive the cancer immunoediting process and point to the future potential that cancer genome sequencing may have on the fields of tumor immunology and cancer immunotherapy.

CHAPTER 1

Introduction to Cancer Immunoediting

AN INTRODUCTION TO CANCER AND GENERAL MECHANISMS OF CANCER SUPPRESSION

The fundamental mechanisms of cellular division and DNA replication carry the inherent danger that the replication machinery will inevitably make mistakes, which could compromise the integrity of the genome and potentially results in cancer formation. Extensive research over the last half-century has revealed cancer to be a genetic disease that arises by an evolutionary process where somatic cells acquire multiple mutations overwhelming the barriers that normally restrain their uncontrolled expansion. The devastation wreaked by cancer cells can be lethal, but fortunately, a number of intrinsic and extrinsic tumor suppressor mechanisms exist to prevent their development.

A variety of intrinsic tumor-suppressor mechanisms attempt to repair genetic mutations and will trigger senescence or apoptosis should repairs fail and cellular proliferation become aberrant. Cellular senescence, a state characterized by permanent cell-cycle arrest with specific changes in morphology and gene expression that distinguish it from quiescence (reversible cell-cycle arrest), is induced by a number of cellular proteins (e.g., p53) that sense genomic disturbances caused by mutagenic insults (1). In addition, cellular senescence is also triggered by activated oncogenes and it is now becoming more evident that escape from oncogene-induced senescence is a prerequisite for cellular transformation such that cancer cells must acquire cooperating lesions that uncouple mitogenic Ras signaling from senescence to proliferate indefinitely (2). Other intrinsic tumor suppressor mechanisms, including p53, sense the activity of oncogenes and initiate the programmed cell-death machinery. In response to cellular stress, injury

or lack of survival signals, alterations in mitochondria integrity results in the release of pro-apoptotic effectors that trigger cell death by terminal activation of executioner caspases (3). In contrast, a second cell-death pathway is activated through ligation of cell-surface death receptors such as tumor necrosis factor receptor (TNFR), tumor necrosis factor apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2, DR5), and Fas/CD95 (4) with their corresponding ligands of the TNF superfamily to induce the formation of a signaling complex that activates the apical caspase 8 to initiate apoptosis. Additionally, increasing attention is being placed on alternative cell death pathways such as necrosis, autophagy and mitotic catastrophe that may halt the transformation process (3). In general terms, both senescence and apoptosis prevent the acquired capability of cells to proliferate without environmental cues and act as a potent barrier to the further development of any pre-neoplastic cell. These cell-intrinsic prerequisite steps for the transformation of normal cells into cancer cells were graphically illustrated and included along with sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis by Hanahan and Weinberg in their landmark review “The Hallmarks of Cancer” (5).

Since this famous review, at least three general extrinsic tumor suppressor mechanisms have been identified by which cells and their adjacent tissues 'sense' the presence of cancerous cells. All of these, to some extent, can be included under the umbrella of mechanisms that prevent cancer cells from invading and spreading to other tissues in the host. The first rests upon the mandatory dependency of cells for specific trophic signals in the microenvironment that quell their innate suicidal tendencies such as the epithelial cell – extracellular matrix association that when disrupted results in cell

death (6). A second appears to involve key links between cell polarity genes that control cellular junctions and proliferation, preventing cell cycle progression in the face of dysregulated junctional complexes (7). A third extrinsic tumor suppressor mechanism involves the limitation of transformation or tumor cell growth by effector leukocytes of the immune system.

The immune system has three primary roles in the prevention of tumors. First, it can protect the host from virus-induced tumors by eliminating or suppressing viral infections. Second, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumorigenesis. Finally, the immune system can specifically identify and eliminate tumor cells in certain tissues on the basis of their expression of tumor-specific antigens. This third process, referred to as cancer immunosurveillance, occurs when the immune system identifies transformed cells that have escaped cell-intrinsic tumor suppressor mechanisms and eliminates them before they can establish malignancy. These effector immune cells employ extremely diverse mechanisms to control tumor targets including the induction of tumor cell death by mitochondrial and cell death receptor pathways and thus, evasion of immunosurveillance is acknowledged to be an additional hallmark of cancer (8-11). However, the immune system not only acts as an extrinsic tumor suppressor, but paradoxically, also promotes cancer outgrowth. Together, the dual host-protective and tumor-promoting actions of immunity are referred to as cancer immunoediting.

A MODERN HISTORY OF CANCER IMMUNOSURVEILLANCE AND CANCER IMMUNOEDITING

The idea that the immune system, which so effectively protects the host from microbial pathogens, might also recognize and destroy tumor cells was conceived 50-100 years ago (12-14). For over a century, the concept of cancer immunosurveillance has been wrought with controversy (reviewed in detail in (8)) and by the early 1990s, little attention was paid to the idea that natural immunity could eliminate tumors *de novo*. However, interest in this aspect of tumor immunology was rekindled in the mid-1990s by the observations that transplanted tumors grew more robustly in mice treated with neutralizing monoclonal antibodies specific for interferon- γ (IFN- γ) (15) and that immunodeficient mice which lacked either IFN- γ responsiveness (IFNGR1, a component of the IFN- γ receptor) or an intact T cell compartment were more susceptible to 3'-methylcholanthrene (MCA)-induced sarcoma formation (16-18).

In the last decade, work from many laboratories including our own have validated the concept of cancer immunosurveillance, demonstrating, unequivocally, that the immune system can indeed protect mice from outgrowth of many different types of primary and transplantable tumors (15, 16, 19-25). An important study in 2001 provided evidence that the immune system not only controlled tumor quantity but also tumor quality (i.e., immunogenicity) (19). Immunodeficient mice lacking either IFN- γ responsiveness or recombination activating gene-2 (RAG2) (the latter fail to generate T, B, and natural killer T lymphocytes) develop more spontaneous neoplasia upon aging and are more susceptible to MCA carcinogen-induced sarcomas compared to wild-type mice.

In addition, a significant portion (40%) of MCA sarcomas derived from immunodeficient *Rag2^{-/-}* mice were spontaneously rejected when transplanted into naïve syngeneic WT mice, while all MCA sarcomas derived from immunocompetent WT mice grew progressively when transplanted into naïve syngeneic WT hosts (19). Thus, tumors formed in the absence of an intact immune system are, as a group, more immunogenic than tumors that arise in immunocompetent hosts. These results show that the immune system not only protects the host against tumor formation, but also “edits” tumor immunogenicity. These new data prompted a refinement of the cancer immunosurveillance concept and led to the formulation of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumor-sculpting actions of immunity on developing tumors.

We now view cancer immunoediting as a dynamic process comprised of three distinct phases: elimination, equilibrium and escape (8, 26-32). Elimination is a modernized view of cancer immunosurveillance where molecules and cells of both innate and adaptive immunity work together to detect the presence of a developing tumor and destroy it long before it becomes clinically apparent. In some instances, where tumor cell destruction goes to completion, the elimination phase represents an endpoint of cancer immunoediting. However, tumor cell variants may sometimes not be completely eliminated but rather enter into an equilibrium phase where the immune system controls net tumor cell outgrowth. In this equilibrium phase, tumor cells can become functionally dormant and remain clinically unapparent for the life of the host. Thus, equilibrium also represents a potential second stable endpoint of cancer immunoediting. Finally, either as a result of changes occurring (a) in the tumor cell population due to an active

immunoediting process or (b) in the host immune system, resulting from increases in cancer-induced immunosuppression or immune system breakdown due to the natural aging process, the functional dormancy of the tumor cell population may be broken, leading to progression of these cells into the escape phase, where they begin to grow in an immunologically unrestricted manner and emerge as clinically apparent disease. The concept of cancer immunoediting is thus, a comprehensive interpretation of previous and current clinical and experimental data, which integrates the immune system's capacity to both protect the host from cancer and promote cancer outgrowth through a multitude of mechanisms. The observations that have led to the concept of cancer immunoediting are reviewed here, with a particular focus on experimental data from various mouse models of cancer and clinical data from human cancer patients.

THE ELIMINATION PHASE: CANCER IMMUNOSURVEILLANCE

Immune-mediated Cancer Elimination in Mice

In the first phase of the cancer immunoediting process, the elimination phase, immune cells locate, recognize and destroy nascent transformed cells and prevent the development of malignancy. This process has never been visualized *in vivo*, but rather has been inferred from the earlier onset or greater penetrance of neoplasia in mice defective for certain immune cell subsets, recognition molecules, effector pathways or cytokines. Predominantly through the use of gene-targeted mice or by employing neutralizing monoclonal antibodies (mAbs) in wild-type mice, this approach has demonstrated that a number of immune effector cells and pathways are important for the

suppression of tumor development. For the purposes of this introduction we will not discuss a large literature where such mice have been challenged by transplanting a bolus of tumor cells derived from wild-type (WT) mice, since these tumor cells originated by escaping host immunity and therefore have already undergone cancer immunoediting. There are three basic mouse models of cancer that are relevant to the discussion of cancer immunoediting that illustrate the important role of immunity in eliminating developing tumors: 1) carcinogen-induced tumors; 2) spontaneous tumors that arise upon aging; and 3) tumor development in mice genetically predisposed to cancer.

Carcinogen-induced Tumors in Immunodeficient Mice

Historically, the concepts of cancer immunosurveillance and immunoediting have predominantly been demonstrated by exposing WT and immunodeficient mice to carcinogens and comparing their relative tumor incidences. The advantages of regulating tumor penetrance, tissue involvement and location in carcinogen-induced tumor models is one reason why these models are widely employed by researchers and in some cases they represent good mouse models of human cancer (e.g., asbestosis). The two most commonly employed carcinogen-induced tumor models are sarcomas induced using 3'-methylcholanthrene (MCA) and skin papillomas induced by a combination of 7,12-dimethylbenz[a]-anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA). To date, a number of mice with defined immunodeficiencies have been tested for their susceptibility to carcinogens (31).

Cells of both the innate and adaptive immune system have been shown to be critical for the elimination (i.e., cancer immunosurveillance) of primary MCA-induced sarcomas.

Lymphocyte-deficient *Rag1*^{-/-}, *Rag2*^{-/-}, severe combined immunodeficient (SCID) and nude mice all display an increased susceptibility to tumor induction after MCA exposure (17-19, 33). Interestingly, 40% of tumors derived from *Rag2*^{-/-} mice are rejected when transplanted into WT recipients, but grow progressively in either *Rag2*^{-/-} hosts or WT hosts depleted of CD4⁺ and CD8⁺ T cells, whereas tumors derived from WT mice grow readily when transplanted into either WT or *Rag2*^{-/-} hosts. These observations demonstrate that carcinogen-induced sarcomas derived from immunodeficient mice are more immunogenic than those arising in mice with a functional immune system and formed the basis for the cancer immunoediting concept (19). Subsequent studies found that mice deficient for either $\alpha\beta$ or $\gamma\delta$ T cells display increased susceptibility to tumor induction, indicating that both lymphocyte populations are important in suppressing MCA-induced tumors (23, 34). In addition, the innate-like lymphocytes are also critical players in eliminating transformed cells. For example, mice lacking CD1d-restricted T cells (*Cd1d*^{-/-}) are more susceptible to MCA-induced sarcomas (35) suggesting that these cells, which bridge the innate and adaptive arms of the immune system, also have a role in suppressing MCA-induced sarcomas. Furthermore, mice lacking the J α 18 T cell receptor (TCR) component are unable to generate the semi-invariant V α 14-J α 18-containing TCR expressed by natural killer T (NKT) cells, resulting in the absence of NKT cells, and rendering these mice more susceptible to MCA-sarcoma induction (36). Consistent with a role for the innate immune cells in cancer immunosurveillance, mice chronically depleted of NK cells displayed increased tumor incidence (37). One striking study revealed that CD8 α ⁺ dendritic cells (DCs) of the innate immune compartment are absolutely required for antitumor immunity and mice lacking these cells (*Batf3*^{-/-})

displayed similar antitumor deficiencies as *Rag2*^{-/-} mice (38). This may be explained in part, due the role of CD8 α ⁺ DCs in cross-presenting antigens to lymphocytes. Even eosinophils, whose role is more clearly defined in host-defense against helminths, can protect the host from tumor development. Mice deficient in eosinophils (more specifically eotaxin-, CCL11- and/or IL-5-deficient or *AdblGATA*) were more susceptible to MCA-induced sarcoma formation than WT mice in both C57BL/6 and BALB/c backgrounds (39). Remarkably, mice transgenic for IL-5 have greater circulating numbers of eosinophils and were more resistant to MCA-sarcomas compared to WT mice, strongly suggesting an immunosurveillance role for these innate immune cells (39).

A number of mice deficient for specific immune effector molecules and recognition pathways have also been examined in the context of MCA-induced tumor susceptibility, including mice lacking perforin (40), IFN- γ (40), IFNGR1 (16, 19), IFNAR1 or IFNAR2 (components of the type I IFN receptor) (24, 41, 42), TRAIL (43, 44), IL-12 (45), TNF- α (42), and (DNAX accessory molecule-1) DNAM-1 (46). Each of these mouse strains demonstrated enhanced susceptibility to sarcoma induction after MCA treatment, suggesting that interferons and cytotoxic lymphocytes suppress tumor initiation *in vivo*. Although WT mice treated with blocking antibodies specific for NKG2D (an activating receptor expressed by CD8⁺ T cells, $\gamma\delta$ T cells and NK cells) was reported to increase the incidence of MCA-induced sarcomas in two different mouse strains (47), C57BL/6 NKG2D-deficient mice had comparable numbers of MCA-induced sarcomas to WT mice (48). In addition, although the rate of MCA-induced tumor formation was similar in the presence or absence of the NK cell natural cytotoxicity receptor NKp46, the expression of its unknown ligands was NKp46-dependent, suggesting some level of immunoediting

by cells expressing this receptor (49).

Interferons can contribute to antitumor effects in a number of ways. IFN- γ can exert direct effects on tumor cells (16) and a major effect of IFN- γ on these cells is to enhance MHC class I expression, rendering them better targets for tumor-specific CD8⁺ T cells (19, 50). In addition, IFN- γ signaling in host immune cells (51) and host stroma cells (52) also plays an important role in the elimination of tumor cells, indicating that IFN- γ 's effects in multiple cellular compartments generates antitumor immunity. Others have proposed that IFN- γ contributes to an inflammatory foreign body reaction that results in the encapsulation of injected MCA, limiting its spread and thereby reducing its carcinogenic effects (53). However, this mechanism does not explain the findings of IFN- γ preventing the formation of lymphomas induced by the soluble carcinogen N-methyl-N-nitrosourea (54), where encapsulation of the carcinogen is not possible. Furthermore, a recent report demonstrated that MCA exposure induced more squamous cell carcinomas (SCC) in the skin of IFN- γ -deficient mice than WT controls (55), indicating that MCA delivery in a different tissue type than the previous subcutaneous injections also supports a role for IFN- γ in mediating cancer immunosurveillance. In contrast to the antitumor effects of IFN- γ occurring at both the level of the tumor and the host, the antitumor effects of type I IFNs (IFN- α/β) are mediated only at the level of the host's hematopoietic system (24, 25, 56). Specifically, CD8 α ⁺ DCs of the innate immune compartment are critical responders of type I IFNs such that selective deletion of IFNAR1 in CD8 α ⁺ DCs results in tumor outgrowth of highly immunogenic sarcomas (25). These results suggest that the ability of type I IFNs to induce antitumor activity in immune cells might be the critical mode of action for this cytokine family and that IFN- γ

and IFN- α/β have distinct, potentially non-overlapping mechanisms of action in antitumor immunity.

As will be discussed later in this introduction, recent studies reveal that initiation of MCA-induced sarcomas requires an inflammatory event. Along similar lines, skin carcinomas induced by the topical application of DMBA (tumor initiator) followed by repetitive doses of TPA (tumor promoter) are known to require inflammatory components for tumor initiation and promotion. For example, the induction of DMBA/TPA skin carcinomas is MyD88- (42), mitogen-activated protein kinase-activated protein kinase-2 (MK2)- (57), TNF- α - (58), receptor for advanced glycation end-products (RAGE)- (59), and IDO-dependent (60). Lesions progress from benign papillomas to metastatic SCC, and both the number of lesions and extent of tumor progression is dependent on the mouse strain. Despite an inflammatory component, DMBA/TPA induced tumors are also detected and destroyed by effector cells and molecules of innate and adaptive immunity. For example, $\gamma\delta$ T cells and CD8⁺ T cells confer protection from DMBA/TPA-induced papillomas (23, 61). In contrast, CD4⁺ T cells promote tumor progression, implying opposite roles for $\alpha\beta$ T cell subsets in the protection or promotion of DMBA/TPA skin carcinogenesis (61). One mechanism by which $\gamma\delta$ T cells and activated CD8⁺ T cells might regulate tumor development is through recognition by NKG2D of the stress ligand retinoic acid early transcript 1 (RAE1) that is induced in the skin after DMBA/TPA treatment and has been found to be upregulated in transformed cells by the DNA damage pathway (23, 62). NKG2D-expressing dendritic epidermal $\gamma\delta$ T cells can kill RAE1-expressing targets in vitro (23), but in transgenic mice expressing RAE1 in the skin, NKG2D expression is down modulated on

lymphocytes and consequently these mice are more susceptible to papilloma induction than WT mice (63). A follow up study using inducible RAE1 transgenic mice have provided further insight into the previous observation, where acute upregulation of NKG2D ligands triggered a swift reorganization of the local skin immune compartment, resulting in local $V\gamma 5V\delta 1^+$ T cells limiting carcinogenesis, but unexpectedly Langerhans cells promoted DMBA/TPA carcinogenesis (64). Another innate recognition receptor, DNAM-1 also protects tumor formation as *Dnam1*^{-/-} mice develop more papillomas than their WT counterparts (46).

In addition to cellular subsets and recognition receptors, effector molecules and cytokines also have a critical function in controlling DMBA/TPA-induced skin tumors. For example, although DMBA/TPA-treated TRAIL-R-deficient mice did not show an increase in the number of benign papillomas or the rate of progression to squamous cell carcinoma when compared to WT mice, metastasis to lymph nodes was significantly enhanced, indicating a role for TRAIL-R specifically in the suppression of metastasis (65). One cytokine, IL-12, has been shown to protect mice against DMBA/TPA-induced tumors, where mice that lack functional IL-12 (*Il12a*^{-/-}) develop increased numbers of papillomas compared to WT mice (66, 67). Interestingly, mice that lack functional IL-23 (*Il23a*^{-/-}) are resistant to tumor development (66, 67), however, the mechanism by which IL-23 suppresses innate immunity and promotes tumor growth requires further clarification since it was unexpectedly IL-17A-independent (67). Nevertheless, IL-17A-deficient mice also develop fewer skin papillomas than WT mice after DMBA/TPA exposure, suggesting a tumor-promoting role for this cytokine (67). One peculiarity of the DMBA/TPA model is that a loss of IFN- γ or IFNGR1 unexpectedly results in reduced

tumorigenesis, hence playing an opposite role than in the MCA tumor model (68). These observations demonstrate the pleiotropic effects that a single immune cell or molecule can have during carcinogenesis and stress the importance of a multimodal analysis. The interplay between antitumor immunity and cancer-promoting inflammation suggested by the above studies is discussed at greater length below.

In addition to the demonstration of cancer immunosurveillance by immune effector cells and molecules against tumors induced by chemical carcinogens, tumors induced by physical carcinogens such as ultraviolet (UV) radiation also seem to be controlled by the immune system (69). Interestingly, UV-induced immune suppression is an important factor in the development of UV-induced tumors, and these tumors are often immunogenic when transplanted into naïve hosts but grow in immunosuppressed or CD8⁺ T cell-depleted (70). These data show that immunoeediting can also be observed in the UV radiation tumor model as well as the MCA chemical carcinogen model.

Spontaneous Tumor Development In Immunodeficient Mice

An elegant approach to examine the role of the immune system in controlling tumor development is to simply remove specific components of the murine immune system and monitor mice as they age for the development of spontaneous tumors. Mice have long telomeres and display a very low incidence of spontaneous tumor development. For example, we observe incidences of cancer in a variety of inbred WT mouse strains that range from only 0-20% over a two-year period. While many immunodeficient mice also do not develop cancers over a two-year observation period, ageing studies have clearly demonstrated a critical role for certain cytotoxic pathways,

lymphocyte cellular subsets, and cytokines in the prevention of spontaneous tumor development. One striking example is the penetrance of immunogenic B cell lymphomas in aged mice (>1 yr) on either C57BL/6 or BALB/c backgrounds that increases from 0-6% in wild-type mice to 40-60% in perforin-deficient mice (20, 22). Mice lacking this key T cell and NK cell cytotoxic effector pathway develop an even greater prevalence of B cell lymphomas with an earlier onset when they additionally lack the MHC class I accessory molecule β 2-microglobulin (β 2m) or IFN- γ compared with perforin alone (22, 71). The absence of other lymphocyte cytotoxic pathways such as TRAIL or FasL also increased the susceptibility of mice to spontaneous lymphomas (72, 73). Collectively, these data provide very strong evidence that critical cytotoxic molecules in lymphocytes protect the host from spontaneous tumor development. Intriguingly, human patients with specific mutations in perforin that develop adult onset familial hemophagocytic lymphohistocytosis (FHL) have recently been identified to also develop leukemia and lymphoma, suggesting the possibility that perforin may protect against hematological malignancies in humans (74).

Ageing experiments have also been performed in mice that lack one or more lymphocyte subsets. Although, early studies in athymic nude mice did not document an increase in spontaneous tumor development (75), one later study suggested that germ-free nude mice did develop a low frequency of B cell lymphoma compared with heterozygote littermates (76). Unlike other genetic models of immunodeficiency (e.g., SCID mice), the absence of RAG-2 does not affect DNA damage repair pathways in non-immune cells undergoing transformation. *Helicobacter*-negative 129/Sv *Rag2*^{-/-} mice aged in a specific pathogen-free mouse facility and maintained on broad-spectrum antibiotics developed

significantly more spontaneous epithelial tumors (35% gastrointestinal and 15% lung of all mice analyzed at 15–16 months of age) than their WT counterparts (19). Consistent with these observations, 129/Sv RAG-2-deficient mice that also lack STAT1 (an important mediator of signaling induced by both type I and type II IFNs) showed an earlier onset and broader spectrum of malignancy, including the development of colon and mammary adenocarcinomas (19). The role of specific lymphocyte subsets in the prevention spontaneous tumor development has yet to be reported in mice lacking NKT cells, $\gamma\delta^+$ T cells or NK cells, but C57BL/6 $\beta 2m$ -deficient mice that lack NKT cells and many $CD8^+$ T cells did not have elevated tumor formation upon aging (71), suggesting that distinct lymphocyte populations may play distinct roles, if any, during cancer immunosurveillance of spontaneous tumors.

Similar to chemical carcinogen models of tumor induction, cytokines are critical for the activation of immune effector mechanisms that limit spontaneous tumor development. In one study, a small proportion (<15%) of BALB/c IFN- γ deficient mice developed lung adenocarcinomas whereas almost half the IFN- γ -deficient mice on a C57BL/6 background developed a spectrum of various T cell lymphomas, indicating strain-specific differences in the contribution of IFN- γ to prevent spontaneous tumors from occurring (22). In addition, C57BL/6 mice deficient for both perforin and IFN- γ develop more B cell lymphomas with earlier onset than *Pfp*^{-/-} mice, suggesting that in the absence of perforin, IFN- γ can play a role in controlling lymphomas (22). Finally, female mice deficient for the IFN- γ -inducible immunoproteasome subunit LMP2 develop spontaneous uterine neoplasms with a disease prevalence of approximately 36% by 12

months of age (77). This observation suggests that IFN- γ inducible proteasome function may be essential for MHC class I-mediated tumor rejection.

In addition to the role of cytokines in cancer immunosurveillance of spontaneous tumors, a possible link between tumor immunity and autoimmune or infection-induced inflammation has been raised by several studies. For example, 50% of mice lacking the $\beta 2$ subunit of the IL-12 receptor (IL-12R $\beta 2$) develop plasmacytomas or lung carcinoma concurrently with immune complex mesangial glomerulonephritis upon aging (78). It is presently unclear why IL-12p40-deficient mice on the same genetic background as the IL-12R $\beta 2$ -deficient mice do not display either autoimmunity or spontaneous tumor development (22). Furthermore, mice deficient for both IFN- γ and GM-CSF have been found to develop spontaneous tumors in a variety of tissues with age and, in this case, tumor development is associated with acute or chronic inflammatory lesions (79).

Maintaining *Gmcsf*^{-/-}*Ifng*^{-/-} mice on a regimen of antibiotics delays tumor onset suggesting that in addition to potentially eliminating tumor cells directly, the immune system might also prevent tumor growth by the timely elimination of infections, thereby limiting inflammation, which is known to facilitate tumor development (80). However, this finding cannot be generalized to all immunodeficient mice that develop spontaneous malignancies since heightened tumor incidence was observed in *Rag2*^{-/-} and *Rag2*^{-/-}*Stat1*^{-/-} mice maintained on the same antibiotics regimen (as mentioned above).

Genetic Tumor Models in Immunodeficient Mice

Data supporting the ability of the immune system to suppress tumor development in genetic models of mouse cancer are accumulating rapidly. Mice heterozygous for the

tumor suppressor p53 (*Trp53*^{+/-}) are genetically predisposed to tumor development, but in the additional absence of IFNGR1 (16), perforin (20), TRAIL (72), or NKT cells (35), more aggressive tumors develop with an earlier onset, providing very strong evidence that these immune components participate in the elimination of nascent transformed cells. More recently, a key role for perforin in immunosurveillance of B cell malignancies has been validated in three different genetic models of B cell malignancies in C57BL/6 mice. Similar to *Pfp*^{-/-}*Trp53*^{+/-} mice, perforin-deficient mice also heterozygous the tumor-suppressor Mlh1 developed more B cell lymphomas with faster kinetics than mice lacking perforin alone (81). Additionally, perforin protects against the development of oncogene-driven tumors on a transgenic background, including v-abl-driven plasmacytomas, and bcl2-driven follicular lymphomas (81).

Other transgenic mice that express oncogenes under the control of tissue-specific promoters have also revealed immune-mediated protection from tumor formation. In one example, IFN- γ suppresses tumor development in mice expressing the human T cell leukemia virus (HTLV) type 1-derived oncogene Tax under the control of a granzyme B promoter (HTLV-Tax transgenic mice) (82). Loss of a single TRAIL-R allele on the lymphoma-prone E μ -myc genetic background significantly reduced median lymphoma-free survival corroborating an extrinsic tumor suppressor role for this cell death pathway (83). The conclusion that NKG2D plays a critical role in cancer immunosurveillance is further supported by the fact that mice defective in NKG2D are more susceptible to E μ -myc driven pre-B cell lymphomas (48). More recently, in a study using transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, investigators assessed whether NKG2D controlled the growth of spontaneous oncogene-driven prostate cancer. NKG2D-

deficient mice developed more aggressive tumors than WT mice and interestingly, these aggressive tumors arising in NKG2D-deficient mice expressed higher amounts of NKG2D ligands than did similar tumors in wild-type mice, suggesting an NKG2D-dependent immunoediting mechanism (48). Also in the same prostate cancer model, the lack of NKT cells in TRAMP *Ja18*^{-/-} mice correlated with more aggressive adenocarcinoma development (84). Despite the presence of a traceable tumor antigen-specific T cell response in these mice, no evidence was found to support a correlation between the presence of NKT cells and the efficacy of CTL responses in this setting. Nevertheless, this study extends the list of spontaneously arising tumors in mice in which NKT cells are critical for natural immune surveillance.

In summary, various cell types including $\alpha\beta$ T cells, $\gamma\delta$ T cells, NKT cells and NK cells have all been implicated in the processes of elimination and immunoediting, along with a number of effector molecules, including perforin and TRAIL, as well as the cytokines IFN- γ , type I IFNs, and IL-12. More is known about the physiologically relevant targets of IFN- γ 's actions than the other effector molecules or cells during cancer immunosurveillance. Both host and tumor cells are important targets of IFN- γ during the development of protective antitumor immune responses and the data substantiating this conclusion has already been extensively reviewed (8, 26, 27). It is important to note that the effector cells and cytokines thought to be involved in elimination and immunoediting differ among models, demonstrating that the success of immunoediting and the evidence of its occurrence varies among experimental systems. Indeed, there are models in which the immune system seems to have little influence on the rate of tumor onset or progression (85) and models in which the immune system has a distinct protective role,

such as the carcinogen-induced and genetically-predisposed tumor models outlined above. The level of immune regulation and tolerance (a state of non-responsiveness to specific antigens) imparted by the tumors in each of these models might explain, at least in part, why in some cases the effect of subtracting immune elements on tumor progression is less overt. Blocking these tolerance mechanisms might reveal the true mechanisms of tumor suppressor immunity. Moreover, recent studies have lent great support for the cancer immunoediting hypothesis by validating the existence of the equilibrium phase in multiple models and demonstrating that the immune sculpting actions on tumor immunogenicity occur during this phase.

THE EQUILIBRIUM PHASE: IMMUNE-MEDIATED TUMOR DORMANCY

Historically, tumor dormancy is the term used to describe latent tumors present in patients for decades that may eventually recur as local lesions or form distant metastases (86). Tumors in the equilibrium phase are a subset of dormant tumors that are specifically controlled by components of the immune system. In the equilibrium phase, the host immune system and tumor cells enter a dynamic balance, wherein powerful antitumor immunity contains, but does not fully eradicate, a heterogeneous population of tumor cells, some of which have acquired means of evading immune-mediated recognition and destruction. The equilibrium phase was originally hypothesized to exist in order to explain the long latency period from the initial transformation event to the escape phase and emergence of malignant disease. In this manner, equilibrium may be the longest of the immunoediting phases where sculpting forces of immunity select for the tumor cells

acquiring the most immunoevasive mutations, potentially leading to clinically detectable disease.

Using a low-dose regimen of the carcinogen MCA, we reported the first experimental demonstration that immunity maintains primary occult cancer lesions in an equilibrium state (87). Treatment of naïve WT mice with low doses of MCA led to overt tumors in only a low proportion of mice. When the remaining carcinogen-treated mice were rendered immunodeficient via depletion of CD4⁺ and CD8⁺ cells and/or neutralization of IFN- γ , sarcomas rapidly grew out at the original carcinogen injection site in approximately 50% of the group. Strikingly, tumor outgrowth was not observed to any significant extent (<10%) in similar MCA treated WT or RAG2-deficient mice injected weekly with control mAb starting at day 200. Subsequent analyses revealed that mAbs that depleted cells of adaptive immunity (such as CD4⁺ and CD8⁺ T cells) or blocked cytokines that promote adaptive immunity (such as IFN- γ and IL-12) caused dormant tumor cells to grow out. In contrast, mAbs that deplete NK cells (anti-NK1.1), block NK cell recognition (anti-NKG2D) or inhibit NK cell effector function (anti-TRAIL) failed to cause the emergence of progressively growing tumors (87). These results support the conclusion that adaptive immunity, but not innate immunity, is responsible for maintaining the equilibrium phase. They also help to mechanistically distinguish this phase from elimination, where both innate and adaptive immunity are required. Histological examination of occult tumors revealed the presence of atypical fibroblasts surrounded by a dense infiltration of leukocytes. These atypical fibroblasts were truly transformed since they formed progressively growing tumors when transplanted into immunodeficient *Rag2*^{-/-} mice. Moreover, occult tumors controlled by

immunity displayed fewer Ki67⁺ atypical fibroblasts and more terminal deoxynucleotidyl transferase dUTP end nick labeling (TUNEL) staining cells than progressively growing sarcomas. The visualization of fewer proliferating tumor cells accompanied by more cells undergoing apoptosis is supportive of an active immune response controlling equilibrium tumors. Occult tumors arising after immunodepletion were, on the whole, highly immunogenic with 40% of the cell lines rejecting after transplantation into WT mice. In contrast, the rare spontaneous tumors that grew out of mice treated with control mAbs were poorly immunogenic and grew progressively when transplanted into WT recipients. Thus, tumor cells held in equilibrium by adaptive immunity remained highly immunogenic and displayed an unedited phenotype while dormant sarcoma cells that spontaneously escaped immune control to become actively growing tumors displayed reduced immunogenicity, indicating that they had undergone editing.

In hindsight, these findings explain previously reported models of immune-mediated tumor dormancy. In the past, most experimental models of tumor dormancy relied heavily on a vaccination-and-challenge strategy with tumor cell lines to induce latent tumor cells. For example, in the BALB/c B cell leukemia/lymphoma 1 (BCL₁) model of tumor dormancy, mice were immunized with BCL₁-derived Ig to create an anti-idiotypic vaccine against the B cell Receptor (BCR) expressed by the lymphoma cells. Naïve, non-immunized mice injected with BCL₁ tumor cells succumbed to malignancy within 30 days. In contrast, mice initially immunized with BCL₁-derived Ig and subsequently challenged with BCL₁ tumor cells did not develop malignancy although tumor cells could be detected in the circulation of cancer-free mice hundreds of days after transplantation (88). Over an extended period of time, vaccinated mice challenged with

live BCL₁ tumor cells spontaneously developed malignancy, suggesting an escape from dormancy. Interestingly, when mice harboring dormant BCL₁ tumor cells were depleted of CD8⁺ T cells or IFN- γ using mAbs, the incidence and duration of dormancy were reduced, suggesting that the immune system plays an important role in controlling these dormant tumor cells (88).

Using a BCR-ABL mouse model of leukemia, dormancy was also achieved via a vaccination-and-challenge strategy. The longer the DA1-3b tumor cells remained dormant within the vaccinated host, the greater the expression of programmed cell death 1 ligand 1 (PD-L1) on the tumor cells, which acted to confer resistance to cytotoxic T lymphocyte (CTL)-mediated killing (89). Consistent with the concept of the equilibrium phase, DA1-3b tumor cells acquired advantageous changes over time such that those cells which remained dormant longer were more resistant to attack by CD8⁺ T cells.

Recently, two additional studies using different mouse models of cancer corroborated our findings for the existence of the equilibrium phase by additionally demonstrating that immunity can control primary carcinomas and metastases for extended periods of time. The first study involved a new mouse model of cancer immunosurveillance and equilibrium using ultraviolet B (UVB)-radiation to induce squamous cell carcinomas of the skin. Here, the authors used mice genetically deficient in E3 ligase Casitas B-lineage lymphoma b (Cbl-b), which is known to limit the effector functions of CTLs (90). Thus, mice lacking Cbl-b exposed to UVB-radiation developed fewer spontaneous squamous cell carcinomas compared to WT mice due to the enhanced antitumor activity of CD8⁺ T cells that lack the Cbl-b regulator. *Cblb*^{-/-} mice that failed to form carcinomas 400 days after UVB treatment were then divided into two

experimental groups. One group received mAbs that depleted CD8⁺ T cells, while the other group received control mAbs. Only 10 days after starting mAb treatment, nearly 50% of mice depleted of CD8⁺ T cells developed rapidly growing tumors whereas none of the mice receiving control mAb developed detectable tumors (90). It will be interesting to determine in the future whether WT CD8⁺ T cells can also maintain occult UVB-induced carcinomas in an equilibrium state.

A second study demonstrates that immunity can prevent the outgrowth of micrometastases for an extended period of time in an oncogene-driven model of melanoma. In this model, transgenic mice that express the human *RET* oncogene and a chimeric mouse/human MHC antigen (91) specifically in melanocytes were found to develop extensive disseminated metastases (92). Depletion of CD8⁺ T cells in RET.AAD mice significantly accelerated the outgrowth of metastatic lesions to visceral organs, indicating that immunity is one significant barrier disseminated tumor cells must overcome in order to establish metastatic disease (92). Interestingly, these CD8⁺ T cells did not seem to directly kill the tumor cells, but rather mediated cytostatic effects on the disseminated tumor cells. One likely mechanism underlying control of disseminated tumor cell outgrowth may be via IFN- γ produced by tumor antigen-specific T cells, which has been shown in other systems to inhibit cellular proliferation and curtail angiogenesis (93-95). For example, in a pre-clinical model of pancreatic cancer using RIP-Tag2 mice, the transfer of IFN- γ producing TNFR1⁺ CD4⁺ T cells specific for Tag prevented the progression of pancreatic islet cancer (95). In this study, transferred Tag-specific T cells arrested tumor cell proliferation and prevented angiogenesis, curtailing tumor growth and resulting in the inhibition of multistage carcinogenesis and induction of

a period of extended tumor dormancy. In the absence of either TNFR1 signaling or IFN- γ receptor signaling, the same T cells paradoxically promoted angiogenesis and multistage carcinogenesis. Currently, the adoptive transfer of cancer-reactive T cells into human cancer patients is an experimental therapy with promising results (96) and it will be interesting to see if these therapies can be optimized as a viable therapeutic endpoint to induce an equilibrium state in some patients.

THE ESCAPE PHASE: FAILURE OF CANCER IMMUNOSURVEILLANCE

While the processes of cancer elimination and equilibrium largely occur “behind the scenes”, a more dramatic result of cancer immunoediting can occur when tumors escape immune control, leading to the appearance of overt cancer. Thus, the escape phase represents the failure of the immune system to either eliminate or control transformed cells, allowing surviving tumor cell variants to grow in an immunologically unrestricted manner. Cancer cells undergoing stochastic genetic and epigenetic changes generate the critical modifications necessary to circumvent both innate and adaptive immunological defenses. Moreover, the immune system contributes to tumor progression by selecting more aggressive tumor variants, suppressing the antitumor immune response, or promoting tumor cell proliferation. The interaction between a heterogeneous population of cancer cells undergoing rapid genetic modifications and the constant immunological pressure exerted by immune cells allows for the Darwinian selection of the most fit tumor variants to survive and form overt cancer in immunocompetent hosts. Thus, nearly all human cancers and experimental cancer cell lines are those that have evaded

immunological control. The focus of this section on tumor escape is not to provide an exhaustive list of escape mechanisms that have been extensively reviewed elsewhere (8, 9, 26, 27, 29, 97) but rather to shape the framework of how tumor cells achieve immunological escape. Although, the mechanisms for tumor escape are varied, they can be categorized generally as cell-autonomous modifications at the level of the tumor cell that directly evade immune detection and destruction or modifications in immune cells effected by tumor cells to generate an immunosuppressive network.

Tumor Cell Modifications to Evade Immune Detection or Destruction

Tumor escape can result from changes that occur at the level of the tumor by directly inhibiting tumor recognition or cytolysis by immune effector cells. In some cases, immune evasion by tumors is absolute and the immune system has little impact on tumor progression, while in other cases tumor growth is delayed before the immune system is overwhelmed, leading to tumor progression. In its simplest form, tumor cells that express very weak antigens can evade detection due to the induction of central or peripheral tolerance. Central tolerance is a process whereby self-reactive T cells are eliminated or converted to a regulatory phenotype in the thymus (98). In this case, and in the absence of neoantigen expression, tumors may remain “invisible” to the adaptive immune system and are free to grow unhindered. Peripheral tolerance is an important process whereby T cells reactive with self-antigens not expressed in the thymus are deleted or rendered non-responsive in the periphery. In this case, some level of antitumor immune response may be initiated transiently before tolerance is induced leading to tumor progression (85, 99).

In addition to tolerance induction, tumor cells can acquire defects in antigen processing and presentation pathways that facilitate evasion from adaptive immune recognition. Specifically, loss of TAP1, MHC class I molecules, β 2-microglobulin, LMP2, LMP7 and the development of IFN- γ or IFN- α/β insensitivity by tumor cells prevents T cell-mediated elimination, resulting in tumor progression (28, 100-102). An extreme version of this escape process occurs when tumors lose the ability to respond to IFN- γ either through mutation or epigenetic silencing of genes encoding the IFN- γ receptor signaling components (IFNGR1, IFNGR2, JAK1, JAK2 and STAT1) (103). In this case, the affected tumor cells not only fail to upregulate MHC class I proteins but also are unable to produce the intracellular machinery that facilitate antigen processing and presentation (i.e., TAP1, TAP2 and components of the immunoproteasome). In addition, genomic instability within tumor cells may result in the loss of tumor-specific antigens creating antigen loss variants that are no longer detectable by antigen-specific CD8⁺ T cells. Similarly, tumors can become unrecognizable to cells of the innate immune system through loss of ligands for the NK cell effector molecule, NKG2D (104), or suppressing the production of proinflammatory danger signals to impair dendritic cell maturation (105). Thus, tumor cells may avoid recognition by adaptive or innate immune cells by multiple mechanisms.

Additionally, tumor cells that are unable to avoid immune cell detection may develop mechanisms to evade immune-mediated killing. Even when antigens continue to be expressed, tumors can evade effector lymphocytes by upregulating expression of anti-apoptotic molecules such as FLIP and BCL-XL (106, 107). Alternatively, resistance to lysis by immune cells can be acquired through expression by tumors of mutated inactive

forms of death receptors including the TRAIL receptor, DR5 (108), and Fas (109).

The above strategies of immune escape can be viewed as passive, involving the loss of recognition or reduced sensitivity to apoptosis. However, tumor cells can take a more active and direct role in subduing immunity through expression of immune-inhibitory ligands on their surface that inhibit the cytotoxic actions of immune cells after tumor cell recognition in a cell-contact mediated manner. The expression of B7-H1 (PD-L1) (110), HLA-G (111), and HLA-E (112) on the cell surface of tumor cells interacts with receptors on the cell surface of T cells to dampen the cytotoxic actions of T cells or induce apoptosis within the T cell itself. In addition, tumor cell expression of HLA-E or HLA-G can modify the actions of innate immune cells by inducing tolerance in antigen-presenting cells and inhibiting NK cell-mediated killing (111). The actions of tumor cells to impede the development of antitumor immune responses is not limited to changes that occur directly at the level of the tumor, but also result from the elaboration of cytokines and molecules that act at a distance to generate an extensive immunosuppressive network that facilitates tumor progression.

Generating an Immunosuppressive Tumor Microenvironment

The development of an immunosuppressive environment concomitantly with tumor development is evidenced by observations in which protective responses against transplantable tumors can be generated when immunotherapies are delivered prior to tumor challenge, but fail against established tumors (113). Importantly, failure of therapy against established tumors seems to be due to local immunosuppression in the tumor microenvironment since tumor-bearing mice can often respond normally to other antigens

(114). The development of an immunosuppressive state is achieved by tumor cells that inhibit the function of effector immune cells or recruit the efforts of regulatory immune cells to evade immunological elimination in a paracrine or endocrine manner.

Tumor cells secrete factors to directly inhibit the function of sentinel immune cells of both the innate and adaptive arms of immunity. For example, tumor cells can block T cell and NK cell function through secretion of soluble forms of ligands for effector molecules, as has been reported for shed ligands of NKG2D (115). In addition, antitumor immunity can be subverted at an early stage by tumor-derived factors that inhibit dendritic cell (DC) function. In response to danger signals and cellular stress, DCs are stimulated to mature, migrate, and carry tumor antigens to lymph nodes to alert the adaptive arm of immunity to the presence of transformed cells. To inhibit this initial immune priming event, tumor cells secrete sterol metabolites to suppress the expression of CCR7 on the cell surface of DCs, thereby disrupting DC migration to the lymph nodes (116). A recent study demonstrates that unknown tumor-derived factors induce the expression of scavenger receptor A on DCs, resulting in excessive uptake of extracellular lipids that reduces their capacity to process antigens (117). Furthermore, many tumors produce VEGF, which is critical for the establishment of one of the hallmarks of cancer development, angiogenesis, but also prevents endogenous DC function. Targeted monoclonal antibodies against VEGF improve DC function in vivo and improve the efficacy of cancer immunotherapies (118).

Simultaneous inhibition of multiple stages in the development of antitumor immunity can be achieved through the liberation of immunosuppressive cytokines by tumor cells. For example, TGF- β secretion by tumor cells leads to inhibition of DC

activation as well as direct inhibition of T cell and NK cell function (119). Similarly, IL-10 present within tumors can suppress DC function and skew T cell responses toward a type 2 immune response that is less effective against malignant cells (120). However, the role of IL-10 in tumor immunity remains somewhat obscure because it has also been shown to enhance immune destruction of tumors (121).

Other tumor-derived factors can be more selective in inhibiting particular components of immune responses but can still effectively suppress immunity. For example, production of galectin can impede T cell activity and survival, and blocking this factor can aid tumor rejection in mice (122). In addition to using cytokines and lectins to down-regulate immune responses, tumors can secrete enzymes that metabolize amino acids within the tumor microenvironment. Specifically, expression of indoleamine 2,3-dioxygenase (IDO) by tumor cells metabolizes tryptophan to generate kynurenines and inhibits CD8⁺ T cell proliferation and promotes CD4⁺ T cell apoptosis (123). Two potential mechanisms of immune inhibition include starvation, where depletion of this important amino acid weakens T cells, and metabolite cytotoxicity, where metabolic products of tryptophan degradation inhibit T cell function (124).

In addition to the mechanism described above, a variety of immunosuppressive regulatory leukocytes can suppress immune function leading to tumor escape. Regulatory T cells (Tregs), largely expressing CD4, CD25 and Foxp3 and have been demonstrated to inhibit CTL function in a number of ways including IL-10 and TGF- β production, CTLA-4 and PD-L1 expression and IL-2 consumption (125). This regulatory lymphocyte is the critical mediator of peripheral tolerance under physiological settings, but is often recruited to the tumor site where it suppresses antitumor immunity. Furthermore, TGF- β

production by tumor cells can induce effector T cells into regulatory T cells that now suppress other effector T cells infiltrating the tumor mass (126). Experimental tumor models that eliminate regulatory T cells, results in robust antitumor immune responses and the rejection of transplanted or primary tumors (127). In addition to Tregs, other regulatory lymphocyte populations can be found in subsets of natural killer T cells and B cells that inhibit effector responses against transformed cells (128, 129).

The production and elaboration of GM-CSF, IL-1 β , VEGF, and PGE₂ by tumors leads to expansion of myeloid-derived suppressor cells (MDSCs) and their accumulation within the tumor mass (130). MDSCs are a heterogeneous group of myeloid progenitor cells and immature myeloid cells that can inhibit lymphocyte function by a number of mechanisms including the production of immunosuppressive cytokines (TGF- β) (131), the depletion or sequestration of amino acids arginine or cysteine that are required for T cell function (132), the inhibition of T cell activation by TCR nitrosylation (133) and the induction of regulatory T cells (134). The multiplicity of mechanisms that inhibit lymphocytes in either an antigen-specific or antigen-nonspecific manner most likely reflects distinct cellular subsets within the MDSC heterogeneous population (135).

In addition to MDSCs, plasmacytoid dendritic cells (pDCs) are recruited to the tumor mass and become key players in the immunosuppressive network. Ovarian cancer cell products activate pDCs, which in turn, induce the expansion of IL-10 producing CD8⁺ regulatory T cells (136). A potentially novel subset of DCs, sometimes referred to as vascular leukocyte cells (VLCs) or Tie2⁺ monocytes, is recruited to the tumor bed by β -defensins and induce their endothelial-like specialization, where they in enhance vasculogenesis and suppress conventional DC function through the secretion of VEGF

and other pro-inflammatory cytokines (137). A recent study by Shields et al. identified lymphoid tissue inducer (LTi) cells that are recruited by CCL21-secreting melanomas and contribute to the development of an immunosuppressive tertiary lymphoid structure within the tumor mass that recruits MDSCs, regulatory T cells, and polarizes monocytes to M2 macrophages (138). Many tumors attract tumor-associated macrophages (TAMs) by IL-4 and IL-13. M2 macrophages can inhibit antitumor immunity through the production of TGF- β and IL-10, and can promote stromal development and angiogenesis through secretion of platelet-derived growth factor (PDGF) (139).

Together, these examples demonstrate that, in addition to central and peripheral tolerance, failure of antitumor immunity can be due to the development of an immunosuppressive microenvironment. Any one, or combination of several, of the above cellular and molecular mechanisms can contribute to suppression of tumor immunity. The balance between these inhibitory mechanisms and immune stimulating conditions determines whether or not tumors escape immune responses and the rate of tumor progression. In human cancer patients, immunosuppression of lymphocytes within the tumor microenvironment has also been widely observed for a variety of cancer types (140). In the next section we will discuss the evidence for cancer immunoediting in humans, with particular emphasis on the elimination and equilibrium phases.

EVIDENCE FOR CANCER IMMUNOEDITING IN HUMANS

The extensive studies discussed above clearly demonstrate that the immune system not only protects against tumor development, but also shapes tumor

immunogenicity in mouse models of cancer. The question therefore naturally arises whether cancer immunoediting occurs in humans. Humans are not clean models of immune deficiency such as those that exist in experimental mice that live in controlled environments. Nevertheless, compelling clinical data support the existence of cancer immunoediting in humans. Here, we will not discuss the mechanisms of tumor escape in humans since they greatly overlap with those observed in mice (discussed above) and have been extensively reviewed elsewhere (8, 9, 26, 27, 29, 97), but rather we will review data supporting an active immune response eliminating or controlling cancer in humans.

Acquired Immunodeficiency and Cancer Risk

While severely immunodeficient humans succumb to infections relatively early, advances in the management of acquired immunodeficiencies have led to extended survival of patients with partly compromised immune systems. Evidence for immunosurveillance can be found in patients with AIDS who have an increased frequency of malignancies (141). Most often, these malignancies are virus-associated and initiated by viral oncogenes, including lymphomas (Epstein-Barr virus), Kaposi's sarcoma (herpesviruses) and urogenital cancers such as cervical cancer (human papilloma viruses) (142). While the antigenic targets of the above malignancies are not fully characterized, viral antigens can certainly be expressed, and an argument can be made that the increased frequency of virus-associated cancers reflects a breakdown in anti-viral immunity rather than reduced immunosurveillance of cancer. However, support for immunosurveillance can be found in malignancies of non-viral origin.

The incidence of non-virally-induced tumors in AIDS patients is less well documented, but, there is evidence of an increased incidence of solid cancers in AIDS patients, particularly lung adenocarcinomas (143). While a large proportion of HIV-infected individuals may be exposed to other lifestyle risk factors, including smoking, the association of lung cancer in AIDS patients has been demonstrated to be independent of smoking (144), with a 3.5-fold elevated risk of lung cancer for AIDS patients compared to the wider population.

Immunosuppressed Organ Transplant Recipients and Cancer Risk

Some level of immunodeficiency can be induced in humans by the use of immunosuppressants following organ transplantation, and an increase in the incidence of malignancies in these patients suggests a role for immunosurveillance in humans. Greater cancer prevalence among transplant recipients has been observed in a range of transplant situations using a variety of immunosuppressants. For example, patients receiving kidney transplants display a 3-fold increase over the general population in the overall incidence of malignancy. While virus-associated malignancies predominate, there was also an increased risk for developing non-infectious cancers of the colon, lung, pancreas, kidney and endocrine system (145). Additionally, a dramatic increase in risk (200-fold) of non-melanoma skin cancers has been demonstrated in renal transplant patients, suggesting a particularly important role for cancer immunosurveillance at this site exposed to ultraviolet irradiation (146). Finally, melanomas have also been observed to increase in frequency in these renal transplant patients, but to a lesser degree (2-10 fold) than other skin cancers (146, 147).

Interestingly, incidences of some cancers including breast, prostate, ovarian, brain and testicular have not been observed to increase in the context of pharmacologically-induced immunosuppression, but it is not clear if these malignancies are less immunogenic or simply take longer to develop. These data support the notion that *de novo* malignancies arise due to the permissive environment created by immunosuppressive regimens, which inhibit cancer immunosurveillance mechanisms. Further supporting the link between immunosuppression and malignancy are observations of spontaneous remissions of lymphomas after cessation of immunosuppression (148).

Spontaneous Immune Responses to Cancer

The spontaneous recognition and destruction of human cancers by cells of the adaptive immune system substantiates the occurrence of cancer immunosurveillance in humans. As early as the 1970s, screening cancer cell lines with autologous patient serum identified spontaneous antibody responses to autologous cancers in a subset of patients (149, 150). Antibody responses in patient serum have been reported for over 100 tumor-associated antigens, although only 8 antigens have been identified in multiple reports suggesting that many immunogenic mutations might be unique for each individual cancer (reviewed in (151)). Among the shared antibody responses were those against the cancer-testis antigen NY-ESO-1 and the mutant forms of tumor suppressor p53, which are often overexpressed in many different types of human malignancies (152, 153). The high frequency of antibodies specific for tumor-associated antigens in cancer patients compared to healthy individuals suggests that immunity has been induced in response to

malignancy. The reasons for spontaneous antibody responses in cancer patients are not known, but may include an over-abundance of antigen or its enhanced presentation to generate immunogenicity in the malignant setting.

The phenomenon of spontaneously regressing melanoma lesions accompanied by the clonal expansion of T cells is arguably the strongest evidence for the elimination phase of cancer immunoediting in humans (154-156). These responses, observed in the absence of specific immunotherapy, support the ability of the immune system to spontaneously recognize antigens on/in tumors. Specific CD4⁺ and CD8⁺ T cell activity against tumor-associated antigens, including NY-ESO-1, are known to develop spontaneously in human cancer patients (157, 158). However, spontaneous T cell responses specific for some tumor-associated antigens (TAA) such as the MAGE family are very rare (159), while those specific for the melanocyte differentiation antigen MART-1/Melan-A have been found in a relatively high percentage (>50%) of healthy individuals (160). Thus, there is a strong correlation between spontaneous T cell responses and some tumor-associated antigens but not others, and it is not clear whether the presence of TAA-specific T cells in healthy individuals reflects past exposure to transformed cells expressing the antigen. More studies are needed to identify tumor-associated and tumor-specific antigens in a variety of cancers to determine the relative abundance and uniqueness of tumor antigens.

Other spontaneous immune responses against malignant cells have been demonstrated in patients with paraneoplastic autoimmune disorders (PND) caused by cross-reactivity between the antitumor immune response and neurologic antigens (161). In addition to antibody responses, tumor-specific T cells have also been identified in

patients with PND (162). Nearly all patients with PND die from cancer or neurologic disease; however, the few surviving patients have complete tumor remission in response to therapy and no longer manifest any neurological impairment. These dramatic clinical cases demonstrate that the tumor antigens are the driver of both beneficial immune responses against neoplastic tissues and pathological immune responses against normal tissues (i.e., neurons). Interestingly, PND symptoms can precede tumor diagnosis by a number of years (163), indicating that antitumor responses might be primed by undetectable, microscopic tumors early in their evolution. It remains to be determined whether the antitumor immune response substantially delays tumor growth in patients with PND and such analysis is likely to be confounded by the lethality of the neurologic complications. Nevertheless, the presence of anti-neuronal antibodies has been reported to correlate with improved prognosis at least for some neurological malignancies (164), and there are some case reports of spontaneous complete remission in the absence of specific treatment (165). Spontaneous tumor regression accompanied by lymphocyte infiltration has also been noted for a number of other tumor types (reviewed in (30)) however, the role of the lymphocyte infiltrate in tumor regression has not been established in these cases due to their rarity. Even in the absence of spontaneous tumor regression, tumor-infiltrating lymphocytes (TILs) appear to be controlling tumor outgrowth and enhancing patient survival, as discussed below.

Tumor-Infiltrating Lymphocytes as a Prognostic Indicator

Further support for cancer immunoediting can be found in reports that correlate the frequency of tumor-infiltrating lymphocytes (TILs) with patient survival. Tumor

infiltration by T cells, NK cells, or NKT cells have been associated with an improved prognosis for a number of different tumor types (91, 166-172). However, tumor infiltration by some leukocytes, such as macrophages and regulatory T cells, has a detrimental impact on patient survival (173). The initial association between favorable patient prognosis and TILs was first observed in patients with melanoma (166, 167), where it was reported that patients with high levels of CD8⁺ T cell infiltration survive longer than those whose tumors contain low numbers of lymphocytes. Since then, various melanoma-specific antigens have been identified in addition to melanoma-specific T cells in patients with melanoma (reviewed in (174)).

In a landmark study in ovarian cancer, the presence of TILs in ovarian cancer tissue specimens correlated with better prognosis. Specifically, 38% of patients with high numbers of TILs survived over 5 years as compared to 4.5% of patients with low numbers of TILs (168). These findings have been confirmed in subsequent studies for ovarian cancer (169) and for other malignancies including melanoma (175) and colon cancer (170-172). Particularly elegant studies on colon and lung cancers reveal a tight correlation between the quality and quantity of intratumor immune responses and patient survival (171, 172). Remarkably, the type and density of lymphocytes infiltrating these cancers was a more powerful prognostic indicator than previous pathological criteria for tumor staging, underscoring the need for clinical pathologists to consider infiltrating immune cells when determining a patient's prognosis. In fact, the results of these studies provide strong evidence for the equilibrium phase of cancer immunoediting in humans. Enhanced survival of some cancer patients is associated with particular subsets of T cells such as the intratumor localization of CD8⁺ T cells and Tregs (91, 169, 170). A

particularly interesting disagreement concerns the significance of Tregs in tumors, where some groups find a correlation between the presence of Foxp3⁺ Tregs in tumors with a poor prognosis (176, 177), while other studies report better prognosis if Tregs are present in tumor tissue samples (178, 179). Reasons for these different outcomes are not clear, but may be related to the type of malignancy involved.

Immunogenicity of Cancers with Microsatellite Instability

All cancers are inherently genetically unstable and this instability seems to be a contributing factor in the capacity of immune cells to detect and control tumor cells. For example, the infiltration of colorectal cancer by CD8⁺ T cells is associated with a favorable prognosis (170-172), as discussed above, and this association is further strengthened in cases where tumors exhibit high levels of a particular type of genetic instability referred to as microsatellite instability (MSI), where defects in DNA mismatch repair mechanisms lead to the duplication or deletion of short repeated sequences of DNA known as microsatellites (180, 181). Strikingly, MSI-high (MSI-H) tumors are often strongly infiltrated with lymphocytes, including activated CD8⁺ T cells (181), and contain tertiary-lymphoid follicles (180) indicative of a potent local immune response. The high rate of mutation in MSI-H tumors has been shown to result in the generation of a number of novel tumor antigens that can be recognized by B cells, CD4⁺ T cells, and CD8⁺ T cells. Together, these findings suggest that the generation of antigenic peptides as a result of genomic instability might result in the priming of a protective CD8⁺ T cell-mediated immune response in patients with MSI-H colorectal cancers. An interesting possibility is that these findings are not unique to colorectal cancers, but apply to other

human cancers as well (182).

Cancer Equilibrium in Human Patients

A plethora of clinical evidence suggests that occult cancers can lay dormant in patients for many years, sometimes exceeding 20 years, before malignant disease progresses to clinically detectable levels (183). For example, 20-45% of patients with breast or prostate cancer will relapse years or even decades later (184-186). Such a lengthy and protracted period from initial cancer remission to cancer recurrence may, in part, be explained by immunological constraints placed on the remaining cancer cells. In some cases, circulating disseminated cancer cells exist for decades after treatment without the re-establishment of clinical disease from these persistent cancer cells (187). This is known as minimal residual disease and it appears to be a common reservoir of cancer cells for most cancer types after the initial therapeutic intervention, but whose mechanisms for maintenance are poorly understood. Minimal residual disease is of critical importance since the vast majority of morbidity and mortality associated with cancer is due to metastatic lesions that are presumed to be seeded by these persistent cancer cells. There is evidence that immunosuppressive intervention for various conditions can be associated with a greatly increased risk of cancer relapse even after long periods of time. In one study, three out of eight patients (37%) experienced cancer relapse following immunosuppression after more than 10 years of remission while cancer patients in remission for 10 years or more that had not undergone immunosuppressive treatment had only a 2% relapse rate (183).

One remarkable clinical scenario that suggests immunity can prevent the outgrowth of occult lesions is the unintentional transplantation of cancer cells from organ donor to immunosuppressed recipient. In these scenarios, organs were harvested from deceased donors, who either had no previous clinical history of malignancy or were in cancer remission and had no overt signs of disease at the time of organ donation and transplantation into recipients. The recipient patients undergoing immunosuppression for organ engraftment later developed clinically detectable cancers of donor origin (188, 189). A subset of these donor-derived malignancies were from donors with no previous history of cancer, suggesting a state of equilibrium operating between cancer cells in the primary lesion and the donor's immune system that subsequently broke down after transplantation into immunosuppressed recipients.

Clinicians have long-observed that the immune system mounts a response against pre-neoplastic cells in monoclonal gammopathy of unknown significance (MGUS) but does not eliminate them, eventually allowing MGUS to progress to multiple myeloma (190). The ability to detect this premalignant phase of disease allows for immunologic monitoring throughout disease progression and such monitoring has revealed that T cells derived from the bone marrow of patients with MGUS mount strong responses to autologous premalignant cells, but these responses are absent in patients with multiple myeloma (191). These findings are consistent with the idea that T cells may hold pre-malignant cells in check for an extended period of time (i.e., equilibrium) but eventually fail to control some abnormal plasma cell clones that ultimately give rise to multiple myeloma (i.e., escape). Additionally, treatment of low-grade B cell lymphoma by administering antibodies specific for the idiotype expressed by the malignant cells results

in remission of disease without entirely eliminating the tumor cells and that these circulating lymphoma cells are detected up to 8 years after treatment without any other signs of progressive disease (192). These results suggest that equilibrium may be a viable therapeutic endpoint for the treatment of cancer, and in such a case, interventions may be necessary to stabilize the equilibrium phase indefinitely and prevent the immunoselection of tumor cell variants possessing novel mutations that eventuate in resistance to immune attack.

Summary of Human Cancer Immunoediting

As discussed above, there is considerable clinical evidence for the cancer immunoediting process in humans even though cancer patients are a genetically and immunologically diverse population. The confluence of these very complex factors may explain why spontaneous immune responses occur in only a proportion of individuals and why some patients respond better to certain immunotherapies. The differences in an individual's immune repertoire, the capacity to process and present antigens, the quality and quantity of tumor antigens generated as well as the ability of cancer to suppress antitumor immunity all help to determine the overall outcome. Future advances in gene expression and proteomics of human cancers and their antigens will provide greater insight into the mechanism of cancer immunoediting in humans, which may be critical in determining which patients benefit from particular treatments.

CANCER-RELATED INFLAMMATION AND CANCER IMMUNOEDITING: INTERDEPENDENT PROCESSES

Inflammation is a broad and complex physiological process that maintains tissue homeostasis in response to tissue stressors such as infection or tissue damage (193). Rudolph Virchow, who established the cellular basis of pathology, was the first to propose the link between inflammation and cancer in the 1860s when he observed leukocytes infiltrating neoplastic tissues (194). We now appreciate that chronic inflammation can contribute to cancer initiation by generating genotoxic stress, cancer promotion by inducing cellular proliferation, and cancer progression by enhancing angiogenesis and tissue invasion. On the other hand, there is overwhelming evidence that immunity against transformed cells can develop to protect the host from cancer formation as discussed above. Each of the six cell-intrinsic hallmarks of cancer can influence the immune system (9) and the cancer immunoediting process attempts to describe the varied outcomes of tumor-immune system interactions including immunosurveillance (anti-tumor), immunoselection (pro-tumor) and immunosubversion (pro-tumor). We maintain that cancer immunoediting and tumor-promoting inflammation are not mutually exclusive processes, but rather potentially overlapping immune algorithms (195).

This overlap was most clearly demonstrated in the MCA model where sarcoma induction was shown to depend on immune cells and molecules that promote inflammation including MyD88, IL-1 β , IL-10, IL-23 and regulatory T cells (42, 67, 127, 196, 197), but then led to the development of host-protective immune responses that resulted in tumor destruction (e.g., IFN- γ , IFN- α/β , T cells, etc.). For example, the

functionally related heterodimeric cytokines, IL-23 and IL-12, both contain the IL-12p40 subunit but activate distinct receptors that share the IL-12R β 1 subunit and play different roles in response to transformed cells. Specifically, loss of IL-23 reduced the incidence of MCA-induced sarcomas, while IL-12-deficient mice developed more sarcomas when compared to WT mice (67).

Similarly, the DMBA/TPA model of skin carcinogenesis is known to have a major inflammatory component contributing to tumor development, however, $\gamma\delta$ T cells, IL-12 and DNAM-1 all participate in immunosurveillance and prevent skin carcinoma formation (23, 46, 66). Therefore, tumor-promoting inflammation and cancer immunosurveillance can co-exist within the same tumor models at the same tissue site, although they may be temporally distinct. For example, both MyD88 and IL-1 β have been shown to promote tumorigenesis in a number of primary carcinogen models (42, 196, 198, 199), but MyD88 and IL-1 β are also critical in the development of antitumor immunity against established tumors through the recognition of dying tumor cells undergoing 'immunological death' (200-202). Furthermore, the same component of the immune system may promote or prevent tumor formation depending on the biological context in which it acts. For example, mice genetically-deficient for TNF- α develop more sarcomas than WT mice after exposure to MCA (42), indicating a host-protective role for this cytokine, while TNF- α -deficient mice develop fewer skin carcinomas than wild-type mice after exposure to DMBA/TPA (58), indicating a tumor-promoting role for TNF- α . One mechanism for TNF- α 's ability to protect the host against tumor formation is the priming, proliferation, and recruitment of tumor-specific T cells that was observed in an oncogene-driven pancreatic cancer model (203).

Finally, inflammation participates in the cancer immunoediting process during the tumor escape phase, where inflammatory cells and regulatory immune cells are recruited and activated by cancer-derived products to dampen antitumor immunity and subvert immune cells to promote cancer progression. To develop more effective immunotherapies, immunologists must identify the cellular and molecular players that either eliminate or promote cancer development and what conditions influence that fate. For this reason, inhibitors of the pro-inflammatory transcription factors NF- κ B and STAT3 may be therapeutically useful in switching the nature of the tumor microenvironment from one of tumor-promoting inflammation to that of tumor-eliminating immunity (204, 205).

LESSONS FROM CANCER IMMUNOEDITING

As our molecular understanding of cancer immunoediting increases, strategies can be developed to harness the power of immunity to protect against cancer development. Targets for therapeutic intervention can be found at each stage of the immunoediting process from elimination to equilibrium to escape. The identification of key immune molecules and cells important in the elimination of nascent transformed cells may provide opportunities to boost specific aspects of immunity to induce tumor regression. Furthermore, development of therapeutic strategies that stabilize tumor masses by inducing an equilibrium state is a viable clinical endpoint that has not been fully implemented by oncologists, but could greatly enhance patient survival. Another potential strategy targeting the equilibrium phase are those that attempt to stabilize tumor

cell genetic instability, thereby halting progression from tumor equilibrium to tumor escape. The inhibition of tumor escape mechanisms may render tumor cells visible for immune-mediated destruction and thus many pharmacological agents have been generated for this end.

Targets of tumor escape mechanisms currently in clinical trials or in the pipeline include antibody blockade of the immunosuppressive moieties CTLA-4, PD-L1, and PD-1. In the case of CTLA-4 blockade, a recent Phase 3 clinical trial reported that patients with metastatic melanoma survived longer after treatment with CTLA-4 blocking antibodies, making this drug one of the most successful immunotherapies that targets the immune system (206). Furthermore, strategies to inhibit immunosuppressive cytokines such as VEGF, enzymes such as IDO and anti-apoptotic molecules such as Bcl-2 are also being pursued. Undoubtedly, chronic inflammation contributes to both cellular transformation and tumor progression, but less is known about what aspects specifically induce cancer formation. Inhibitors of pro-inflammatory transcription factors may reduce tumor development and switch the tumor microenvironment from tumor-promoting inflammation to tumor-eliminating immunity.

Ultimately, high-throughput screening of cancer genomes and proteomes are required to identify polymorphisms and mutations in immune pathways that limit human cancer development and progression. Insights gained from deciphering the molecular underpinnings of the cancer immunoediting process could lead to strategies for manipulating the cellular and molecular microenvironment of tumors in the hope of inducing immune-mediated eradication or stabilization of malignant disease.

CHAPTER 2

Global Neutralization of IFN- γ Confirms its Role in Preventing Primary Tumors and Shaping Tumor Immunogenicity

INTRODUCTION

IFN- γ in Tumor Immunity

IFN- γ Protects the Host from Transplantable and Primary Tumors

Over a decade ago, renewed interest in cancer immunosurveillance came from several studies that revealed a critically important role for IFN- γ in host tumor immunity. The first was the demonstration that endogenously produced IFN- γ protected the host against the growth of transplanted tumors and the formation of primary chemically induced and spontaneous tumors. Using tumor transplantation approaches, the LPS-dependent rejection of the MethA fibrosarcoma was abrogated in mice treated with neutralizing antibodies specific for IFN- γ (clone H22) (15). Second, using models of MCA-induced tumor formation, 129/SvEv mice lacking either the IFN- γ receptor or STAT1 were found to be approximately 10-20 times more sensitive than wild type mice to tumor formation after carcinogen exposure. Specifically, IFN- γ insensitive mice developed more tumors than their wild type counterparts and showed a shortened tumor latency period (16). These results were confirmed by subsequent independent experiments using mice on a different genetic background that lacked the gene encoding IFN- γ itself (40). Similarly, mice lacking both the p53 tumor suppressor gene and IFNGR1 formed a wider spectrum of tumors compared to IFN- γ -sensitive mice lacking only p53 (16). In addition, *Ifng*^{-/-} mice on a C57BL/6 background showed an increased incidence of disseminated lymphomas despite the presence of a normal p53 tumor suppressor gene (22). Taken together, data from transplantation approaches as well as

chemical and spontaneous tumor induction studies clearly demonstrate a physiologically important role for IFN- γ in host-protection against tumors.

IFN- γ Acts Directly on Tumor Cells to Exert its Antitumor Effects

The demonstration that endogenous IFN- γ is critical to protect the host from both transplantable and primary tumors prompted a search for the physiologically important targets of the antitumor actions of IFN- γ . Due to fact that nearly every host cell expresses the IFN- γ receptor and that IFN- γ is very pleiotropic, there are many likely cellular targets *in vivo* that are important for the direct actions of IFN- γ in the tumor rejection process. A recently published study from our lab used bone-marrow chimera approaches to demonstrate that IFN- γ sensitivity in both non-hematopoietic and hematopoietic host tissues are contributing to the antitumor response of IFN- γ (25). A fellow graduate student in lab, Sang-hun Lee, has recently generated a conditional floxed IFNGR1 mouse to address which hematopoietic and non-hematopoietic cells of the host are targets of IFN- γ 's antitumor effects. Future studies using this mouse will elucidate which host cells require IFN- γ sensitivity to mediate tumor rejection, but there is also evidence that IFN- γ acts directly on tumor cells to exert its antitumor effects. In one study, MethA tumor cells described above were engineered to be unresponsive to IFN- γ by overexpression in these cells of a mutant dominant-negative IFNGR1 (IFNGR1 Δ IC) (15). The paralysis of cellular IFN- γ responsiveness abrogated the LPS-dependent rejection of MethA when transplanted into syngeneic wild type mice. In a second study, an opposite approach was employed using a tumor generated in an *Ifngr1*^{-/-} mouse. When transplanted into wild type mice, the *Ifngr1*^{-/-}-derived tumor, RAD.gR28, grew progressively. However, the

reconstitution of IFNGR1 expression in this tumor resulted in its rejection upon transplantation into syngeneic immunocompetent mice (16). These results have subsequently been corroborated by the observed increases in tumorigenicities of the SCK mammary adenocarcinoma and K1735 melanoma when both tumors were significantly impaired in their IFN- γ responsiveness using the dominant-negative IFNGR1 Δ IC (207). Taken together, these data from different tumor systems show that IFN- γ acts directly on tumor cells to exert its antitumor effects.

Although there is a plethora of data demonstrating that IFN- γ is critical to mediate protection against primary and transplantable tumors, there is less direct evidence of IFN- γ as an immunoeditor. In this study, we globally neutralize IFN- γ in wild type mice with an anti-IFN- γ mAb (clone H22) and treat these mice with the chemical carcinogen, MCA, to generate sarcomas that developed in the absence of IFN- γ , but retain sensitivity to IFN- γ (as opposed to tumors generated in *Ifngr1*^{-/-} mice). A subset of these sarcomas are rejected in wild type mice and thus are highly immunogenic tumors that resemble unedited tumors derived from *Rag2*^{-/-} mice (19). These results provide definitive evidence that IFN- γ edits primary MCA sarcomas.

MATERIALS AND METHODS

Mice. *Ifngr1^{-/-}* mice (208) on a 129/Sv background were originally provided by Dr. Michel Aguet and were bred in our specific pathogen-free animal facility. Wild type and *Rag2^{-/-}* mice were purchased from Taconic Farms. All mice were on a C57BL/6 background and were housed in our specific pathogen-free animal facility. For all experiments, mice were 8-12 weeks of age and performed in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University in St. Louis.

Generating primary MCA sarcomas. 3-methylcholanthrene (MCA) (Sigma) was dissolved in corn oil at a concentration of 0.66 µg/ml by placing a polystyrene container in a water bath that is just below boiling temperature for 3-4 hrs. Stock MCA was then diluted with additional corn oil to desired concentration and injected into the shaved flanks of mice in a volume of 150 µl as previously described (16).

Tumor cell lines. 3-methylcholanthrene (MCA) induced sarcomas used in this study were generated in C57BL/6 strain wild type or *Rag2^{-/-}* mice and banked as low passage tumor cells as previously described (19). Tumor cells derived from frozen stocks were propagated *in vitro* in RPMI media (Hyclone, Logan, UT) supplemented with 10% FCS (Hyclone) and injected subcutaneously in 150 µl of endotoxin-free PBS into the flanks of recipient mice. Tumor cells were >90% viable at the time of injection as assessed by trypan blue exclusion and tumor size was quantified as the average of two perpendicular diameters.

Antibodies. Anti-IFN- γ (H22), anti-CD4 (GK1.5), anti-CD8 α (YTS169.4) mAbs and control immunoglobulin (PIP, a mAb specific for bacterial glutathione S-transferase) were produced from hybridoma supernatants and purified in endotoxin-free form by Protein G affinity chromatography (Leinco Technologies, St. Louis, MO). IFNGR1-PE, H-2D^b-PE, H-2K^b-PE and purified anti-CD16/32 were purchased from BioLegend (San Diego, CA).

Expression vectors. The dominant negative version of the IFNGR1 subunit (IFNGR1 Δ IC) was expressed into H31m1 and d42m1 tumor cells as previously described (15).

Measurement of MHC class I expression. Tumor cells were treated with 100U/ml IFN- γ for 48-72 hrs. MHC class I expression on the cell surface was analyzed by flow cytometry using H-2K^b or H-2D^b antibodies conjugated to PE.

Flow cytometry. For flow cytometry, cells were stained for 20 minutes at 4°C with 500 ng of Fc block (anti-CD16/32) and 200 ng of H-2K^b, H-2D^b, or IFNGR1 in 100 μ L of staining buffer (PBS with 1% FCS and 0.05% NaN₃ (Sigma)). Propidium iodide (PI) (Sigma) was added at 1 μ g/mL immediately before FACS analysis. Events were collected on a FACSCalibur (BD Biosciences) and analyzed using FloJo software.

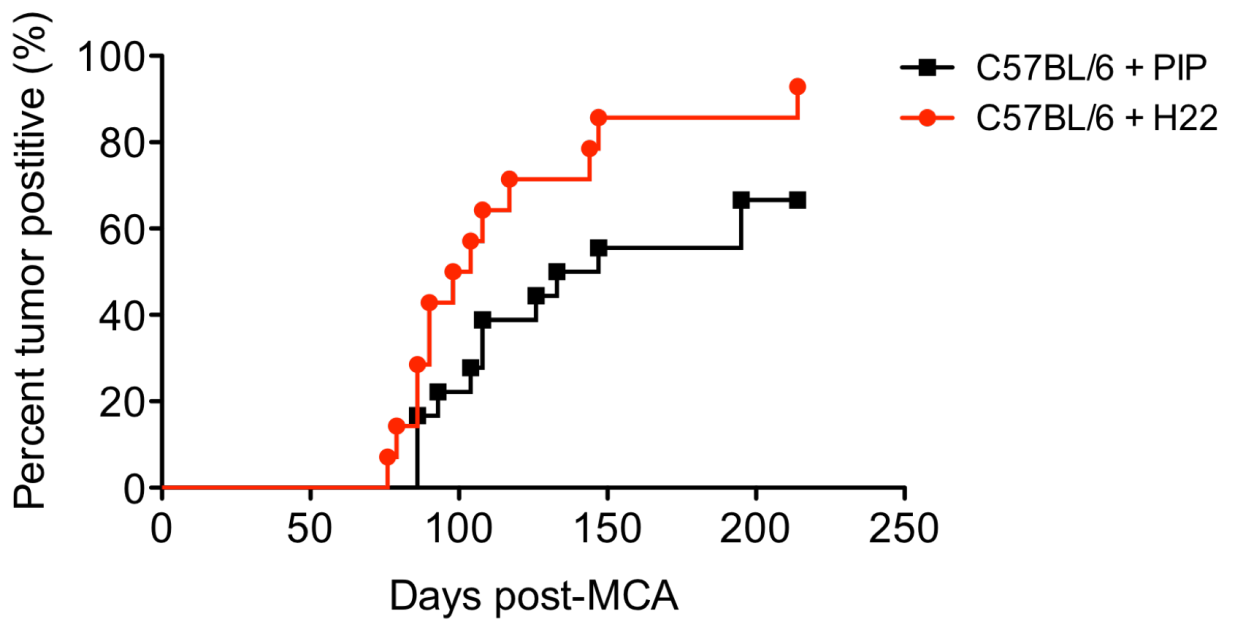
Statistical Analysis. Samples were compared using an unpaired, two-tailed Student's *t* test and Mantel-Cox for tumor incidence.

RESULTS

Global Neutralization of IFN- γ Prevents Primary MCA Sarcoma Formation

Using IFN- γ neutralizing antibodies (H22), we sought to corroborate the finding that mice lacking IFN- γ are more susceptible to MCA-sarcoma induction. A key distinction from the previous studies that used gene-targeted mice lacking either IFN- γ responsiveness (*Ifngr1^{-/-}*) or IFN- γ itself (*Ifng^{-/-}*) is that tumors generated in anti-IFN- γ mAb treated mice will be of wild type origin and have normal IFN- γ sensitivity. Two cohorts of 15 female C57BL/6 mice were injected with 25 μ g of the MCA chemical carcinogen. One cohort was treated with 750 μ g of anti-IFN- γ mAb (H22) on Day -1 and then 250 μ g of anti-IFN- γ mAb weekly for the duration of the experiment. The second cohort was treated with a similar dosing schedule, but was treated with control immunoglobulin (PIP). When wild type C57BL/6 mice injected with 25 μ g of MCA were chronically treated with the control Ig, 67% (10/15) of mice developed progressively growing sarcomas. In contrast, wild type C57BL/6 mice chronically treated with neutralizing anti-IFN- γ mAbs developed more MCA sarcomas (14/15; 93%) with a shorter latency than control mice, confirming that IFN- γ protects the host from primary carcinogenesis (Mantel-Cox $p=0.0373$) (Figure 1). This result verifies that IFN- γ is a critical player in the immunosurveillance against primary MCA sarcomas.

Figure 1. Global neutralization of IFN- γ protects against primary MCA sarcoma formation. Two cohorts of 15 C57BL/6 mice were each treated with 25 μ g of the carcinogen MCA. One cohort received weekly injections of control antibodies (PIP) (black) while the other cohort received weekly injections of neutralizing IFN- γ antibodies (H22) (red). Tumor incidence was monitored weekly and compared using Mantel-Cox ($p=0.0373$).



Global Neutralization of IFN- γ Shapes MCA Sarcoma Immunogenicity

To test whether IFN- γ edits MCA sarcomas from wild type mice, tumors from mice treated with control mAbs or anti-IFN- γ mAbs were harvested, disaggregated, collagenase-treated, and cultured to generate tumor cell lines. All MCA-sarcoma cell lines were frozen after four *in vitro* passages, subsequently thawed and injected into both wild type and *Rag2*^{-/-} recipients. All of the tumors derived from control Ig treated wild type mice grew progressively when transplanted into both wild type and *Rag2*^{-/-} hosts (data not shown). Similarly, all tumors derived from IFN- γ -neutralized mice grew progressively when transplanted into *Rag2*^{-/-} mice (Figure 2). In contrast, 30% (3/10) of cell lines derived from IFN- γ -neutralized mice were spontaneously rejected upon transplantation into wild type mice, indicating that these tumors are highly immunogenic unedited sarcomas (Figure 2) similar to MCA sarcomas generated in *Rag2*^{-/-} or *Ifnar1*^{-/-} immunodeficient mice (19, 24). These data suggest that IFN- γ sculpts tumor immunogenicity and, to date, is the best evidence that endogenous IFN- γ can alter tumor immunogenicity during primary tumor development. In addition, this is the first demonstration that unedited sarcomas can be generated using blocking monoclonal antibodies in wild type mice.

Highly Immunogenic Sarcomas that Developed in the Absence of IFN- γ Require CD4⁺ T cells, CD8⁺ T cells and IFN- γ for Their Rejection

Three highly immunogenic sarcomas from wild type mice chronically treated with neutralizing IFN- γ antibodies are spontaneously rejected when transplanted into wild type mice (Figure 2). We have designated these three tumors (H22-28027, H22-28030, and

H22-28032) collectively as H22 regressor tumors since they display an unedited phenotype similar to a subset of tumors derived from immunodeficient *Rag2*^{-/-} mice that we have termed Rag2 regressors (19). The fact that these H22 tumors grow progressively in *Rag2*^{-/-} mice, strongly suggests that lymphocytes are critical mediators of their rejection. We next explored what specific components of adaptive immunity are required to reject each of these highly immunogenic H22 regressor tumors. For the first tumor, wild type mice were transplanted with 1x10⁶ H22-28027 tumor cells and subsequently treated with antibodies that neutralize IFN- γ (H22), deplete CD4⁺ T cells (GK1.5) or CD8⁺ T cells (YTS-169.4) and were monitored for tumor growth. The unedited H22-28027 tumor grows progressively in wild type mice that were treated with antibodies that neutralize IFN- γ , deplete CD4⁺ T cells or CD8⁺ T cells, suggesting that all of these adaptive immune components are required for tumor rejection (Figure 3). Similar results were obtained for the other two H22 regressor tumors H22-28030 and H22-28032 (Figure 3), suggesting that IFN- γ , CD4⁺ and CD8⁺ T cells are required for the rejection of all three immunogenic tumors derived from MCA-treated, IFN- γ -neutralized wild type mice. The requirement of adaptive immunity to specifically target these highly immunogenic sarcomas is identical for the subset of unedited sarcomas derived from *Rag2*^{-/-} mice, providing further evidence that these H22 regressors are unedited due to the blockade of IFN- γ signaling in the host.

Figure 2. Tumors from IFN- γ -neutralized mice show an unedited phenotype. In a panel of 10 MCA sarcomas from wild type mice that were chronically treated with anti-IFN- γ mAbs (H22), three (30%) tumor cell lines were spontaneously rejected upon transplantation into wild type recipients (highlighted in blue).

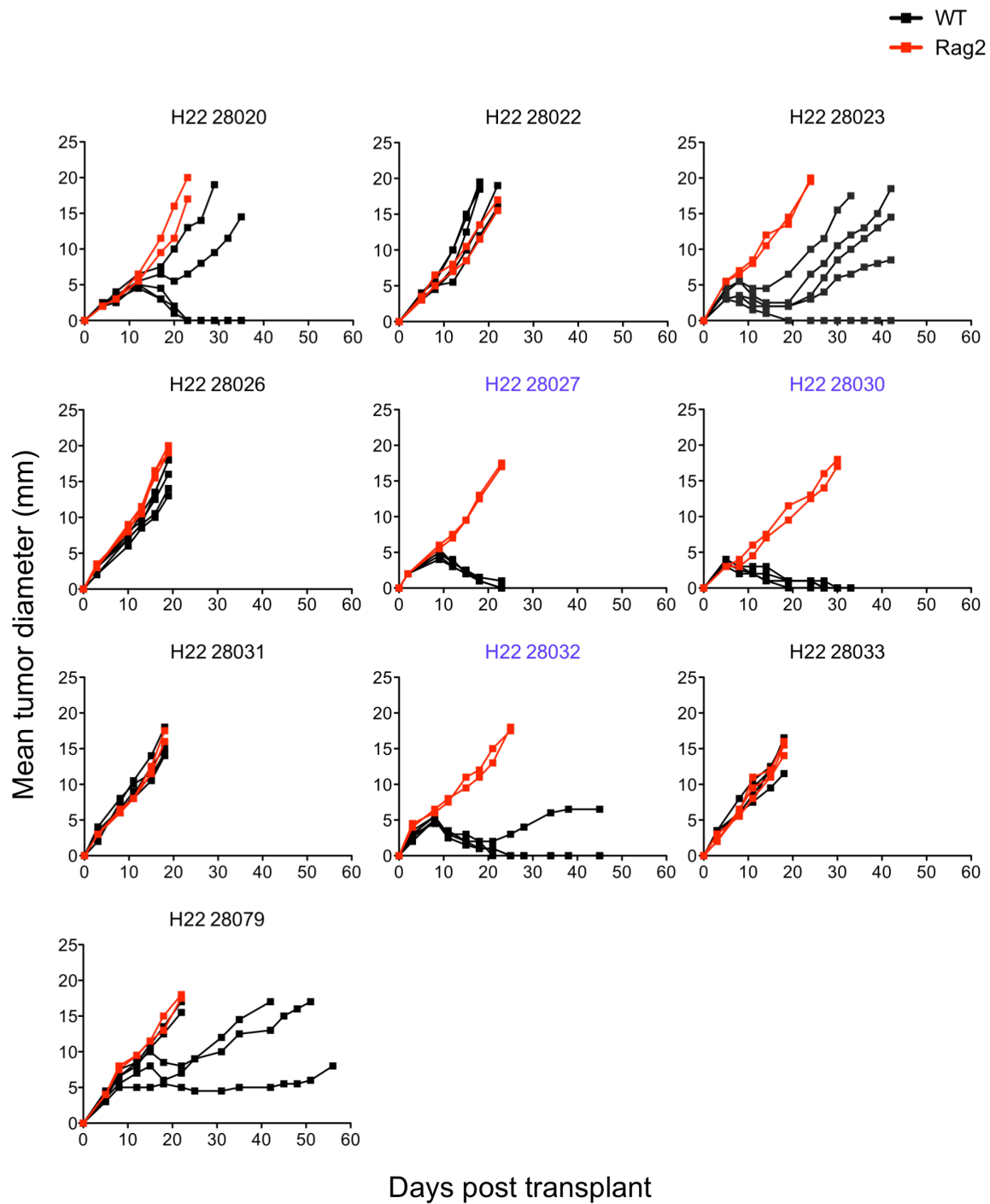
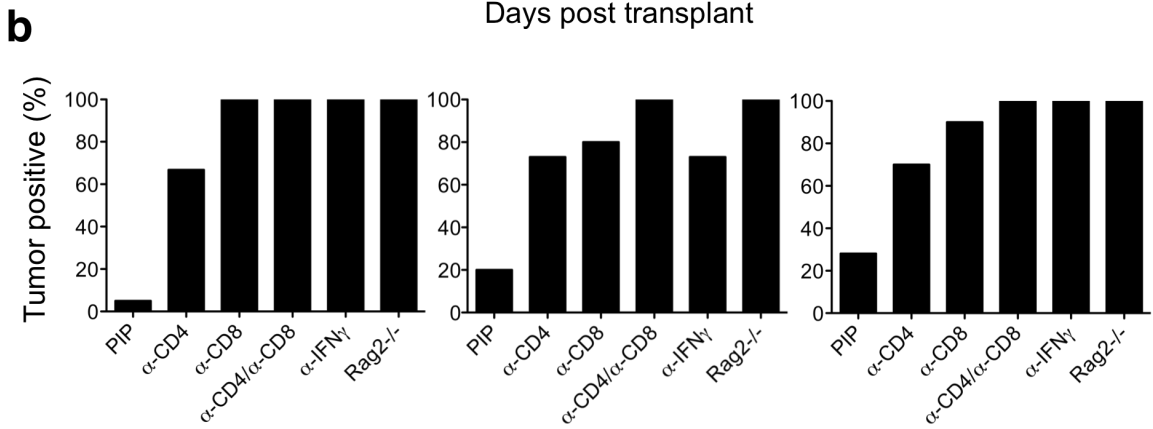
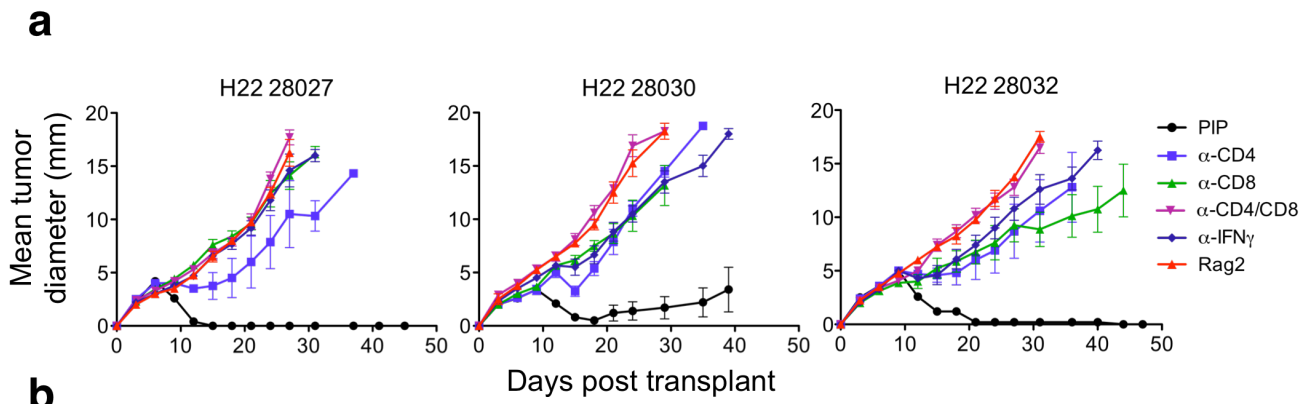


Figure 3. Highly immunogenic sarcomas require components of adaptive immunity for their rejection in wild type hosts. **a**, All three highly immunogenic H22 regressors, H22-28027, H22-28030, and H22-28032 grow progressively when transplanted (1×10^6 cells) into wild type mice that are treated with mAbs that deplete CD4⁺ T cells (GK1.5), deplete CD8⁺ T cells (YTS-169.4), or neutralize IFN- γ . **b**, Percentage of mice with progressively growing tumors from 2-3 independent tumor transplantation experiments.



Differential Requirements for Tumor Cell and Host Cell IFN- γ Responsiveness for the Rejection of Each Individual H22 Regressor Tumor

The finding that highly immunogenic H22 regressors grow progressively in wild type mice treated with IFN- γ neutralizing antibodies (Figure 3) established that IFN- γ is critical for the rejection of these tumors. However, IFN- γ can exert its antitumor effects on both cells of the host and well as the tumor cell itself (15, 16, 25). To test whether IFN- γ responsiveness was required at the level of the host, tumor cells were transplanted into *Ifngr1*^{-/-} recipients. Both H22-28027 and H22-28030 were mostly rejected upon transplantation into mice lacking host IFN- γ responsiveness. In contrast, H22-28032 grew progressively when transplanted into *Ifngr1*^{-/-} mice (Figure 4). These results suggest that H22-28032 requires host IFN- γ sensitivity for its rejection while H22-28027 and H22-28030 tumors do not require IFN- γ sensitivity at the level of the host alone for their rejection. Given that global neutralization of IFN- γ through the use of anti-IFN- γ mAbs results in the growth of all three H22 regressors, but only H22-28032 requires IFN- γ sensitivity at the level of the host for its rejection, we next addressed whether IFN- γ sensitivity at the level of the tumor is required for tumor rejection.

To generate IFN- γ -insensitive variants of the H22 regressors, each tumor was transduced with a dominant negative version of the IFNGR1 subunit, which lacks the intracytoplasmic domain required for IFN- γ receptor mediated signaling (IFNGR1 Δ IC) that has been previously described (15). Each tumor cell line expressing IFNGR1 Δ IC upregulates H-2D^b and H-2K^b class I proteins in response to recombinant murine IFN- α ₅ and IFN- β , but fails to express any class I proteins on its cell surface after treatment with recombinant murine IFN- γ , indicating that IFNGR1 Δ IC tumors are selectively and

completely insensitive to IFN- γ (Figure 5). H22-28027 tumor cells lacking IFN- γ responsiveness (H22-28027 Δ IC) grow progressively when transplanted into wild type mice in a manner that is indistinguishable from growth in *Rag2*^{-/-} mice (Figure 6). Thus, IFN- γ responsiveness at the level of tumor, but not the host, is necessary for the rejection of H22-28027 tumor cells. Interestingly, H22-28030 Δ IC tumor cells are rejected upon transplantation into wild type mice suggesting that neither IFN- γ sensitivity at the level of the host nor at the level of the tumor alone is sufficient for tumor rejection. To test whether IFN- γ sensitivity at the level of the host and tumor are both required for tumor rejection, H22-28030 Δ IC tumor cells should be transplanted into *Ifngr1*^{-/-} mice. Unlike H22-28027 and H22-28030 tumors, which are rejected upon transplantation into *Ifngr1*^{-/-} mice, host IFN- γ responsiveness alone is required to mediate tumor rejection of H22-28032 as this tumor grows progressively in *Ifngr1*^{-/-} mice. Nevertheless, we investigated whether IFN- γ responsiveness at the level of the tumor was also contributing to rejection of H22-28032 tumors. H22-28032 Δ IC tumor cells that are insensitive to IFN- γ display a partial growth phenotype when transplanted into wild type mice (Figure 6). Overall, in two independent experiments, H22-28032 Δ IC tumors grew in half of wild type mice (5/10) upon transplantation (Figure 6), suggesting that there is a role for IFN- γ sensitivity at the level of the tumor and the host. Similar to H22-28030, IFN- γ responsiveness at the level of the tumor and the host may both be required to achieve maximal protection from H22-28032 tumors.

Figure 4. H22-28032 grows progressively in *Ifngr1*^{-/-} hosts. Mice deficient for IFNGR1 and thus lack IFN- γ responsiveness in host cells were injected with 1×10^6 cells of H22-28027, H22-28030, or H22-28032. (Experiments performed by Sang Hun Lee, a pre-doctoral candidate in the Schreiber laboratory).

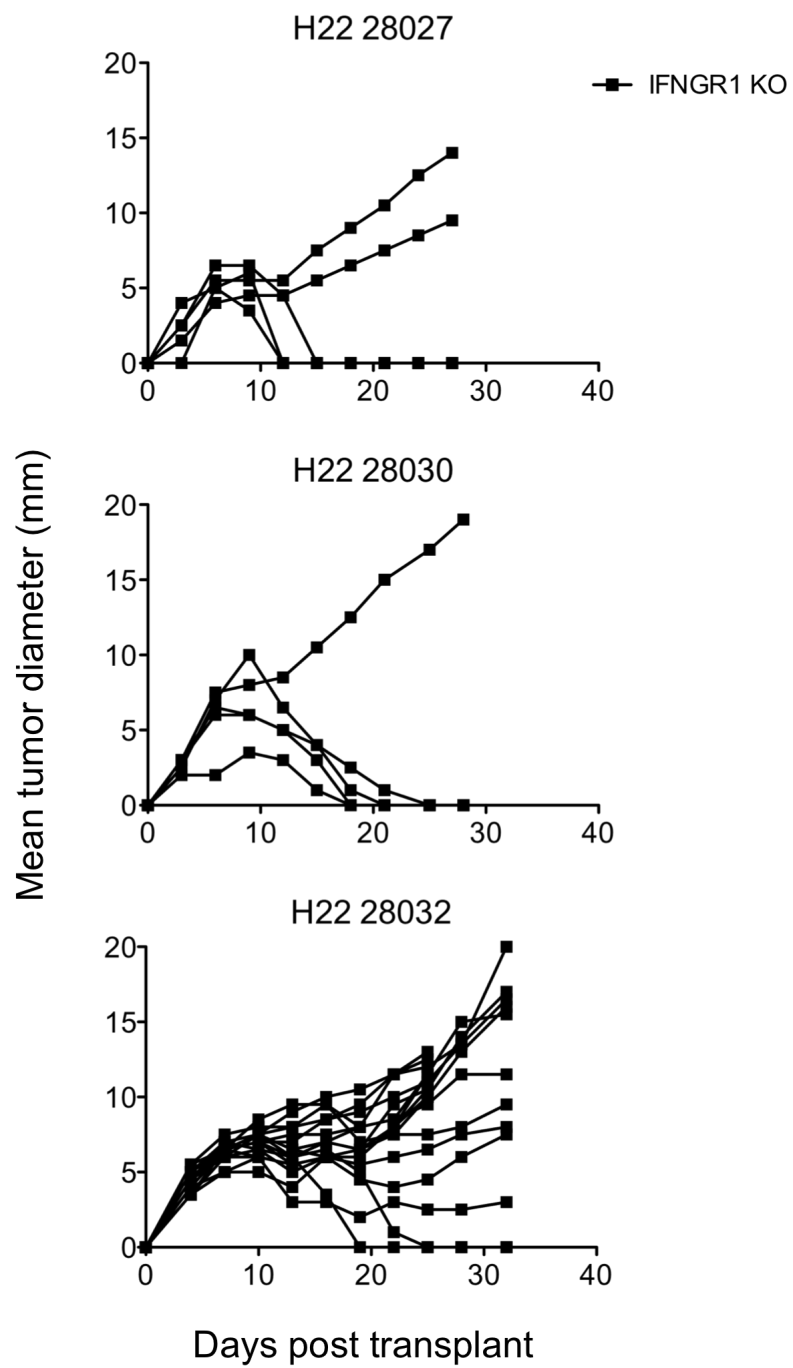


Figure 5. H22 regressor sarcoma cells rendered insensitive to IFN- γ . H22-28027, H22-28030, H22-28032 tumor cells were transduced with a dominant negative version of the IFNGR1 subunit (IFNGR1 Δ IC) or control retrovirus (RV) and tested for capacity to upregulate expression of cell surface MHC class I molecules (H-2D^b) using flow cytometry after the addition of murine recombinant IFN- α_5 , IFN- β , or IFN- γ for 48 hrs *in vitro*.

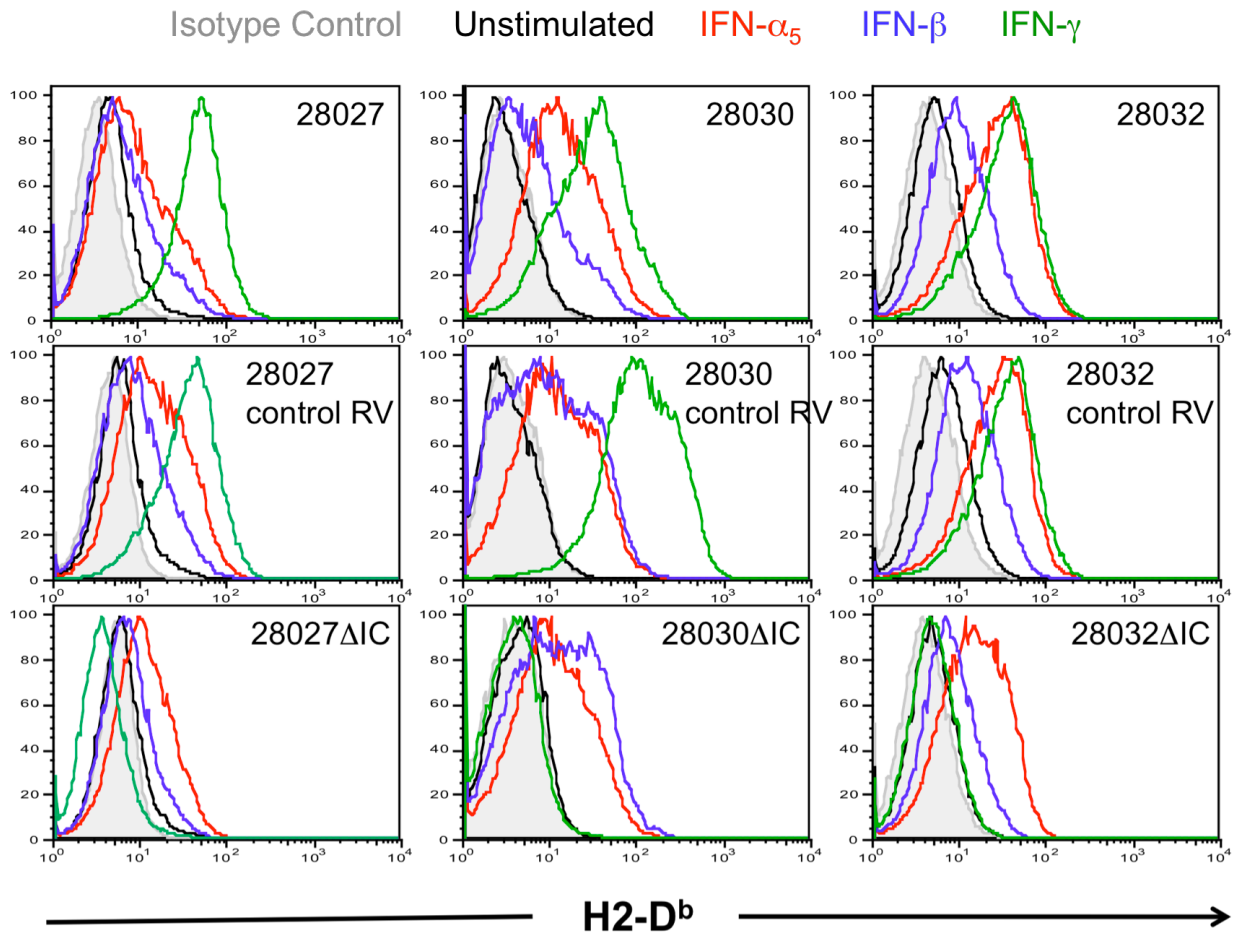
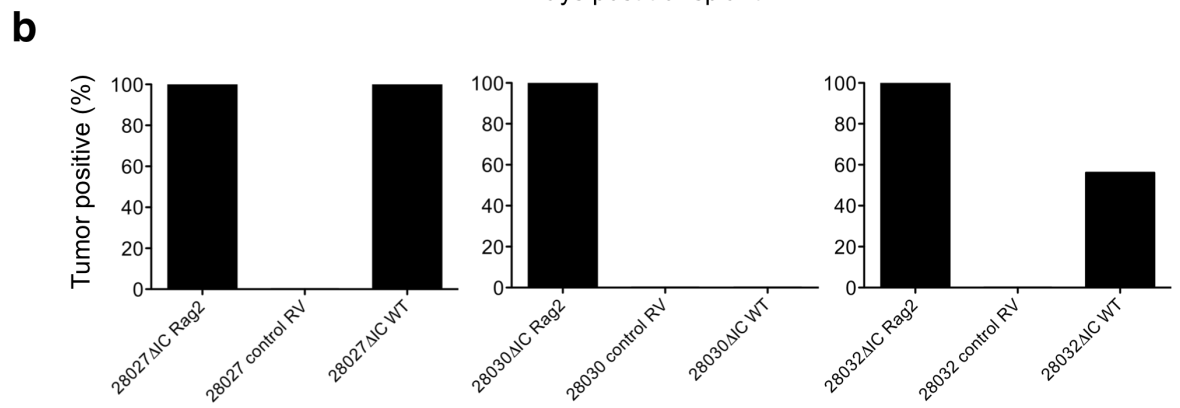
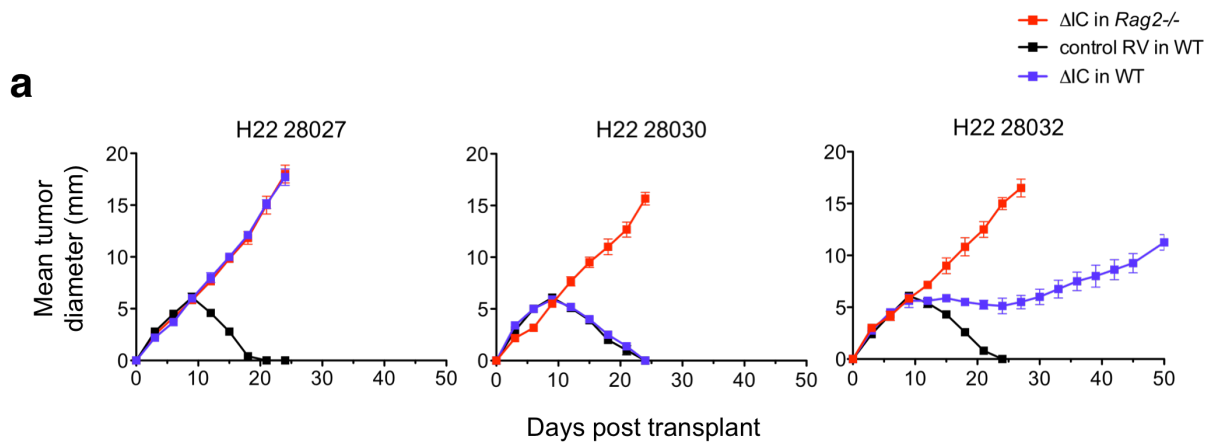


Figure 6. H22-28027 and H22-28032 require tumor cell responsiveness to IFN- γ for tumor rejection. **a**, Dominant negative versions of the IFNGR1 receptor subunit were expressed in H22-28027, H22-28030, and H22-28032 unedited sarcomas and transplanted into wild type mice (blue) or *Rag2*^{-/-} mice (red). In addition tumors transduced with an empty retrovirus vector were transplanted into wild type mice (black) as a control. **b**, Percentage of mice with progressively growing tumors from two independent tumor transplantation experiments.



DISCUSSION

Several studies from our lab and others have demonstrated that IFN- γ is critical in mediating anti-tumor immunity, especially from primary tumor formation. Mice deficient in IFN- γ (*Ifng*^{-/-}), IFN- γ responsiveness (*Ifngr1*^{-/-}), or IFN- γ signaling (*Stat1*^{-/-}) all develop more chemically induced MCA sarcomas and spontaneous tumors upon aging than their wild type counterparts (16, 19, 22, 34). Recently, a new study reported that IFN- γ also protects from primary skin carcinomas induced by intradermal injection of MCA (55). This study confirms earlier reports that IFN- γ is necessary for the prevention of skin carcinomas and papillomas in the DMBA/TPA inflammatory model (23). Due to the powerful antitumor actions of IFN- γ , one would expect that tumors would evolve mechanisms of immune escape by rendering components of IFN- γ signaling ineffective. Indeed, many human and mouse cancers have lost responsiveness to IFN- γ due to mutations in signaling components downstream of the IFN- γ receptor (16, 97). For example, JAK1 is epigenetically silenced in the human prostate cancer cell line LNCaP and thus is unable to upregulate class I antigen processing and presentation machinery in response to IFN- γ and IFN- α/β (103).

In addition to the tumor-preventing roles of IFN- γ , there are a few studies that also provide evidence for the tumor-sculpting roles of IFN- γ on developing cancers. One such study generated cell lines from MCA-treated *Ifngr1*^{-/-} mice and showed that reconstitution of these cell lines with IFNGR1 results in tumor rejection when transplanted into naïve wild type mice (16). Subsequent studies demonstrated that these cell lines could also be rendered immunogenic by enforcing expression of TAP1 (19) or

H-2D^b (A.T. Bruce and R.D. Schreiber, unpublished results). However, spontaneous lymphomas that formed in C57BL/6 *Ifng*^{-/-} mice were found not to be immunogenic and grew progressively when transplanted into wild type recipients (22). In contrast, spontaneous lymphomas from perforin-deficient mice were immunogenic and were rejected when transplanted into wild type mice (20, 22). Thus, although there is evidence that IFN- γ is important for sculpting tumor immunogenicity, it is mostly inferred either from (a) already edited tumors that have lost IFN- γ sensitivity, or (b) tumors that lack functional IFN- γ receptors rendered immunogenic by reconstitution of functional receptors. Here, we unequivocally demonstrate that IFN- γ is involved in editing tumor immunogenicity in a primary tumor model and confirm its actions on both host cells and tumor cells to mediate host-protective, anti-tumor functions.

Using neutralizing antibodies against IFN- γ , we chronically treated wild type mice exposed to MCA and documented that these mice develop more primary MCA sarcomas than control Ig treated mice, further validating IFN- γ 's role in preventing primary tumors. Importantly, we generated 10 tumor cell lines from these mice and found that 3/10 (30%) sarcomas spontaneously rejected in wild type mice—a finding that is remarkably similar to *Rag2*^{-/-} mice and *Ifnar1*^{-/-} mice where 40% and 36% of the MCA sarcomas are highly immunogenic regressors, respectively (19, 24). In addition, these highly immunogenic H22 regressors resemble typical unedited sarcomas generated in *Rag2*^{-/-} mice in that they require IFN- γ , CD4⁺ T cells and CD8⁺ T cells for their rejection. As these tumors require IFN- γ for tumor rejection, we next explored whether host cells or tumor cells were the critical targets for IFN- γ . In support of previous studies, we found IFN- γ responsiveness is required on both tumor cells and host cells for tumor rejection

(15, 16, 25). However, each of the three H22 regressors had different combinations of host and tumor cell requirements for IFN- γ responsiveness to mediate tumor rejection, a result that is in contrast to *Rag2*^{-/-} MCA sarcomas generated on a 129/Sv background where either IFN- γ responsiveness at the level of the host or the tumor alone is sufficient to mediate tumor rejection (25) (C.M. Koebel and R.D. Schreiber, unpublished results).

Taken together, these data suggest that IFN- γ sculpts tumor immunogenicity and, to date, is the best evidence that endogenous IFN- γ can alter tumor immunogenicity during primary tumor development. In addition, this is the first demonstration that unedited sarcomas can be generated using blocking monoclonal antibodies in wild type mice. Although we do not yet know the host cell targets and thus the mechanisms of action for IFN- γ , one likely mechanism is IFN- γ 's role in facilitating the recognition of tumor-specific antigens by lymphocytes that leads to tumor cell destruction. The remainder of this thesis dissertation will explore the role that tumor antigens play in driving the cancer immunoediting process.

CHAPTER 3

**Exome Sequencing of the Highly
Immunogenic, Unedited d42m1 Sarcoma
Identifies its Major Rejection Antigen**

INTRODUCTION

A central tenet of tumor immunology in general, and the cancer immunoediting process in particular, is that tumor cells express antigens that distinguish them from their non-transformed counterparts, thus permitting their recognition by T cells and their ultimate destruction by immunological mechanisms. Since the first human tumor antigen was identified in 1991 (209), several tumor antigens have been cloned and can be classified in the following five categories: 1) differentiation antigens (e.g., melanocyte differentiation antigens, tyrosinase); 2) mutational antigens (e.g., abnormal forms of p53); 3) overexpressed/amplified normal proteins (e.g., HER2/neu); 4) cancer-testis antigens (e.g., NY-ESO-1); and 5) viral antigens (e.g., human papilloma virus) (8). Subsequent studies have also identified tumor antigens in the murine system (210, 211).

Inherent genomic instability of tumor cells allows some variants to acquire additional mutations that are able to evade immunological detection. Specifically, tumor cells may lose sensitivity to the interferons, resulting in the reduction of tumor antigen processing and presentation (97, 101). Furthermore, numerous studies have demonstrated that the immune system can select for tumor variants better suited for survival in an immunologically intact environment. Specifically, when P815 mastocytoma or ultraviolet-induced 1591 fibrosarcoma cells are serially transplanted into immunocompetent hosts, tumor variants emerge with reduced immunogenicity (211, 212).

Although a deep understanding of human and mouse tumor antigens currently exists (32), it comes nearly entirely from analyses of tumor cells derived from

immunocompetent hosts which were likely subjected to the sculpting forces of cancer immunoediting. Little is known about the antigens expressed in nascent tumor cells, whether they are sufficient to induce host-protective, anti-tumor immune responses or whether their expression is modulated by the immune system. We realized that these questions might be answered by defining the antigens expressed in unedited sarcoma cell lines derived from 3'-methylcholanthrene (MCA) treated, immunodeficient *Rag2*^{-/-} mice since these tumors phenotypically resemble highly immunogenic, nascent primary tumor cells (87). However, current methods to identify tumor antigens using expression cloning approaches (209, 211) are time and effort intensive and are not well suited to establishing a tumor's antigenic profile. Recent advances in the field of genome sequencing have made possible rapid and cost effective methods to define cancer genomes and have established that whereas cancer cells acquire some mutations involved in the transformation process (driver mutations), they also express many passenger mutations that develop, in part, as a consequence of genomic instability (213). These tumor-specific mutant proteins have been proposed, but never proven, to represent tumor-specific antigens for T cells (214).

Herein, we use a modified form of exome sequencing to define the mutational profile of two independent, unedited MCA sarcomas (d42m1 and H31m1). This technique, involving cDNA capture by biotinylated mouse exome probes (Agilent) followed by deep sequencing (hereafter referred to as cDNA Capture Sequencing or cDNA CapSeq) showed that the two tumor cell lines display largely non-overlapping patterns of mutations. By pipelining the cDNA CapSeq data for one of these tumors (d42m1) into MHC class I epitope prediction algorithms, we identify a potential major

antigen of this unedited tumor, validate its identity as the major rejection antigen using expressing cloning techniques, and show that antigen-loss via a T cell dependent immunoselection process represents the mechanism underlying cancer immunoediting of this tumor. This study, thus, provides mechanistic insights into the process of cancer immunoediting and points to the future potential that cancer genome analysis may have on the fields of tumor immunology and cancer immunotherapy.

This study was performed in a combined and equal effort with Dr. Hirokazu Matsushita, a post-doctoral fellow in the lab and thus, his data and my own are presented here together for clarity.

MATERIALS AND METHODS

Mice. *Ifngr1^{-/-}* mice (208) and *Ifnar1^{-/-}* mice (215) on a 129/Sv background were originally provided by Dr. Michel Aguet and were bred in our specific pathogen-free animal facility. Wild type and *Rag2^{-/-}* mice were purchased from Taconic Farms. All mice were on a 129/Sv background and were housed in our specific pathogen-free animal facility. For all experiments, mice were 8-12 weeks of age and performed in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University in St. Louis.

Tumor transplantation. 3-methylcholanthrene (MCA) induced sarcomas used in this study were generated in 129/Sv strain wild type or *Rag2^{-/-}* mice and banked as low passage tumor cells as previously described (19). Tumor cells derived from frozen stocks were propagated *in vitro* in RPMI media (Hyclone, Logan, UT) supplemented with 10% FCS (Hyclone) and injected subcutaneously in 150 μ l of endotoxin-free PBS into the flanks of recipient mice. Tumor cells were >90% viable at the time of injection as assessed by trypan blue exclusion and tumor size was quantified as the average of two perpendicular diameters.

Isolation of normal skin fibroblasts. Skin fibroblasts were isolated from three independent 129/Sv *Rag2^{-/-}* pups by harvesting skin and incubating in 0.25% trypsin (Hyclone) at 37°C for 30 minutes prior to washing in DMEM media (Hyclone). After washing, chunks of skin were filtered to achieve single cell suspensions and cultured *in*

vitro with DMEM media. After 3 passages, skin fibroblasts were harvested to isolate genomic DNA and total RNA.

Extraction of genomic or complementary DNA. Genomic DNA from sarcoma cells and normal skin fibroblasts was extracted using DNeasy Blood & Tissue Kit (Qiagen). For cDNA isolation, total RNA from sarcoma cells and normal skin fibroblasts was isolated using RNeasy Mini kit (Qiagen) and cDNA was synthesized using oligo (dT) primers and SuperScript II Reverse Transcriptase (Invitrogen).

cDNA capture, sequencing, and alignment (cDNA CapSeq). cDNA samples from each tumor (100 ng) were constructed into Illumina libraries according to the manufacturer's protocol (Illumina Inc, San Diego, CA) with the following modifications: 1) cDNA was fragmented using Covaris S2 DNA Sonicator (Covaris, Inc. Woburn, MA) in 1X end-repair buffer followed by the direct addition of the enzyme repair cocktail (Lucigen, Madison, WI). Fragment sizes ranged between 100 and 500 bp. 2) Illumina adapter-ligated DNA was amplified in four 50 µl PCRs for five cycles using 4 µl adapter-ligated cDNA, 2X Phusion Master Mix and 250 nM forward and reverse primers, 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC and 5'CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC, respectively. 3) Solid Phase Reversible Immobilization (SPRI) bead cleanup was used to purify the PCR-amplified library and to select for 300-500 bp fragments. 500 ng of the size-fractionated Illumina library was hybridized with the Agilent mouse exome

reagent. After hybridization at 65°C for 24 hrs, we added 50 µl of DynaBeads M-270 Streptavidin-coated paramagnetic beads (10 mg/ml) to selectively remove the biotinylated Agilent probes and hybridized cDNA library fragments. The beads were washed according to manufacturer's protocol (Agilent) and the captured library fragments were released into solution using 50 µl of 0.125 N NaOH and neutralized with an equal volume of neutralization buffer (Agilent). The recovered fragments then were PCR amplified according to the manufacturer's protocol using 11 cycles in the PCR. Illumina library quantification was completed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA). The qPCR result was used to determine the quantity of library necessary to produce 180,000 clusters on a single lane of the Illumina GAIIx. One lane of 100 bp paired-end data was generated for each captured sample (since cDNA was used as the source for sequencing, we refer to this process as cDNA Capture Sequencing or CapSeq). Illumina reads were aligned to the NCBI build 37 (Mm9) mouse reference sequence using BWA (216) v0.5.5 (with -q 5 soft trimming). Alignments from multiple lanes for the same sample were merged together using SAMtools r599, and duplicates were marked using Picard v1.29.

Mutation detection and annotation. Putative somatic mutations were identified using VarScan 2 (v2.2.4) (217) with the parameters “*--min-coverage 3 --min-var-freq 0.08 --p-value 0.10 --somatic-p-value 0.05 --strand-filter 1*” and specifying a minimum mapping quality of 10. Variants whose supporting reads exhibited read position bias (average read position <10 or >90), strand bias (>99% of reads on one strand), or mapping quality (score difference >30, or mismatch quality sum difference >100) relative to reference

supporting reads were removed as probable false positives. We also required that the variant allele be present in at least 10% of tumor reads and no more than 5% of normal reads. The single nucleotide variants (SNVs) meeting these criteria were annotated using an internal database of Genbank/Ensembl transcripts (v58_73k). In the event that a variant was annotated using multiple transcripts, the annotation of most severe effect was used. Non-silent coding mutations (missense, nonsense/nonstop, or splice-site) were prioritized for downstream analysis.

Mutation rate and overlap comparisons. Mutation rates were estimated for each tumor sample using the number of putative “tier 1” SNVs (missense, nonsense/nonstop, splice site, silent, or noncoding RNA). To account for variability in coverage between samples, the SNV count for each tumor sample (S) was divided by a coverage factor (F), computed as the fraction of all tier 1 SNVs identified in any tumor sample ($n=16,991$) that were covered by at least 4 reads in a given sample. For example, in the d42m1 parental sample, 15,852 of 16,991 tier 1 SNV positions were covered, for a coverage factor of 93.30%. The number of coverage-adjusted mutations in each sample was divided by the total size of tier 1 space in the mouse genome (43.884 Mbp) to determine the number of coding mutations per megabase (R).

$$R = (S / F) / (43.884 \text{ Mbp})$$

For the mutation overlap comparisons and relatedness-to-parental-tumor analysis, only high-confidence missense mutations were used (i.e., 20X or above). A mutation was considered “shared” between two samples if both samples had a predicted mutation at the same genomic position. For the comparison of mutated genes between d42m1 and

H31m1 parental lines, a gene was considered “shared” if both d42m1 and H31m1 samples had a predicted missense mutation in that gene, even if the mutations did not occur at the same position.

MHC class I epitope prediction. All missense mutations for each d42m1 tumor variant were analyzed for the potential to form MHC class I neoepitopes that bind to either H-2D^b or H-2K^b molecules. The artificial neural network (ANN) algorithm provided by the Immune Epitope Database and Analysis Resource (www.immuneepitope.org) was used to predict epitope binding affinities (218) and the results were ultimately expressed as “Affinity Values” (Affinity Value = $1/IC_{50} \times 100$).

Antibodies. Anti-H-2K^b (B8-24-3) and anti-H-2D^b (B22/249) mAbs were generously provided by Dr. Ted H. Hansen (Washington University School of Medicine). Anti-CD4 (GK1.5), anti-CD8 α (YTS169.4) mAbs and control immunoglobulin (PIP, a mAb specific for bacterial glutathione S-transferase) were produced from hybridoma supernatants and purified in endotoxin-free form by Protein G affinity chromatography (Leinco Technologies, St. Louis, MO). Purified Rat IgG was purchased from Sigma (St. Louis, MO). CD45-FITC, CD45-PE, CD8-APC, and purified anti-CD16/32 were purchased from BioLegend (San Diego, CA).

cDNA library construction and screening. To generate a d42m1 tumor cell cDNA library, mRNA was isolated from parental d42m1 tumor cells using a QuickPrep mRNA Purification kit (Amersham), converted into cDNA using SuperScript II First Strand

Synthesis System (Invitrogen) and inserted into the EcoRI site of the expression vector pcDNA3 (Invitrogen). The cDNA library was divided into pools of 100 bacterial colonies with 200-300 ng of DNA from each pool transfected into 2.5×10^4 monkey COS cells engineered to ectopically express mouse H-2D^b (COS-D^b) cells using Lipofectamine 2000. After 48 hr, 5×10^3 C3 CTL cells were added, and supernatants were assayed for IFN- γ release 24 hrs later by ELISA. A single positive cDNA clone was isolated after screening 120,000 cDNA colonies. The putative H-2D^b-binding peptide VAVVNQIAL was predicted using the algorithm available at the Immune Epitope Database and Analysis Resource, <http://www.immuneepitope.org/>. The peptides were kindly produced by Dr. Paul Allen and Steve Horvath (Washington University School of Medicine).

Expression vectors. Full length cDNA encoding wild type spectrin- β 2 and mutant spectrin- β 2 were cloned from parental d42m1 tumor cells by RT-PCR using primer pairs 5'-TGAGACAGTCAAGATGACGACCACGGTAGCCACA-3' and 5'-CGGGACAACAGGGAAGTTCACTTCTTCTTGCCGA-3'. Wild type and mutant spectrin- β 2 cDNA were subcloned from the TOPO-XL vector (Invitrogen) into the RV-GFP vector (219). To generate the RV-RFP vector, full length cDNA encoding RFP was cloned from the pTurboRFP-C vector (Evrogen) by RT-PCR using primer pairs 5'-ATCTCAGAATTCATGAGCGAGCTGATCAAGGA - 3' and 5'-ATCTCAGGATCCTTATCTGTGCCCCAGTTTGCTAG - 3'. RFP cDNA was then cloned into the RV vector. To remove candidate T cell epitopes in RFP, the nucleotide A was replaced by G at position 334 in the cDNA, resulting in amino acid substitution N112D. Coding sequences of the constructs were verified by DNA sequencing (Big Dye

method; Applied Biosciences). The dominant negative version of the IFNGR1 subunit (IFNGR1ΔIC) was expressed into H31m1 and d42m1 tumor cells as previously described (15).

Establishment of CTL lines and clones. To generate the d42m1 specific C3 CTL clone, wild type mice were injected with 1×10^6 parental d42m1 tumor cells. Fourteen days later, the spleen was harvested from a mouse that rejected the tumor and a CTL line was established by stimulating 40×10^6 splenocytes with 2×10^6 parental d42m1 tumor cells pre-treated for 48 hr with 100 U/ml of recombinant murine IFN- γ and irradiated (100 Gy). After CD8⁺ T cell purification using magnetic-beads (Miltenyi Biotec) and limiting dilution, the CTL clone C3 was obtained.

Measurement of IFN- γ production. To generate target cells, tumor cells were treated with 100U/ml IFN- γ for 48 hrs and irradiated with 100 Gy prior to use. The C3 CTL clone was co-cultured at the indicated ratios with target tumor cells (10,000 or 5,000 cells) in 96-well round-bottomed plates overnight. IFN- γ in supernatants was quantified using an IFN- γ ELISA kit (eBioscience). For blocking assays, 10 μ g/ml of α -CD8 (YTS-169.4), α -CD4 (GK1.5), or control immunoglobulin (PIP) were added to the cell culture of effector (C3 CTL clone) and target cells (tumors).

Fluorescence-activated cell sorting analysis. For flow cytometry, cells were stained for 20 minutes at 4°C with 500 ng of Fc block (anti-CD16/32) and 200 ng of CD45, CD4, or CD8 α in 100 μ L of staining buffer (PBS with 1% FCS and 0.05% NaN₃ (Sigma)).

Propidium iodide (PI) (Sigma) was added at 1 $\mu\text{g}/\text{mL}$ immediately before FACS analysis. For quantitative analysis of tumor-infiltrating lymphocytes/leukocytes (TIL) and lymph node populations, a $\text{CD45}^+\text{PI}^-$ gate was used and gated events were collected on a FACSCalibur (BD Biosciences) and analyzed using FloJo software.

Tumor, draining lymph node, and spleen harvest. After tumor cell transplantation, established tumors were excised from mice, minced and treated with 1 mg/ml type IA collagenase (Sigma) in HBSS (Hyclone) for 2 hrs at room temperature. The ipsilateral inguinal tumor-draining lymph nodes and spleen were also harvested and crushed between two glass slides and vigorously resuspended to make single-cell suspensions.

Tetramers. H-2D^b tetramers conjugated to phycoerythrin (PE) were prepared with mutant spectrin- β 2 peptides and produced by the NIH Tetramer Core Facility (Emory University, Atlanta, GA).

Mutation specific RT-PCR and real-time RT-PCR. Total RNA from tumor cells was isolated by RNeasy Mini kit (Qiagen) and cDNA was synthesized from the total RNA using oligo (dT) primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR specific for wild type spectrin- β 2, mutant spectrin- β 2 and GAPDH using the SYBR Green Mastermix kit (Applied Biosystems) were performed on ABI 7000. The primer sequences for used for mutant spectrin- β 2 are 5'-GGTGAACCAGATTGCACT-3' and 5'-TGTCCACCAGTTCTCTGAACT-3'.

Detection of mutation in *spectrin-β2* cDNA. The point mutation in the *spectrin-β2* gene creates a *PstI* restriction site (CGGCAG to CTGCAG, underlined italic letters indicate the site of mutation). To amplify *spectrin-β2* cDNA we used a forward primer (ACCCTGGCCCTGTACAAGAT) and reverse primer (TAGACTCGATGACCTTGGTCT). The PCR conditions used were 94°C for 2 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s. The PCR products were digested for 2 hrs at 37°C with *PstI* restriction enzyme, which cleaved mutant *spectrin-β2*, but not wild-type *spectrin-β2*, and generates a 200 bp fragment from cDNA. The products were resolved by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining.

Isolation of non-transformed cells from frozen primary d42m1 tumor chunk. A frozen d42m1 tumor chunk from the original d42m1 tumor was thawed and treated with 1 mg/ml type IA collagenase (Sigma) in HBSS for 2 hrs at room temperature. After filtration, single-cell suspensions were stained for 20 minutes at 4°C with 500 ng of Fc block (anti-CD16/32) and 200 ng of CD45-PE in 100 μL of staining buffer. Propidium iodide was added at 1 μg/mL immediately before sorting. A CD45⁺PI⁻ gate was used and the top 15% percent and the bottom 15% of gated events were collected using a FACSAria II (BD Biosciences).

Statistical Analysis. Samples were compared using an unpaired, two-tailed Student's *t* test.

RESULTS

cDNA CapSeq of Unedited MCA Sarcomas Reveals a Similarity to Carcinogen-induced Human Cancers

For this study, we chose two representative, highly immunogenic, unedited MCA sarcoma cell lines, d42m1 and H31m1, that grow progressively when transplanted orthotopically into *Rag2*^{-/-} mice, but are immunologically rejected when transplanted into naive wild type mice (19) (Figure 1 and 2). Using cDNA CapSeq, we identified 3,737 non-synonymous mutations in d42m1 cells (3,398 missense, 221 nonsense, 2 nonstop and 116 splice site mutations) and 2,677 non-synonymous mutations in H31m1 cells (2,391 missense, 160 nonsense, 3 nonstop and 123 splice site mutations) (Figure 3a and Figure 4). However, d42m1 and H31m1 share only 119 identical missense mutations when comparing sequences with at least 20X coverage (Figure 3b), thus explaining the unique antigenicity that each cell line displays (Figure 3c). Whereas d42m1 and H31m1 had mutations in 73 and 42 cancer-associated genes (220), respectively, most of these do not correspond to known activating or inactivating gene mutations in human cancers (221). However, d42m1 and H31m1 display mutations in *Kras* (d42m1 *Kras* G12C; H31m1 *Kras* G12D) and in *Trp53* (d42m1: *Trp53* E295^{stop}; H31m1: *Trp53* S152R and S258I) that are frequently observed in human and mouse cancers, including edited MCA sarcomas from wild type mice (222). These same mutant proteins have been shown to induce cancers *de novo* when co-expressed in transgenic mice (223, 224).

Figure 1. d42m1 is a highly immunogenic, unedited tumor. d42m1 tumor cells were injected at a dose of 1×10^6 into syngeneic wild type (**a-d**), *Rag2*^{-/-} (**a** and **c**), *Ifnar1*^{-/-} (**c**), or *Ifngr1*^{-/-} (**d**) mice. **b**, Groups of wild type mice injected with 1×10^6 d42m1 tumor cells were treated with control IgG, anti-CD4, or anti-CD8 α mAbs. **d**, d42m1 tumor cells were rendered insensitive to IFN- γ (d42m1 Δ IC) by expressing a dominant-negative version of IFNGR1 (IFNGR1 Δ IC) and were then transplanted (1×10^6 cells) into wild type mice. Data are presented as average tumor diameter \pm s.e.m. of 3-5 mice per group and are representative of at least three independent experiments. Samples were compared using an unpaired, two-tailed Student's *t* test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

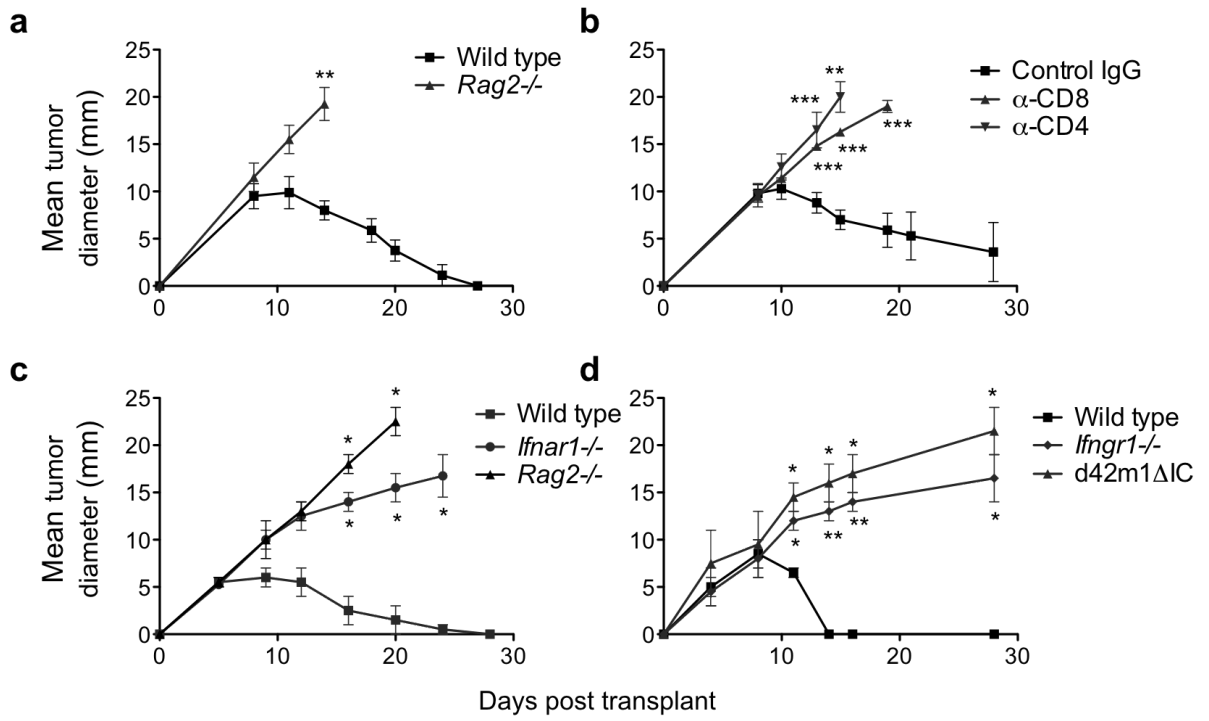


Figure 2. H31m1 is a highly immunogenic, unedited tumor. H31m1 tumor cells were injected at a dose of 1×10^6 into syngeneic wild type (**a-d**), *Rag2*^{-/-} (**a** and **c**), *Ifnar1*^{-/-} (**c**), or *Ifngr1*^{-/-} (**d**) mice. **b**, Groups of wild type mice injected with 1×10^6 H31m1 tumor cells were treated with control IgG, anti-CD4, or anti-CD8 α mAbs. **d**, H31m1 tumor cells were rendered insensitive to IFN- γ (H31m1 Δ IC) by expressing a dominant-negative version of IFNGR1 (IFNGR1 Δ IC) and were then transplanted (1×10^6 cells) into wild type mice. Data are presented as average tumor diameter \pm s.e.m. of 4-9 mice per group and are representative of at least three independent experiments. Samples were compared using an unpaired, two-tailed Student's *t* test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

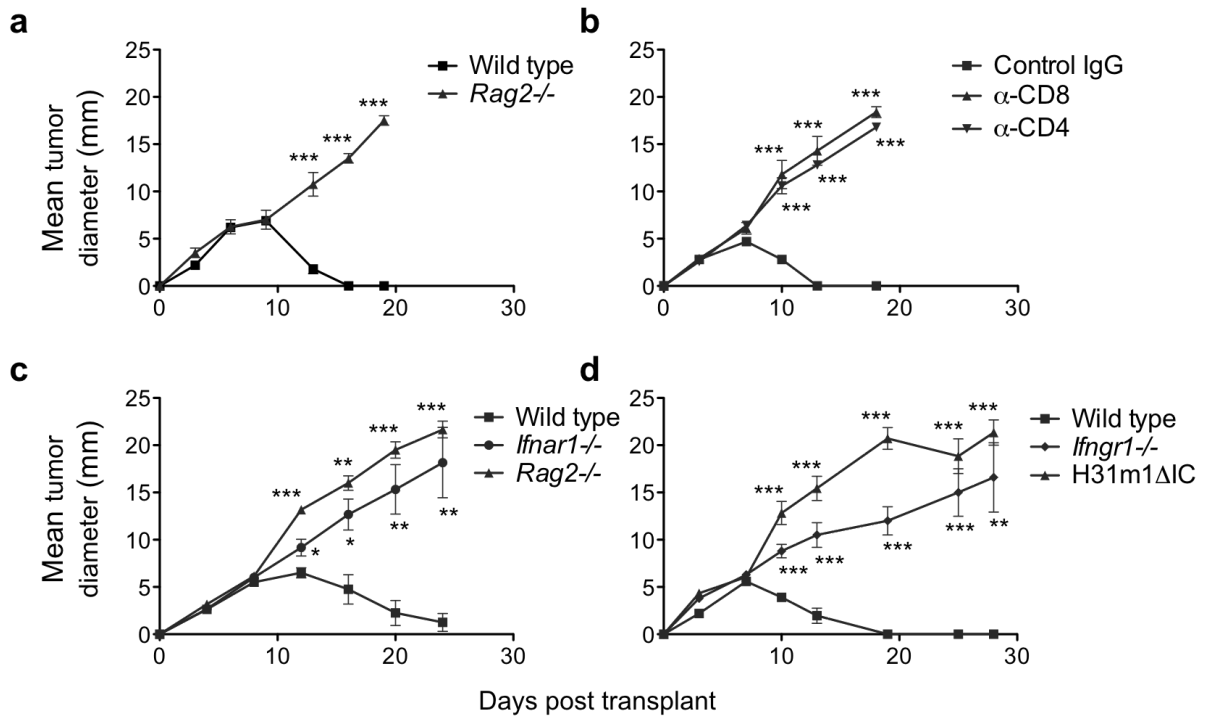


Figure 3. d42m1 and H31m1 are distinct antigenic and immunogenic tumors. a,

Number of somatic, non-synonymous mutations (missense, nonsense, nonstop, and splice site) in d42m1 and H31m1 tumor cells as detected by cDNA CapSeq. **b,** Venn diagram comparing the number of unique and shared missense mutations expressed in d42m1 and H31m1 tumor cells that had at least 20x sequencing coverage for each genomic site. **c,** IFN- γ ELISA assay using the bulk CTL lines developed against either parental d42m1 (left panel) or H31m1 (right panel) and tested against unedited MCA sarcoma cell lines, d42m1, H31m1, F510, 1773, 1782, and 1779 or the edited F244 MCA sarcoma derived from a wild type 129/Sv mouse. Data are representative of at least two independent experiments and are presented as average IFN- γ release \pm s.e.m. Samples were compared using an unpaired, two-tailed Student's *t* test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

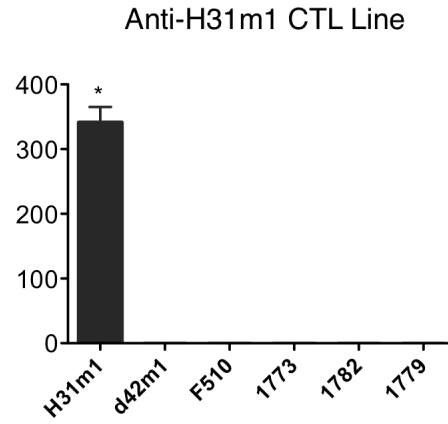
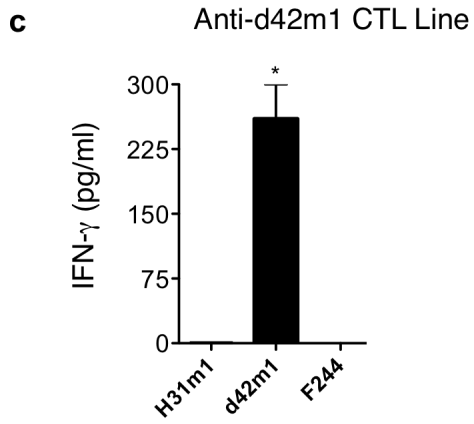
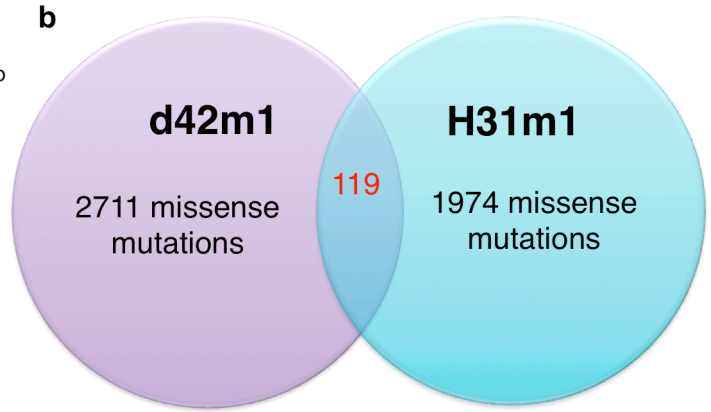
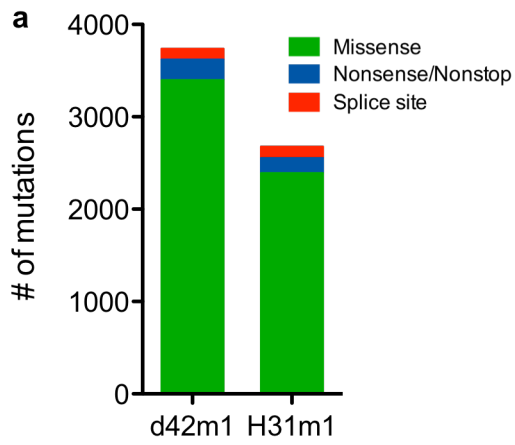
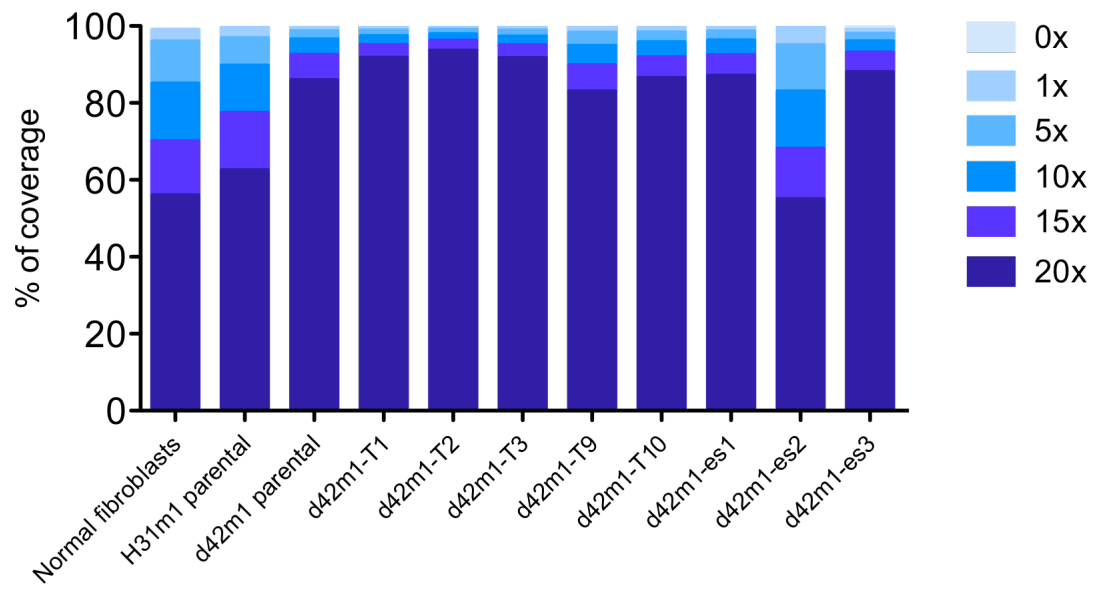


Figure 4. Exome sequencing coverage. Percentage of exome sequence coverage (20x, 15x, 10x, 5x, 1x, 0x) is displayed for the MCA sarcoma cell lines and normal skin fibroblasts that were isolated from three independent syngeneic 129/Sv *Rag2*^{-/-} mice.



When we compared the cDNA CapSeq data of d42m1 and H31m1 sarcoma cells to those of human cancer genomes (225-233), two similarities were observed. First, the overall mutation rates of d42m1 and H31m1 most closely resemble those of carcinogen-induced lung cancers from smokers, and particularly of “hypermutators” whose smoking induced-lung cancers develop mutations in genes encoding DNA repair components (Figure 5a). Interestingly, d42m1 and H31m1 also have mutations in DNA repair genes, including *Trp53*, *Atm*, *Brca2*, *Brip1*, *Fancd2*, *Fancg* and *Xpc*. Second, 46% and 47% of mutations in d42m1 and H31m1, respectively, are C/A or G/T transversions and thus represent typical carcinogen signatures (229, 230) similar to those established for lung cancers from smokers (44-46%), but distinct from those for other human cancers, including lung cancers from individuals who never smoked (15%) (Figure 5b). Thus, the current genomic analyses reveal the oft-proposed, but never documented similarity between MCA sarcomas in mice and carcinogen-induced human cancers.

cDNA CapSeq of d42m1 Tumor Variants

The d42m1 sarcoma cell line displays a sporadic tendency to produce escape variants following transplantation into naïve, syngeneic wild type mice (Figure 6a). In fact, escape variants are observed in about 20% of naïve wild type mice injected with parental d42m1 tumor cells (Figure 6c). Cell lines made from three such escape variants (d42m1-es1, d42m1-es2 and d42m1-es3) consistently formed progressively growing tumors when transplanted into naïve syngeneic recipients (Figure 6d and 7). In contrast, parental d42m1 tumor cells passaged through immunodeficient *Rag2*^{-/-} mice retained

their immunogenicity (Figure 6b, d). Thus, unedited d42m1 tumor cells can undergo immunoediting when transplanted into wild type mice.

To determine the basis for the heterogeneous behavior of d42m1 tumor cells in naive immunocompetent mice, we generated single cell clones from the parental cell line and tested the immunogenicity of each. Whereas 8 of 10 clones were rejected when transplanted into wild type mice, two (d42m1-T3 and d42m1-T10) grew progressively and displayed growth kinetics similar to the d42m1 escape variants (Figure 7). Thus, the parental d42m1 cell line consisted of a disproportionate mixture of regressor and progressor tumor cell clones.

cDNA CapSeq of d42m1 clones and escape variants revealed that all expressed similar numbers of mutations compared to parental d42m1 tumor cells (Figure 8a). Moreover, the missense mutations in clones and escape variants were similar to parental d42m1 cells, but distinct from those in H31m1 parental cells (Figure 8b). However, a greater percentage of mutations in d42m1 regressor clones were shared with parental d42m1 tumor cells (71-78%) than those shared between parental d42m1 tumor cells and d42m1 progressor clones (48%) or escape variants (33-35%) (Figure 8b), a result that further supports the conclusion that the d42m1 cell line consists of a related, but heterogeneous population of tumor cells.

Figure 5. Unedited MCA sarcomas, d42m1 and H31m1, resemble carcinogen-induced human cancers. a, Log mutation rate/10 Mbp for d42m1 and H31m1 sarcomas as compared to previously generated data from a panel of human cancers including acute myelogenous leukemia (225, 226) (AML), chronic lymphocytic leukemia (232) (CLL), breast cancer (breast-lobular (228), breast-basal (227), aromatase inhibitor (AI)-resistant, and AI-sensitive (Ding et al., manuscript in review)), ovarian cancer (OVC1, 3, 4, 5) (manuscript in preparation), liver cancer (Hepatitis C Virus (HCV)-positive) (231), melanoma (ultraviolet (UV)-induced) (233), and lung cancers (non-small cell (NSC) (229), small cell (SC) (230), Never-Smoker, Smoker, and Hypermutator (manuscript in progress)). Only protein-coding alterations (Tier 1 SNVs) were used to calculate mutation rates. **b,** Spectrum of DNA nucleotide transitions and transversions detected in d42m1 and H31m1 sarcomas and human cancers described in **a**.

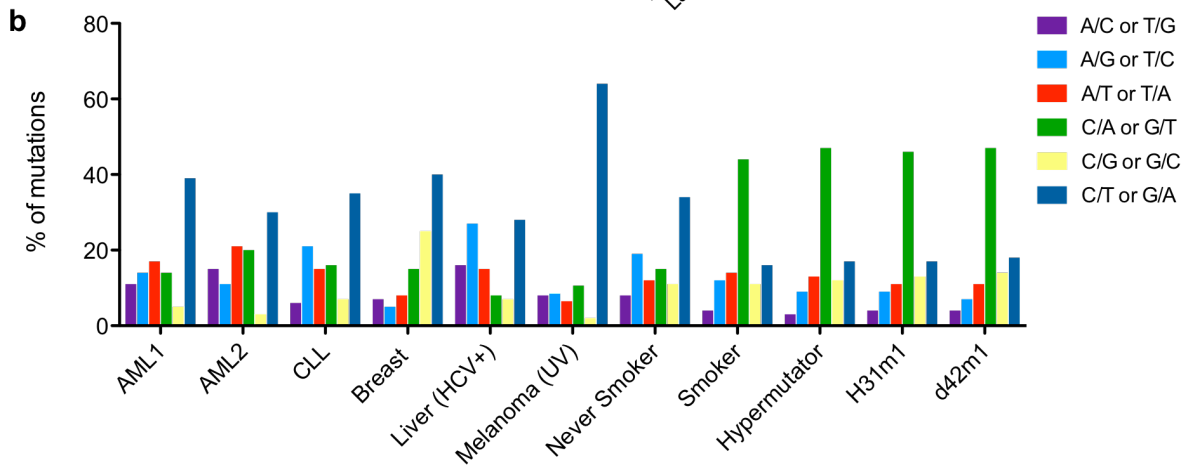
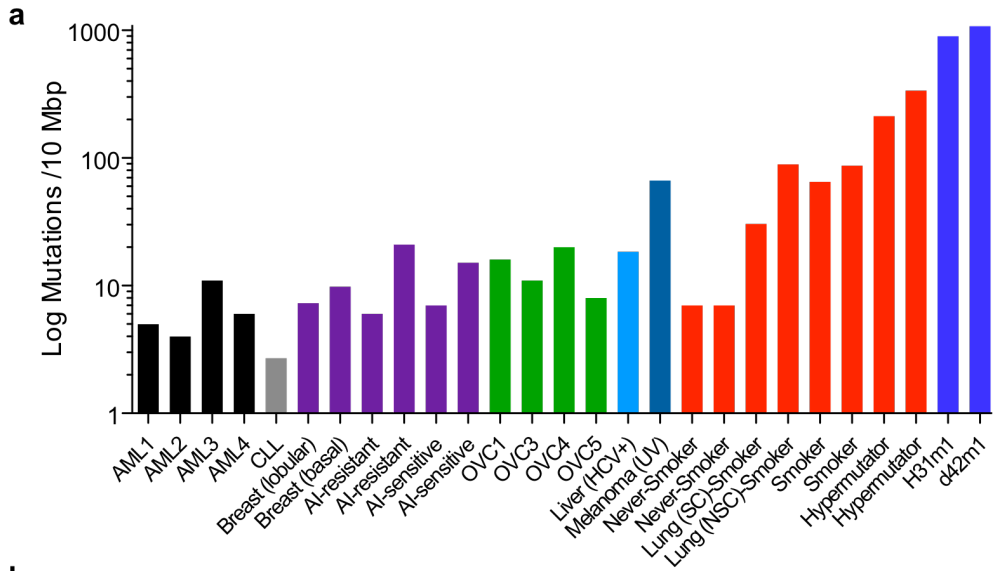


Figure 6. Escape variants of d42m1 display reduced immunogenicity and an edited growth phenotype. **a**, d42m1 tumor cells (1×10^6) were transplanted into wild type (solid lines) or *Rag2*^{-/-} (dashed lines) mice. **b**, The escape variant d42m1-es3 (harvested from the wild type mouse bearing an escaped d42m1 tumor in a and generated into a cell line) (n=5, diamonds) or d42m1-RagPass (n=5, squares) were transplanted (1×10^6 cells) into wild type mice. Data presented as average tumor diameter \pm s.e.m of 5 mice per group over time. **c**, Wild type mice or *Rag2*^{-/-} mice were challenged with 1×10^6 d42m1 tumor cells. Data presented as percent tumor positive from 2-4 independent experiments (n=4-6 mice per group). **d**, Wild type mice were challenged with 1×10^6 d42m1-RagPass, d42m1-es1, d42m1-es2, or d42m1-es3 tumor cells. Data presented as percent tumor positive from two independent experiments (n=4-5 mice per group). Samples were compared using an unpaired, two-tailed Student's *t* test (**p<0.01 and ***p<0.001)

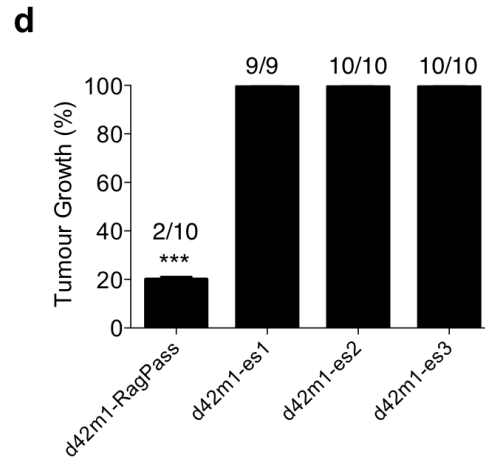
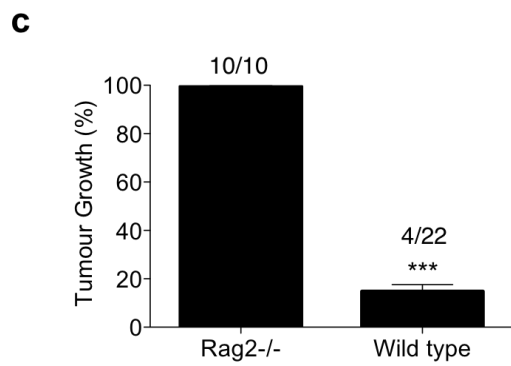
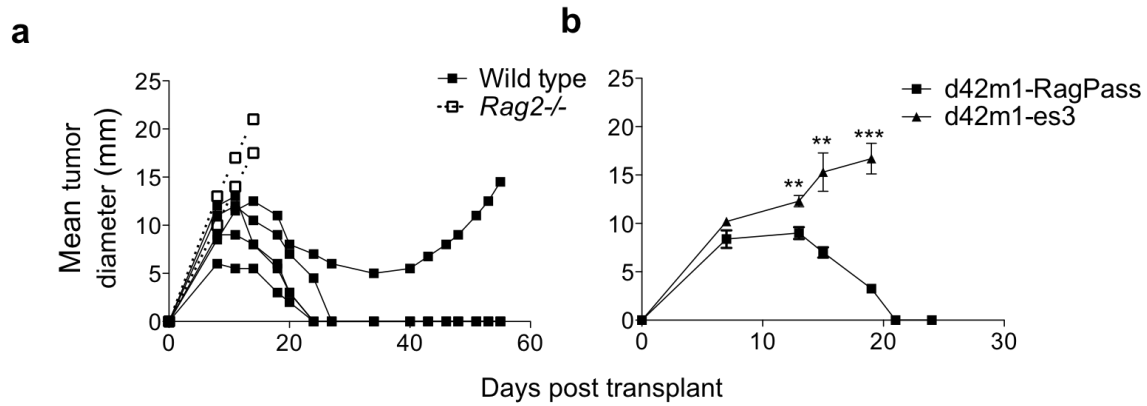


Figure 7. d42m1 tumor cell clones display heterogeneous growth characteristics.

d42m1 tumor clones (d42m1-T1, -T2, -T3, -T4, -T5, -T6, -T7, -T8, -T9, and -T10) and escape variants (d42m1-es1, -es2, and -es3) were transplanted at a dose 1×10^6 cells into wild type mice (n=5, squares). Data are presented as average tumor diameter \pm s.e.m. and are representative of two-three independent experiments.

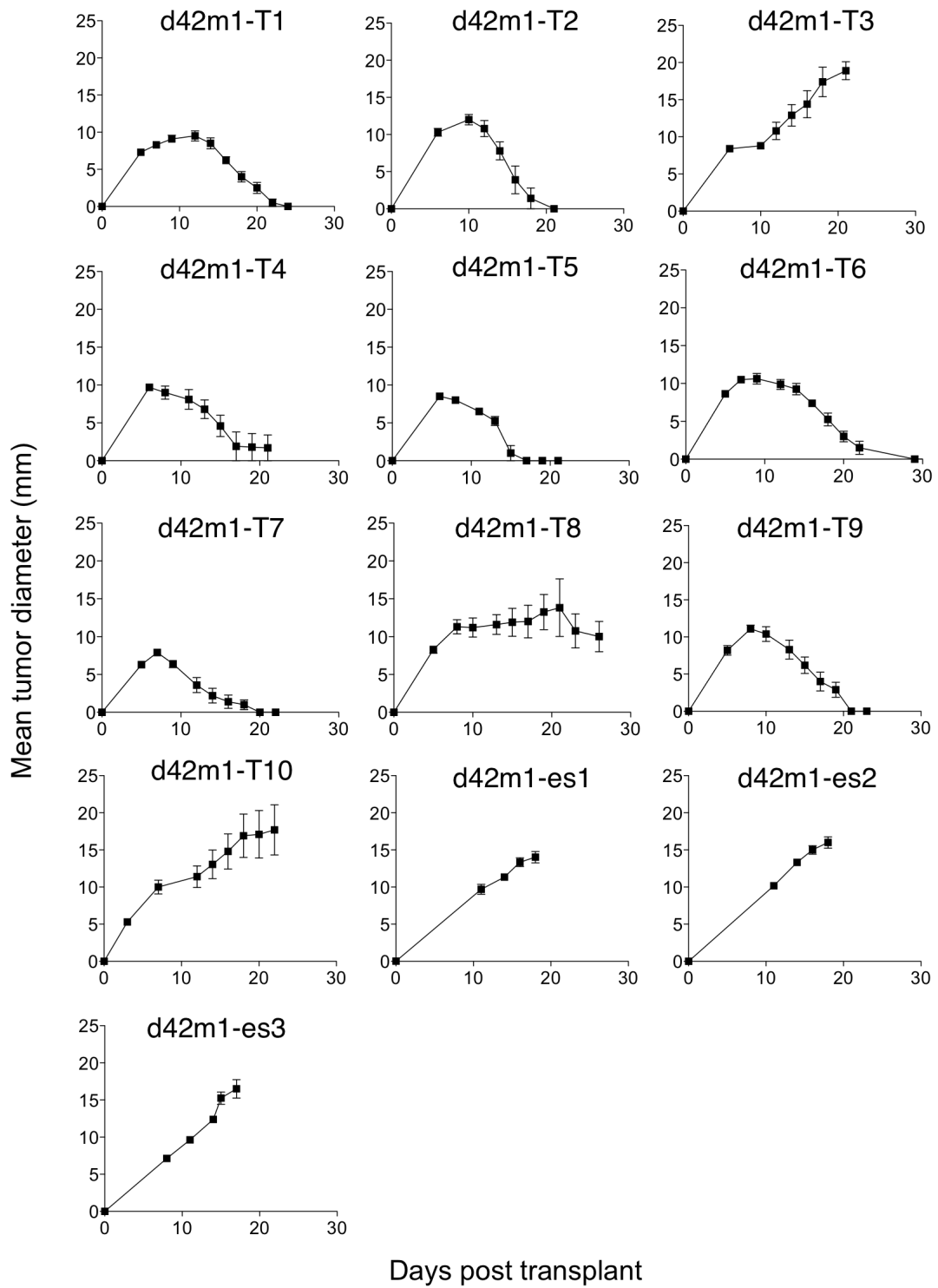
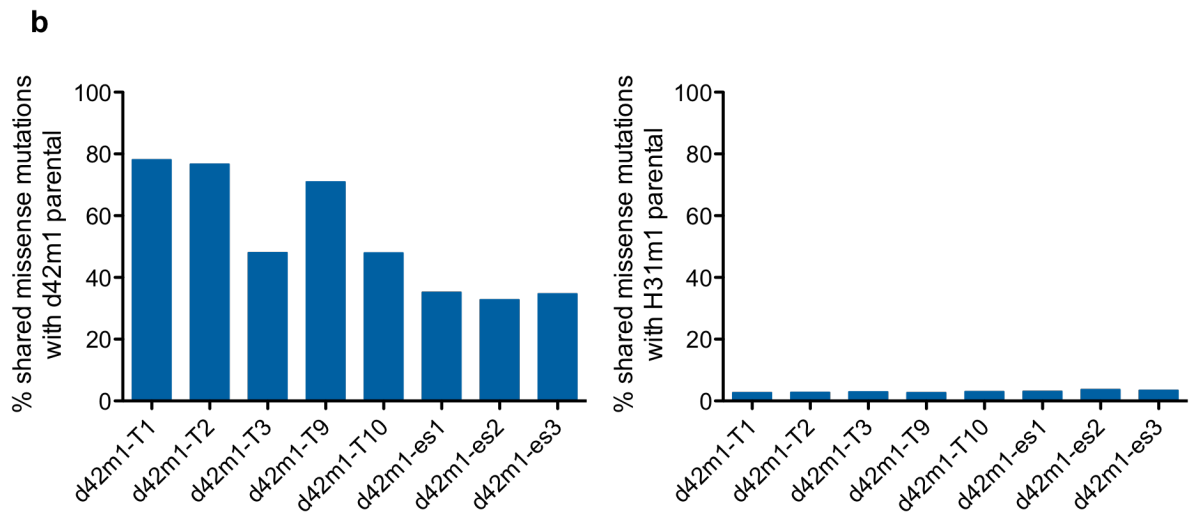
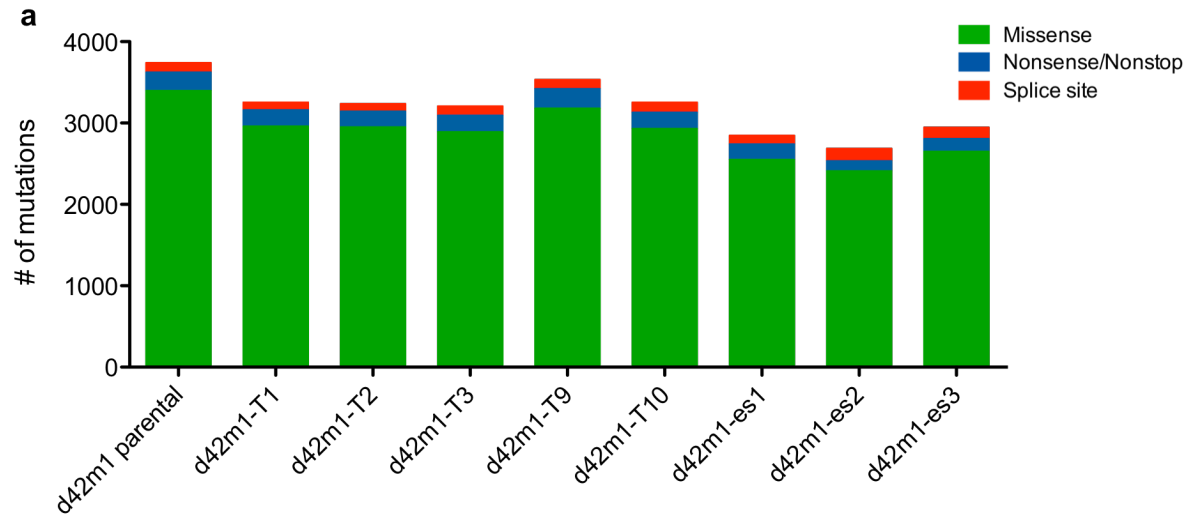


Figure 8. d42m1 tumor cell clones are closely related to d42m1 parental cells. a, Number and type of non-synonymous somatic mutations (missense, nonsense, nonstop, and splice site mutations) in d42m1 tumor variants. **b,** Percent of shared missense mutations expressed in each d42m1 tumor cell variant when compared with d42m1 parental cells (left) or H31m1 parental cells (right).



Identifying Potential d42m1 Tumor Antigens From Genomic Data

We next assessed the theoretical capacity of peptides containing each missense mutation to bind to MHC class I proteins (i.e., to function as neoantigens) by *in silico* analysis (218). As these tumors were generated on a 129/Sv genetic background, we restricted our analysis to H-2D^b and H-2K^b. Remarkably, among the large number of potential mutant epitopes examined for binding to H-2D^b or H-2K^b, only 32 (0.06%) and 138 (0.27%) displayed high affinity binding potential ($IC_{50} < 50\text{nM}$; Affinity Value > 2) to H-2D^b and H-2K^b, respectively (Figure 9).

To further simplify the pattern, we asked whether all of the d42m1 regressor variants shared a common rejection antigen. Using a d42m1 specific CD8⁺ cytotoxic T lymphocyte (CTL) clone (C3) derived from a wild type mouse that had rejected parental d42m1 tumor cells, we observed reactivity *in vitro* (as evidenced by IFN- γ production) with parental d42m1 tumor cells and with regressor d42m1 tumor cell variants, but not with progressor d42m1 tumor cell variants or unrelated MCA sarcoma cells (Figure 10a, b). Together, these results reveal that the regressor d42m1 tumor cell variants, indeed, share a common rejection antigen. This conclusion was supported by the additional finding that the original CD8⁺ T cell line from which the C3 clone was derived displayed a limited oligoclonality, as evidenced by V β usage limited only to V β 6 and V β 8.1/8.2. This result suggests that d42m1 expresses only a limited number of rejection antigens, perhaps even a single immunodominant antigen. Therefore, we focused on the limited number of epitopes that were common to all d42m1 regressor variants (Figure 11). Furthermore, recognition of all d42m1 regressor variants by the C3 clone was restricted by H-2D^b (Figure 12). Based on these results, the R913L mutation in spectrin- β 2

represents the best potential rejection antigen candidate because of its high affinity for H-2D^b (Figure 11). The mutation in spectrin- β 2 most likely represents a passenger rather than a driver mutation since (a) spectrin- β 2 is a cytoskeletal protein whose only known role is maintaining plasma membrane integrity, (b) expression of mutant spectrin- β 2 is not required for the neoplastic phenotype of d42m1 tumor cell clones, and (c) it is not on the list of known human cancer genes (220).

Mutant spectrin- β 2 is a d42m1 Specific Tumor Antigen

To verify the predicted importance of mutant spectrin- β 2 on d42m1 antigenicity, we used a T cell based expression cloning approach (209) to independently identify the tumor antigen recognized by the C3 CTL clone. After three rounds of screening 120,000 cDNA clones, a single positive cDNA was identified encoding a 1,722 bp fragment with a sequence identical to *spectrin- β 2* except for a single G to T point mutation resulting in an amino acid substitution of arginine to leucine at position 913. Thus, conventional antigen expression cloning identified the same sequence that was predicted using the cDNA capture sequencing/*in silico* approach.

To establish whether mutant spectrin- β 2 represents a tumor-specific antigen, we first assessed expression of mutant *spectrin- β 2* mRNA in the various d42m1 cell variant populations by mutation specific qRT-PCR. Mutant *spectrin- β 2* mRNA was expressed in parental d42m1 cells and d42m1 regressor clones, but not in d42m1 progressor clones or escape variants (Figure 13). Moreover, the mutant form of *spectrin- β 2* was not observed in normal tissue derived from the very same mouse as the d42m1 tumor, revealing that the mutation was truly tumor cell specific and not due to a mouse-specific polymorphism.

We then asked whether T cells could discriminate between the mutant and native spectrin- β 2 905-913 peptide sequences when presented on H-2D^b. For this purpose, we synthesized wild type (VAVVNQIAR) and mutant (VAVVNQIAL) 905-913 spectin- β 2 peptides and tested their ability to stimulate IFN- γ production from the C3 clone when presented on an unrelated H-2D^b expressing cell line. Whereas the mutant peptide stimulated C3 CTL cells in a dose-dependent manner, the wild type peptide did not, even when added in 1000-fold excess (Figure 14).

To document that the anti-R913L spectrin- β 2 response occurred under physiologic conditions, we used labeled H-2D^b tetramers generated with the 905-913 spectrin- β 2 mutant peptide to identify CD8⁺ T cells in d42m1 variant tumors. Mutant spectrin- β 2 specific CD8⁺ T cells were detected in parental d42m1 tumors and draining lymph nodes (DLNs) as early as 6 days after tumor injection and gradually increased in numbers until day 11 (just prior to tumor rejection), where they reached a maximum of 5% and 0.5% of the CD8⁺ T cells in the tumor and DLN, respectively (Figure 15). In contrast, no mutant spectrin- β 2 specific CD8⁺ T cells were detected in d42m1-es3 tumors or DLNs (Figure 15). Together these data demonstrate that a mutated gene expressed selectively in unedited d42m1 tumor cells, gives rise to a mutant protein that evokes a naturally occurring T cell response in naïve wild type mice. Thus mutant spectrin- β 2 is an authentic tumor-specific antigen of d42m1 sarcoma cells.

Figure 9. Affinity value profiles of predicted MHC class I epitopes from tumor-specific mutations. All missense mutations for each d42m1 tumor variant were analyzed for the potential to form MHC class I neoepitopes that bind to either H-2D^b or H-2K^b molecules. The artificial neural network (ANN) algorithm provided by the Immune Epitope Database and Analysis Resource was used to predict epitope binding affinities and the results were ultimately expressed as “Affinity Values” (Affinity Value = $1/IC_{50} \times 100$). Mutant epitopes present in each individual d42m1 tumor variant are displayed. Arrow is pointing to one of the H-2D^b epitopes created by the R913L point mutation in spectrin- β 2 that is expressed only in d42m1 cell variants that are rejected in wild type mice.

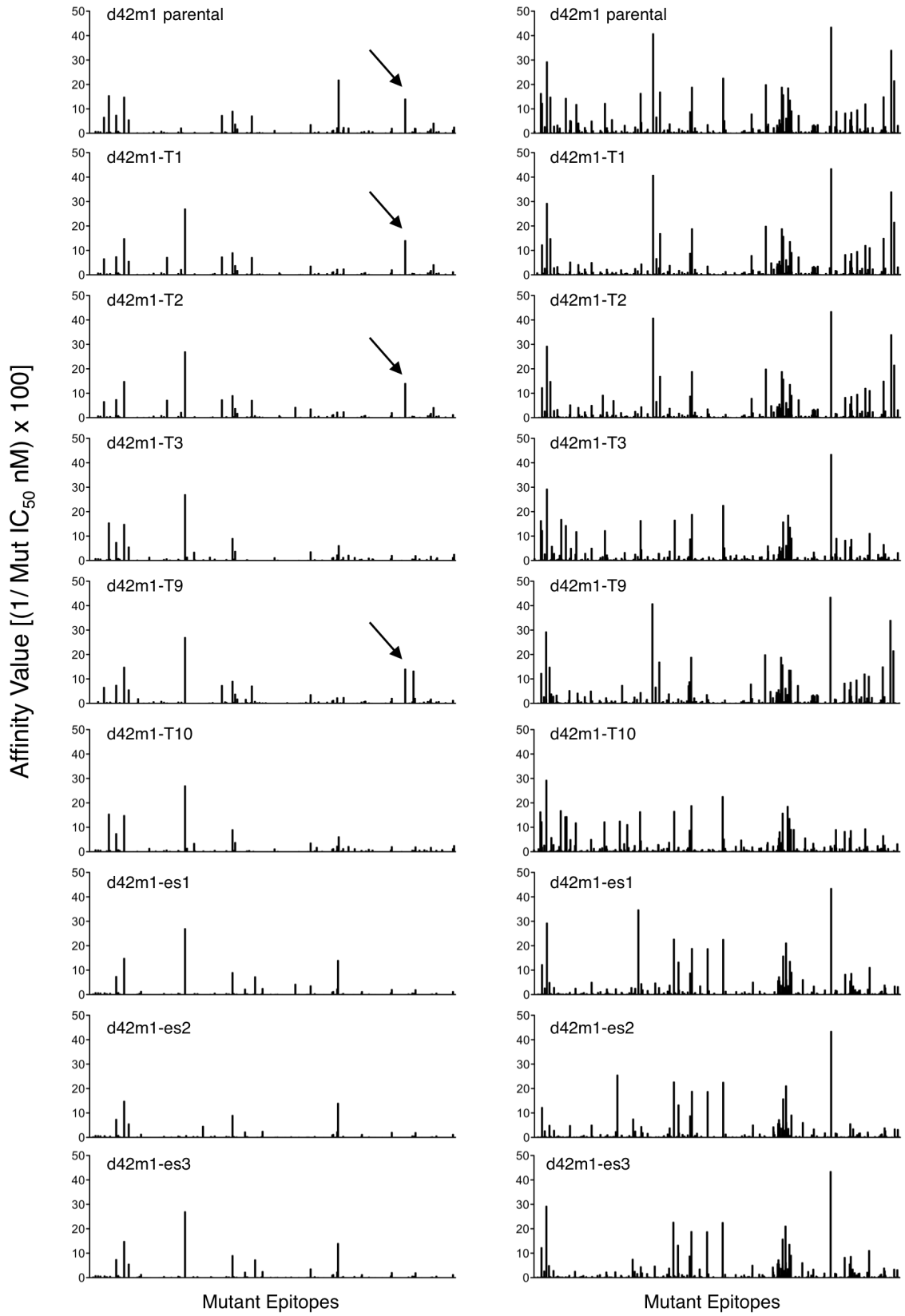


Figure 10. CD8⁺ T cells selectively recognize highly immunogenic d42m1 tumor variants. a, b, IFN- γ ELISA assay of C3 CTL cells generated from splenocytes of a mouse that rejected d42m1, against five different unedited sarcoma cell lines (**a**) or against d42m1 tumor clones and escape variants (**b**). Data are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's *t* test (* $p < 0.05$ and ** $p < 0.01$; n.s. is non-significant).

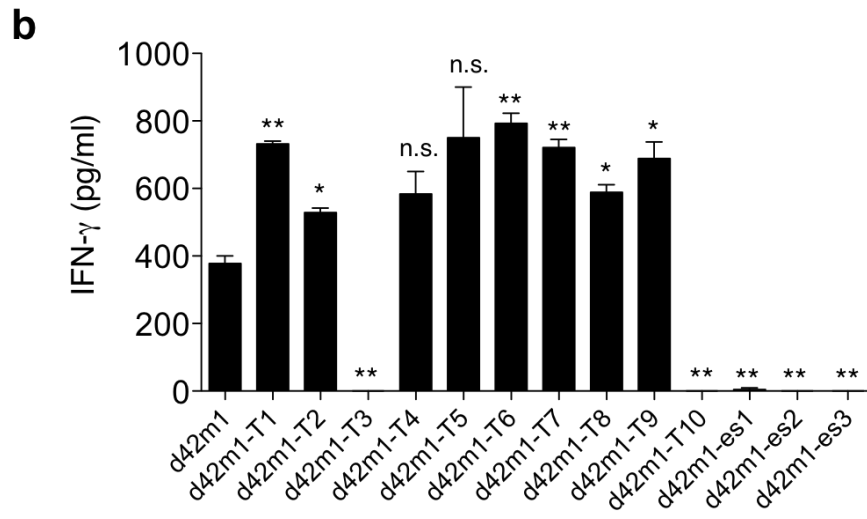
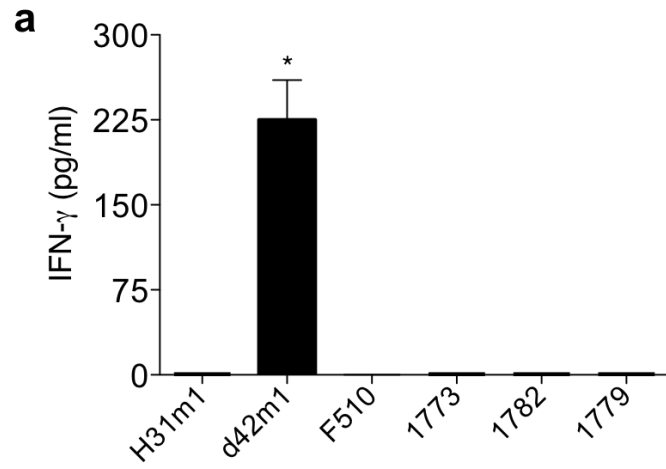


Figure 11. Shared epitopes among highly immunogenic d42m1 variants. MHC class I epitopes predicted to be shared in all of the highly immunogenic d42m1 tumor variants (d42m1 parental, -T1, -T2, and -T9), but not in d42m1 tumor variants that display reduced immunogenicity (d42m1-T3, -T10, -es1, -es2, and -es3) for H-2D^b (top) and H-2K^b (bottom). One of the H-2D^b epitopes created by the R913L point mutation in spectrin-β2 is highlighted in red.

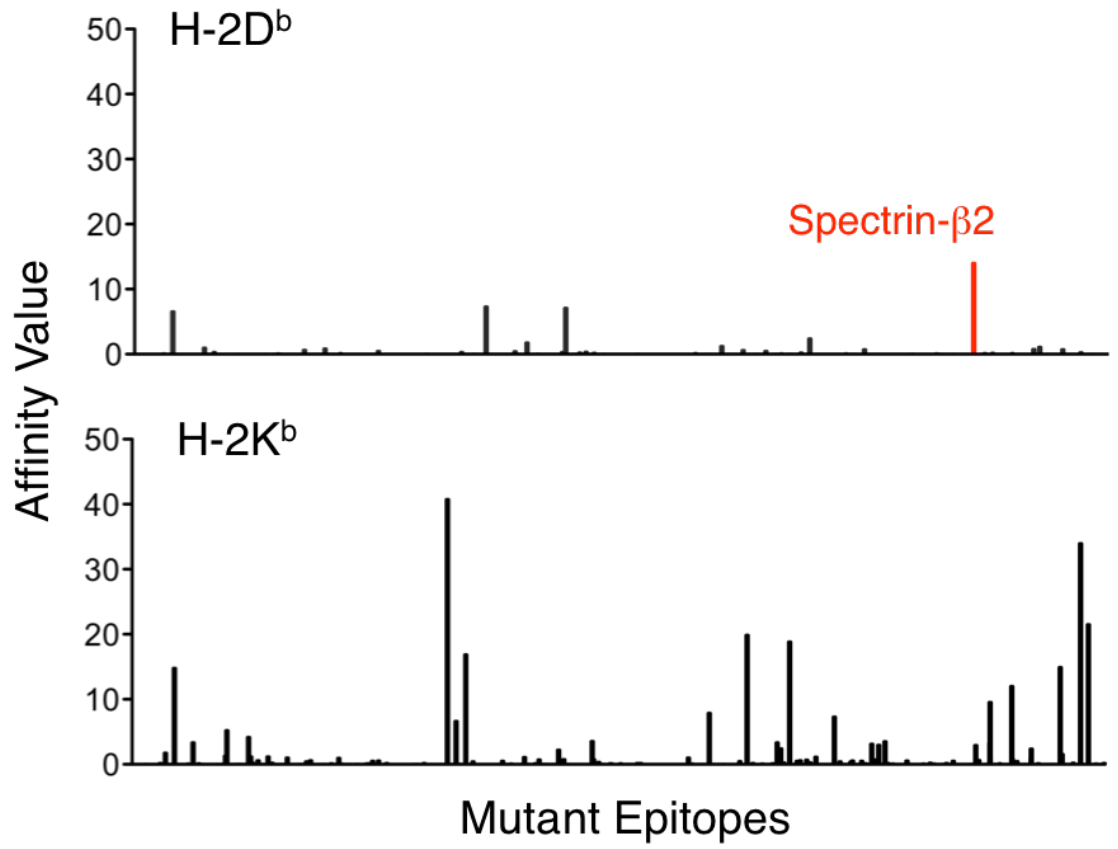


Figure 12. CD8⁺ T cell recognition of d42m1 parental tumor cells is H-2D^b restricted. IFN- γ ELISA assay of C3 CTL cells against d42m1 parental tumor cells with the addition of antibodies that block CD4, CD8 α , H-2D^b, or H-2K^b. Data are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's *t* test (**p<0.01).

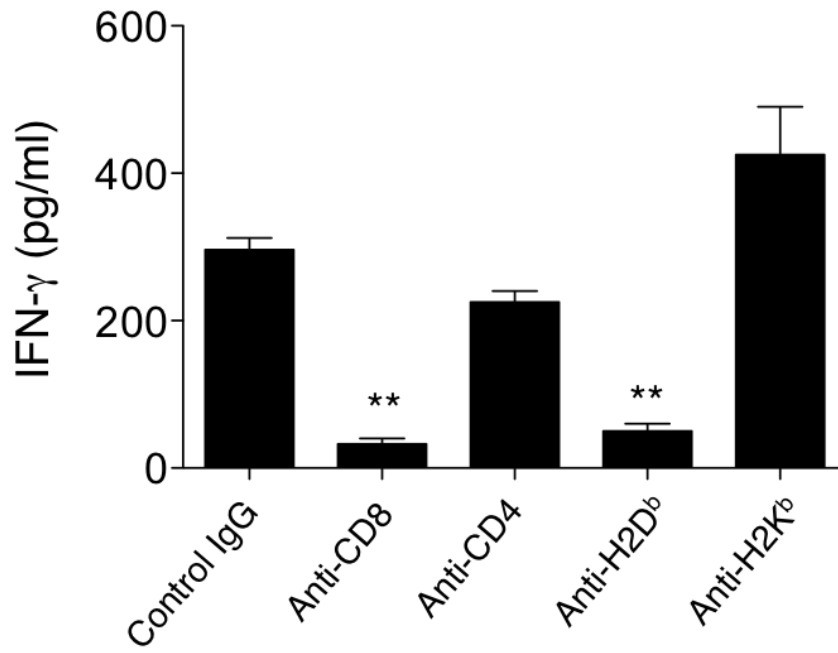


Figure 13. Expression of mutant *spectrin-β2* is restricted to highly immunogenic d42m1 tumor variants. qRT-PCR analyses using primer pairs specific for mutant *spectrin-β2* in d42m1 parental, d42m1 clones and escape variants and 1773, an unrelated unedited sarcoma.

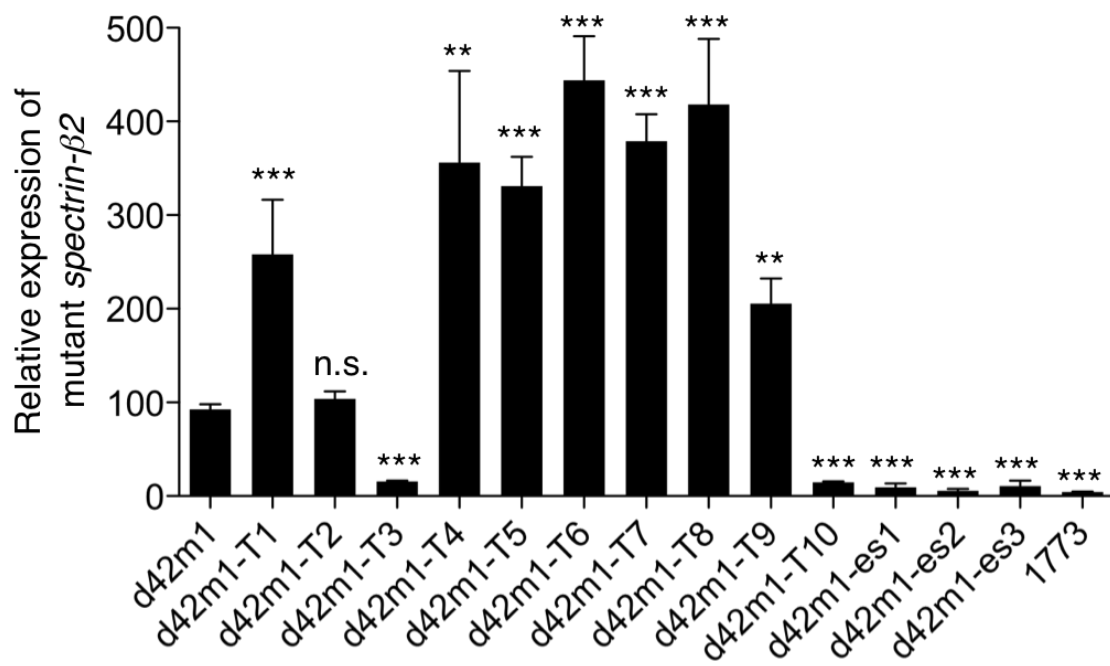


Figure 14. C3 CTL clone recognizes mutant 905-913 spectrin-β2 peptide. a, Estimation of IC₅₀ (nM) for wild type and mutant spectrin-β2 epitopes on H-2D^b by Immune Epitope Database and Analysis Resource. **b,** IFN-γ ELISA assay of C3 CTL cells against COS-D^b cells pulsed with wild type (circles) or mutant (squares) spectrin-β2 peptides. Data are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test (*p<0.05 and **p<0.01).

a

	Peptides	*IC ₅₀ (nM)
Spectrin-β2	VAVVNQIAR (wild type)	5305
	VAVVNQIAL (mutant)	7

*Immune Epitope Database and Analysis Resource

b

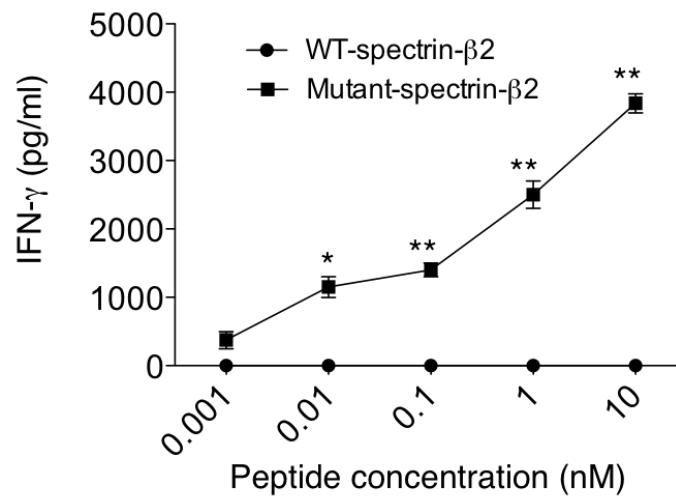
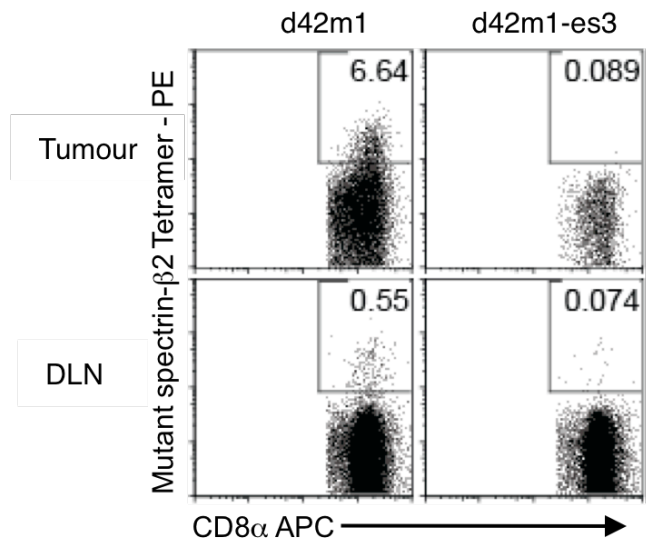
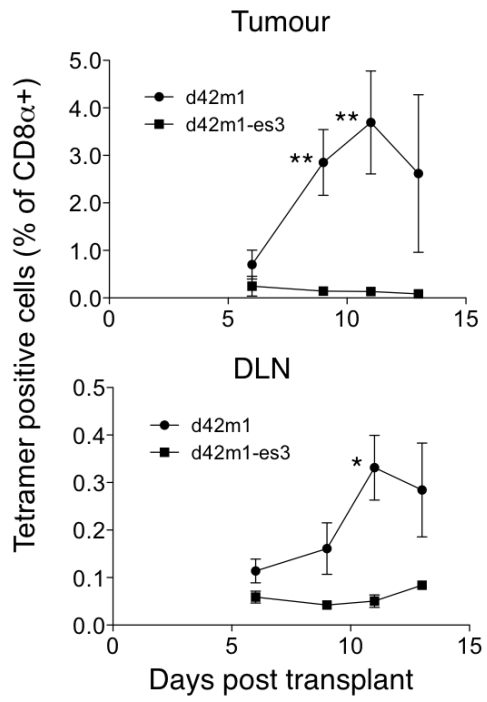


Figure 15. Mutant spectrin-β2 specific CD8⁺ T cells infiltrate d42m1 parental tumors, but not d42m1-es3 tumors. Time course of tetramer positive CD8α⁺ T cells in tumors and DLNs from d42m1 or d42m1-es3 tumor-bearing mice. Tumors and DLNs were harvested from d42m1 (n=3, circles) or d42m1-es3 (n=3, squares) -bearing mice at days 6, 9, 11, and 13. Data includes results from 3 mice per group and is representative of two independent experiments (left). Tumors and draining lymph nodes (DLNs) were harvested at day 11 and CD8α⁺ T cells were stained with mutant spectrin-β2 tetramers (right). Data are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test (*p<0.05 and **p<0.01).



Mutant spectrin-β2 is the Major Rejection Antigen of d42m1 Tumor Cells

In order to explore whether mutant spectrin-β2 represents the major rejection antigen of parental d42m1 tumor cells, we enforced expression of either the mutant or wild type forms of spectrin-β2 into d42m1-es3 cells (Figure 16). When injected into wild type mice, d42m1-es3 tumor cell clones expressing GFP alone or GFP plus wild type spectrin-β2 (WT.1 and WT.3) grew progressively and displayed similar growth kinetics to the parental d42m1-es3 cell line (Figure 17a, c). In contrast, d42m1-es3 tumor cell clones expressing GFP plus mutant spectrin-β2 (mu.6 and mu.14) were rejected in wild type mice, but not in *Rag2*^{-/-} mice (Figure 17b, c, d). CD8⁺ T cells specific for mutant spectrin-β2 did not infiltrate d42m1-es3 tumors expressing wild type spectrin-β2 (WT.3), but were detected by tetramer staining in rejecting d42m1-es3 tumors that had been reconstituted with mutant spectrin-β2 (mu.14) (Figure 17e). The frequency of antigen-specific T cells infiltrating mutant spectrin-β2 expressing tumors was similar to that in mice rejecting parental d42m1 tumors. These results demonstrate that expression of mutant spectrin-β2 is both necessary and sufficient for the rejection of d42m1 tumors and thus, validates it as the major rejection antigen of d42m1 sarcoma cells.

Immunoselection is the Immunoediting Mechanism for d42m1 Tumor Cells

The results presented thus far identified mutant spectrin-β2 as the major rejection antigen of d42m1, established that the d42m1 tumor cell line is heterogeneous in its expression of mutant spectrin-β2, and showed that loss of mutant spectrin-β2 in d42m1 cells following exposure to an intact immune system results in tumor cell variants that escape immune control. Since our cDNA CapSeq analyses were performed on cDNA

derived from tumor cells, we tested whether these observations could be explained by epigenetic silencing of the mutated form of the *spectrin-β2* gene. We therefore, treated clones and escape variants of d42m1 that did not express mutant *spectrin-β2* with methyltransferase and histone deacetylase inhibitors (i.e., 5-azacytidine and trichostatin A, respectively) alone or in combination and assessed expression of mutant *spectrin-β2* mRNA. Mutant *spectrin-β2* expression was not induced in d42m1 progressor clones or escape variants (H. Matsushita and R.D. Schreiber, data not shown). We therefore formulated the hypothesis that T cell dependent immunoselection was a likely mechanism favoring outgrowth of tumor variants that lack strong rejection antigens. This possibility is consistent with our finding that every d42m1 clone that expresses mutant spectrin-β2 was rejected while every clone or variant that lacks mutant spectrin-β2 formed progressively growing tumors. To formally test this hypothesis, we assessed the *in vivo* behavior of a disproportionate mixture of a highly immunogenic d42m1 tumor cell clone expressing mutant spectrin-β2 (i.e., d42m1-T2) and a limiting amount of a d42m1 tumor cell clone lacking mutant spectrin-β2 (i.e., d42m1-T3). To distinguish between these two cell populations *in vivo* we labeled d42m1-T2 with an altered, non-immunogenic form of RFP that lacked class I epitopes and labeled d42m1-T3 with GFP. We first documented that the inherent *in vivo* growth characteristics of the two cell lines were not altered (Figure 18a). We then tested several different ratios of the two clones and found that we could recapitulate the tumor growth phenotype of parental d42m1 at a ratio of 95% regressor d42m1-T2RFP cells to 5% progressor d42m1-T3GFP cells (Figure 18b). At this ratio, 100% of *Rag2*^{-/-} mice developed progressively growing tumors. Similar results were obtained using wild type mice depleted of either CD4⁺ or CD8⁺ T

cells (Figure 18c). In contrast, 5/20 (25%) wild type mice injected with the tumor cell mixture developed escape tumors, a result that closely resembles the behavior of parental d42m1 tumor cells in wild type recipients. Tumors harvested from *Rag2*^{-/-} mice were comprised of 84% RFP⁺ d42m1-T2 tumor cells and 14% GFP⁺ d42m1-T3 cells (Figure 18d) as detected by flow cytometry, a ratio that is very similar to the initial 95:5 mixture that was injected. At tumor harvest, the cell mixture expressed mutant *spectrin-β2* as detected by mutation specific qRT-PCR (Figure 18e). In contrast, tumors that grew out in wild type mice consisted of 98% GFP⁺ d42m1-T3 tumor cells and lacked mutant *spectrin-β2* expression. Thus, escape variants of parental d42m1 tumor cells develop as a consequence of a T cell dependent immunoselection process that favors the outgrowth of tumor cell clones that lack the major rejection antigen.

Figure 16. Enforced expression of mutant spectrin- β 2 in d42m1 escape variant tumor cells. qRT-PCR analysis using a primer pair specific for mutant spectrin- β 2 in d42m1-es3 tumor cell clones that have been engineered to express either wild type or mutant spectrin- β 2. Data is displayed as relative expression after normalization to control GAPDH expression and is representative of two independent experiments

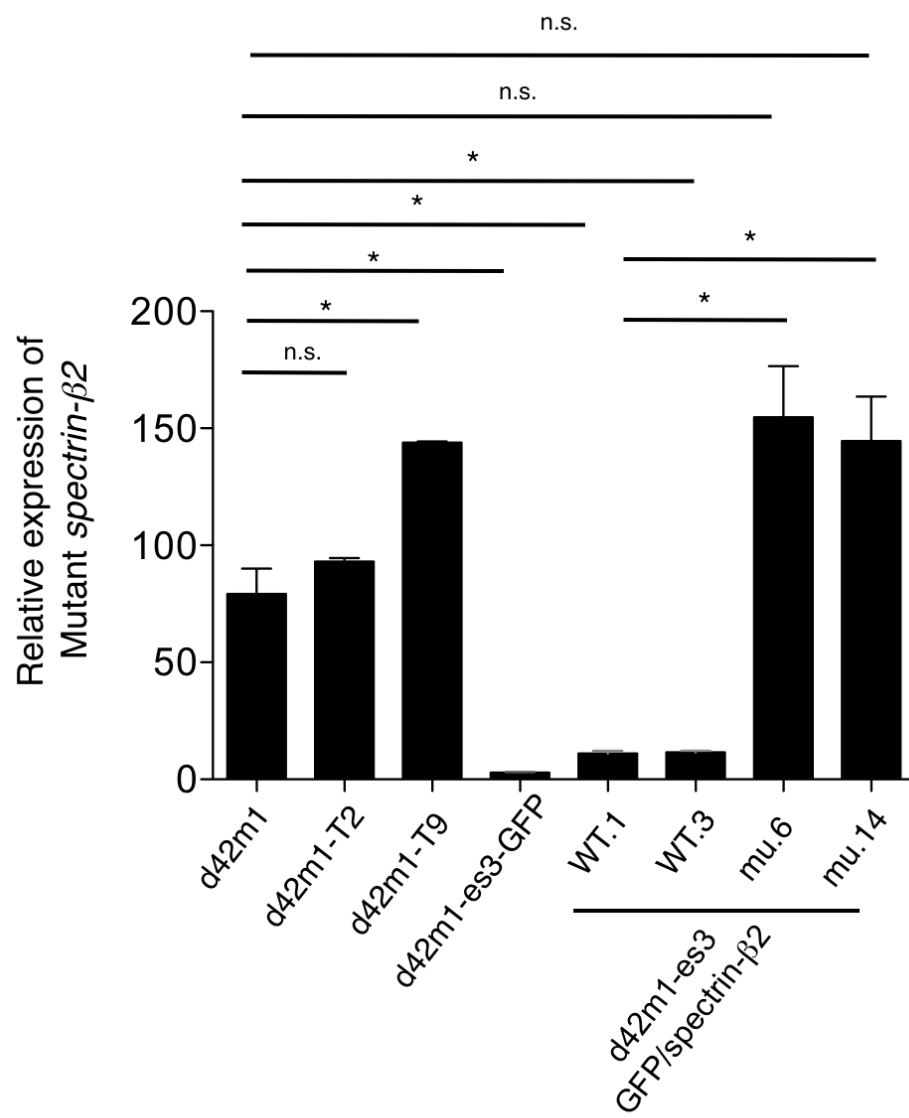


Figure 17. Mutant spectrin- β 2 is the major rejection antigen of d42m1 tumor cells.

a, b, d, d42m1-es3 tumor cell clones reconstituted with wild type or mutant spectrin- β 2 and control d42m1-es3 expressing only GFP were transplanted at a dose of 1×10^6 cells into five-member groups of wild type (**a, b**) or *Rag2*^{-/-} mice (**d**). Data are presented as average tumor diameter \pm s.e.m. over time. **c**, Percent tumor positive with five wild type mice per group from 2-3 independent experiments is shown. **e**, d42m1-es3 tumors reconstituted with wild type (WT.3) or mutant spectrin- β 2 (mu.14) were harvested at day 11 (**a**) and CD8 α^+ T cells were stained with mutant spectrin- β 2 tetramers. Samples were compared using an unpaired, two-tailed Student's *t* test (**p*<0.05, ***p*<0.01, and ****p*<0.001; n.s. is non-significant).

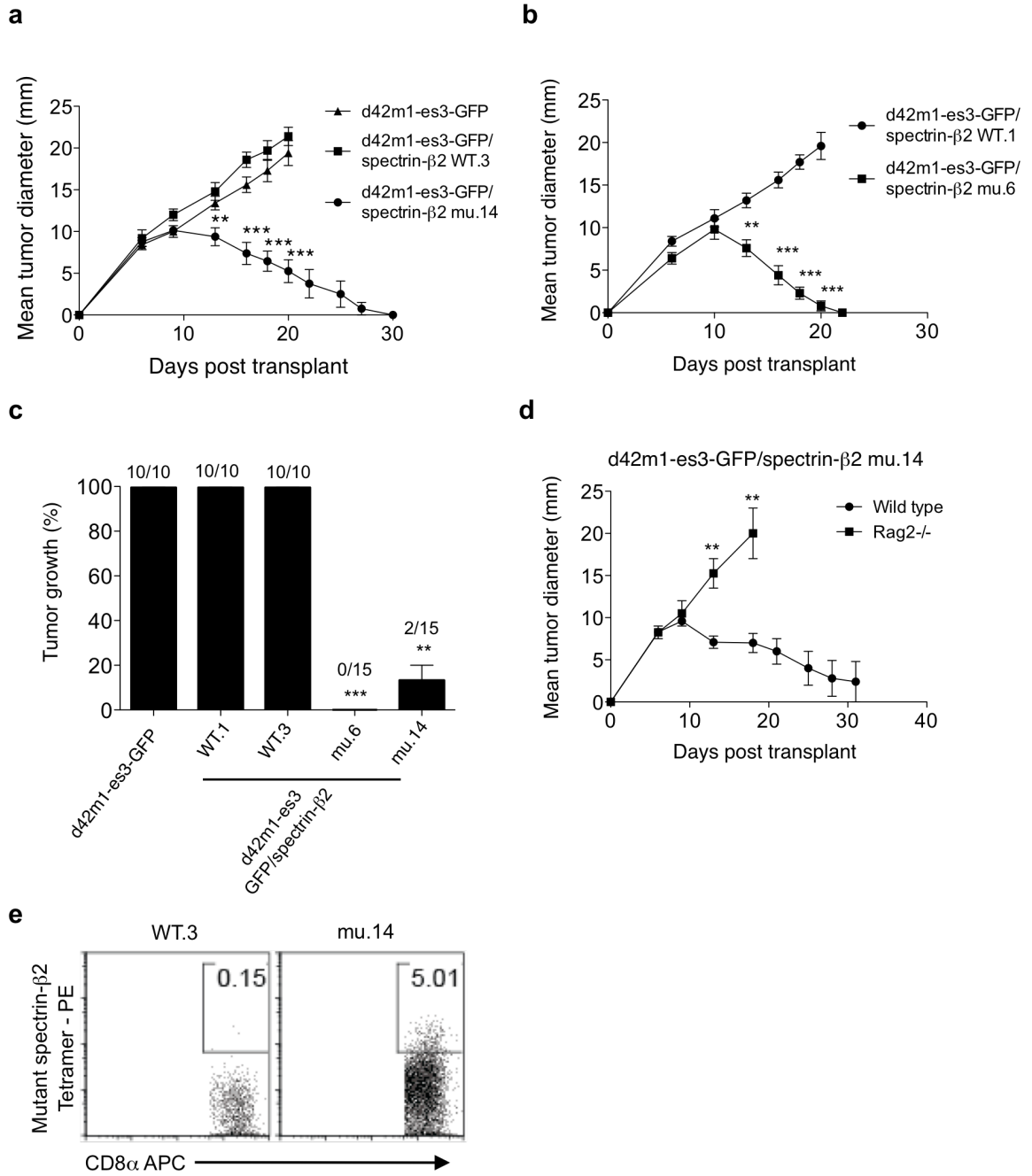
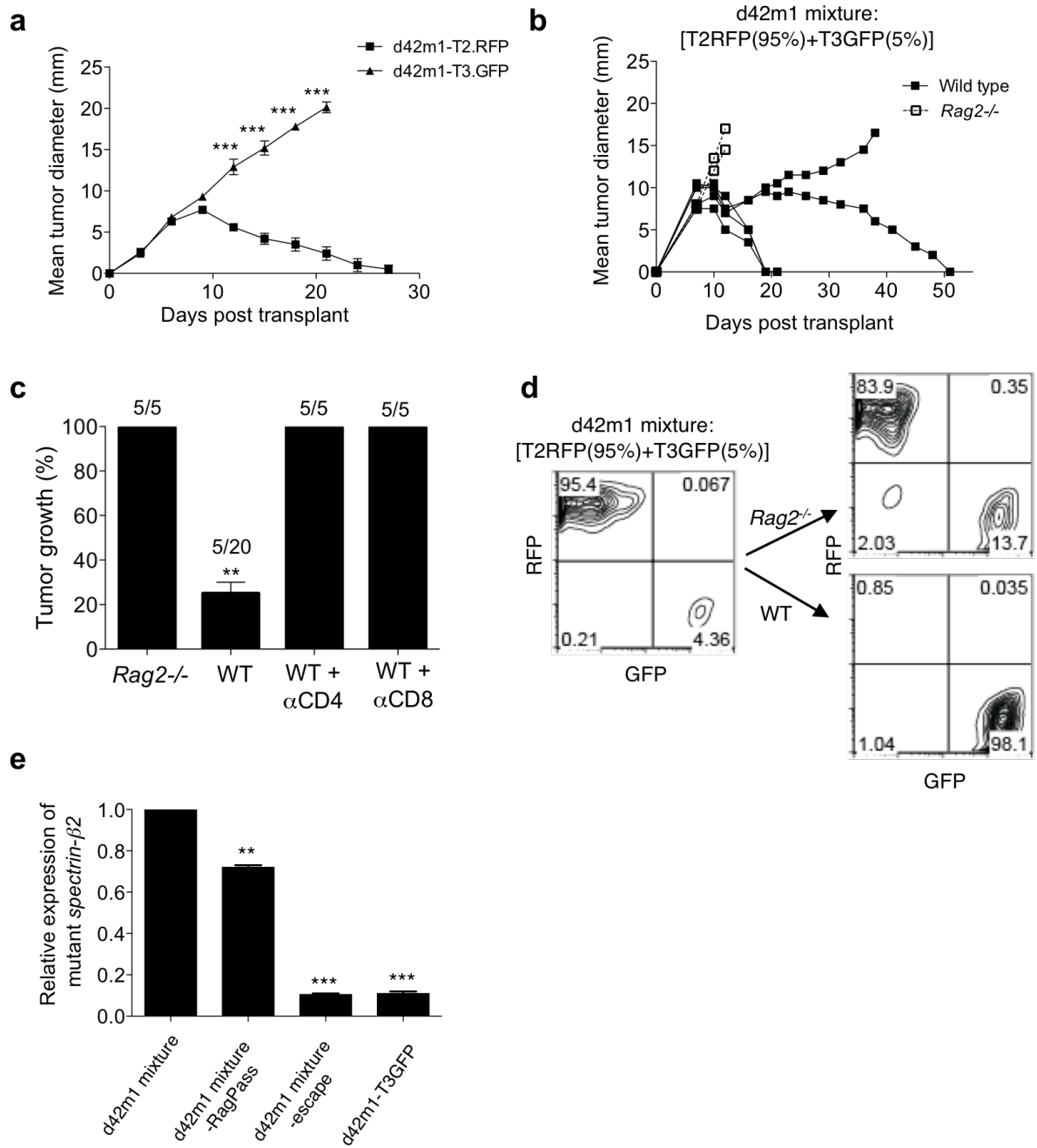


Figure 18. Immunoselection of d42m1 tumor cell clones that lack mutant spectrin- β 2 results in tumor escape. **a**, d42m1-T2 clone labeled with RFP (d42m1-T2RFP) (n=5, squares) or d42m1-T3 clone labeled with GFP (d42m1-T3GFP) (n=5, triangles) were transplanted (1×10^6 cells) into wild type mice. Data are presented as average tumor diameter \pm s.e.m. and are representative of three independent experiments. **b**, A mixture of d42m1-T2RFP (95%) and of d42m1-T3GFP (5%) was transplanted at a total dose of 1×10^6 cells into wild type (solid lines) or *Rag2*^{-/-} (dashed lines) mice. **c**, *Rag2*^{-/-} mice or wild type mice left untreated or treated with antibodies that deplete CD4⁺ or CD8⁺ T cells were challenged with 1×10^6 d42m1 mixture (95% T2RFP and 5% T3GFP). Data presented as percent tumor positive from 2-4 independent experiments (n=2-5 mice per group). **d**, GFP and RFP expression was analyzed in the d42m1-T2RFP/d42m1-T3GFP tumor cell mixture before injection and from tumors that grew out in *Rag2*^{-/-} mice or escaped in wild type mice by flow cytometry. Data are representative of two independent experiments. **e**, Mutation specific qRT-PCR analysis for mutant *spectrin- β 2* in the d42m1-T2RFP/d42m1-T3GFP tumor cell mixture before injection and from tumors that grew out in *Rag2*^{-/-} mice (RagPass) or escaped in wild type mice. Data are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test (**p<0.01 and ***p<0.001).



DISCUSSION

Recent advances in genome sequencing have resulted in unprecedented opportunities to assess genetic influences on disease development. For cancer, most genome sequencing studies have focused on identifying new driver mutations that promote neoplastic development and metastasis in the hope of obtaining insights that lead to novel cancer-targeted therapeutics or that provide prognostic value. However, we show herein that this same technology, when combined with *in silico* epitope prediction algorithms, can be used to identify expressed mutations in cancers that may result in formation of tumor-specific antigens which function as targets for immune-mediated elimination. To our knowledge, the current study is the first to use cDNA capture sequencing (CapSeq) analysis of expressed genes to assess the spectrum of non-synonymous mutations in unedited tumors derived from immunodeficient mice and provide information pointing to potential antigens that may be responsible for immune-mediated tumor rejection. We also demonstrate that the immunoediting of a tumor studied here in detail is the result of T cell dependent immunoselection for tumor cell variants that fail to express this mutation. These results thus not only provide definitive evidence for at least one mechanism underlying the cancer immunoediting process, but also demonstrate the key role that tumor-specific mutations play in development of a tumor's immunogenic phenotype and subsequent fate.

For d42m1 tumor cells, we show that an immunoselection process acting on an oligoclonal parental tumor cell population leads to the outgrowth of tumor cell variants that lack the major tumor rejection antigen—mutant spectrin- β 2. Currently, we are

unable to distinguish between the possibility that d42m1 clones in the parental d42m1 sarcoma line that lack mutant spectrin- β 2 either never expressed this antigen or lost expression. Nevertheless, the immunoselection that occurs upon exposure to an intact immune system is dependent on adaptive immunity since neither parental d42m1 tumor cells nor the mixture of regressor and progressor d42m1 tumor cell clones undergo editing when passaged through *Rag2*^{-/-} mice, but are indeed edited following transplantation into immunocompetent wild type mice. Additional experiments involving depletion of CD4⁺ or CD8⁺ lymphocytes point strongly to T cells as the specific effectors of this editing. These results are consistent with the finding that both T cells and perforin are required for editing of primary MCA sarcomas (87), primary lymphomas (22), and UV-induced tumors (212), although the targets of editing in these earlier studies were not defined. Thus, in the case of d42m1, the target of the immunoselection process has been clearly identified as the major rejection antigen. However, this finding does not rule out the possibility that similar immunoediting mechanisms might select for mutations in other critical components of the MHC class I antigen processing and presentation pathway such as the class I heavy chain (97), β 2 microglobulin (100), or components of IFN- γ receptor signaling (27), all of which are known to regulate tumor cell recognition by tumor-specific CD8⁺ T cells.

We find it surprising that a single mutant protein functions as the sole major rejection antigen in d42m1 tumor cells despite the fact that these tumor cells contain thousands of mutations. Thus, the immunodominance of the mutant spectrin- β 2 derived epitope, in some ways, resembles the known immunodominance of certain viral antigens (234). Many factors may contribute to the immunodominance of mutant spectrin- β 2. On

the basis of *in silico* analysis, the mutant 905-913 sequence is predicted to interact with H-2D^b with very high affinity, as opposed to the corresponding wild type sequence that is predicted to bind only weakly. However, several other factors may also contribute to the immunodominance of mutant spectrin- β 2 including (1) antigen abundance, (2) antigen cross-presentability, (3) T cell repertoire, or (4) presence of epitopes recognized by regulatory T cells. Clearly, more work is needed in order to refine our capacity to accurately predict the ultimate antigenicity of a mutated protein.

Chemically induced tumors have played a critical role in the history of tumor immunology, providing the first unequivocal evidence for the existence of tumor-specific antigens (235-237). It is therefore surprising that so little attention has been given to identifying the transplantation rejection antigens of this class of tumors. Despite the widely held assumption that mutations account for the immunogenicity of these tumors, only one study thus far has identified a mutant protein recognized by CD8⁺ T cells (238). In contrast, other studies of mouse and human tumors have chosen to focus on antigens that are shared among different tumors and sometimes even normal tissues. With the growing recognition of the importance of mutational antigens as tumor-specific targets for immune recognition, there is a critical need to develop experimental systems that facilitate their identification. The approach we have taken in this study, combining deep sequencing, algorithm analysis, and T cell epitope cloning, provides a new use for the powerful data that is rapidly accumulating from analyses of cancer genomes. We predict that this approach may not only provide new insights into basic mechanisms underlying cancer immunoediting, but also new opportunities for individualized cancer immunotherapy. It may also be useful in identifying subsets of cancer patients whose

tumors express antigens that can be most effectively targeted by checkpoint blockade immunotherapy, such as that involving anti-CTLA-4 or anti-PD-1. Finally, this approach may provide a mechanism to longitudinally evaluate changes in a tumor's antigenic profile as a consequence of ongoing immunotherapy.

CHAPTER 4

Exome Sequencing of d42m1 Escape Variants as an Approach to Identify the Antigenic Targets of Checkpoint Blockade Therapy

INTRODUCTION

The previous chapter in this dissertation established that exome sequencing of a highly immunogenic, unedited sarcoma (d42m1) could be used to identify the major rejection antigen of this tumor (R913L spectrin- β 2 mutant). Nevertheless, d42m1 escape variants that lack mutant spectrin- β 2 emerge that can grow in an immunologically unrestricted manner. In this chapter, we will explore whether the many thousands of mutations still expressed by d42m1 escape variants and progressor clones exhibit residual immunogenicity. Specifically, we will use antibodies that block negative co-stimulators that have recently been very successful in human cancer patients (206) to determine whether the potential antigens identified by exome sequencing of d42m1 escape variants and progressor clones can be targeted by this form of cancer immunotherapy.

Checkpoint Blockade Therapy

Mechanisms of tumor escape from immune control may be directly at the level of the tumor, whereby alterations at the tumor cell surface results in decreased recognition (i.e. loss of MHC components) by immune cells or tumors may indirectly impede immunosurveillance through the recruitment of cells (i.e. regulatory T cells) or the liberation of cytokines (i.e. IL-10 or TGF- β) involved in immunosuppression. The negative co-stimulatory molecules cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) and its ligand (PD-L1) are likely candidates that may be facilitating tumor escape due to their potent ability to attenuate cytotoxic T cell responses. The blockade of these negative co-stimulatory molecules to boost anti-tumor

immunity and overcome cancer-induced immunosuppression is termed “checkpoint blockade” therapy.

CTLA-4 in Inhibiting Tumor Immunity

These negative co-stimulatory molecules attenuate T cell responses during the physiological contraction phase of an immune response and help maintain peripheral tolerance. CTLA-4 is a homologue of CD28 binds to the same receptors as CD28, both B7.1 (CD80) and B7.2 (CD86) (239, 240). The profound ability of CTLA-4 to inhibit T cell responses is dramatically displayed by gene-targeted mice deficient for CTLA-4, which succumb to lethal lymphoproliferative disease as a result of excessive T cell activation (241). Subsequent studies have demonstrated that CTLA-4 expression on regulatory T cells (Tregs) and effector T cells is critical to prevent autoimmune lymphoproliferative disease (242, 243). There are two general mechanisms proposed to explain how CTLA-4 is regulating immunity. The first general mechanism involves CTLA-4 reducing the threshold of activation required for T cells to become activated in response to stimuli (threshold model). The second general mechanism involves CTLA-4 reducing or attenuating T cell proliferation after it has become activated (attenuation model) (244). Substantial evidence exists for both models as CTLA-4 prevents binding of CD28 to CD80/CD86, recruits and activates phosphatases to the immunological synapse which degrade T cell receptor mediated signaling and inhibits cell cycle progression (245). These data formed the basis for the concept that CTLA-4 could be blocked in the presence of an established tumor to reduce the threshold for T cell activation, enhance T cell proliferation, and boost anti-tumor activity.

Considerable evidence exists for both mice and humans for using CTLA-4-specific blocking monoclonal antibodies to eradicate or limit the growth of established tumors. Initial studies in mice were promising as CTLA-4 blockade resulted in tumor rejection in a number of pre-clinical models of lymphomas, colon, renal, and prostatic carcinomas (246-249). When CTLA-4 mAb treatment failed to lead to the rejection of poorly immunogenic tumors, synergistic strategies proved very effective. Most often, CTLA-4 immunotherapy was combined with irradiated tumor vaccines engineered to secrete particular cytokines such as GM-CSF, to cause rejection of poorly immunogenic tumors (250). In human cancer patients, both CTLA-4 monotherapy and combinatorial therapies have been successful in reducing melanoma, prostate and renal carcinoma tumor burdens and occasionally eliminating cancer altogether (206, 245).

PD-1/PD-L1 Axis in Inhibiting Tumor Immunity

Similar to CTLA-4, PD-1 and its ligand, PD-L1, regulate the contraction phase of the immune response and maintain peripheral tolerance. Additionally, PD-1 expression by antigen-specific CD8⁺ T cells induces exhaustion during chronic viral infections (251). Initial studies investigating the role of PD-L1 as a mechanism of tumor escape involved enforced expression of PD-L1 on murine tumor cell lines. In these studies it was observed that T cell activation and tumor killing were diminished *in vitro* and tumors grew more aggressively *in vivo* (252, 253). Using the 4T1 mammary carcinoma model, tumor cells were found to upregulate the expression of PD-L1 *in vivo*, rendering the immunotherapy with anti-4-1BB mAb ineffective (254). Furthermore, PD-L1 blockade

using anti-PD-L1 mAb delayed the growth of a myeloma cell line known to endogenously express PD-L1 (253).

Recent studies are now beginning to explore the use of combining PD-1, PD-L1, and CTLA-4 antibodies to boost the anti-tumor immune response and enhance the efficacy of these immunotherapies (255). Although it is well established that these antibodies enhance the proliferation and cytokine secretion of both CD4⁺ and CD8⁺ T cells, the exact mechanism of how these antibodies mediate tumor rejection remains unknown. There is some evidence that pre-existing immunity to tumor antigens prior to CTLA-4 therapy in human patients results in better outcomes (256). This suggests that tumors that express antigens of moderate strength may be capable of eliciting a robust immune response in the absence of immunosuppression or if the threshold for T cell activation is lowered in some manner.

MATERIALS AND METHODS

Mice. *Batf3*^{-/-} mice (38) on a 129/Sv background were originally provided by Dr. Kenneth Murphy and were bred in our specific pathogen-free animal facility. Wild type and *Rag2*^{-/-} mice were purchased from Taconic Farms. All mice were on a 129/Sv background and were housed in our specific pathogen-free animal facility. For all experiments, mice were 8-12 weeks of age and performed in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University in St. Louis.

Tumor transplantation. 3-methylcholanthrene (MCA) induced sarcomas used in this study were generated in 129/Sv strain wild type or *Rag2*^{-/-} mice and banked as low passage tumor cells as previously described (19). Tumor cells derived from frozen stocks were propagated *in vitro* in RPMI media (Hyclone, Logan, UT) supplemented with 10% FCS (Hyclone) and injected subcutaneously in 150 μ l of endotoxin-free PBS into the flanks of recipient mice. Tumor cells were >90% viable at the time of injection as assessed by trypan blue exclusion and tumor size was quantified as the average of two perpendicular diameters.

MHC class I epitope prediction. All missense mutations for each d42m1 tumor variant were analyzed for the potential to form MHC class I neoepitopes that bind to either H-2D^b or H-2K^b molecules. The artificial neural network (ANN) algorithm provided by the Immune Epitope Database and Analysis Resource (www.immuneepitope.org) was used

to predict epitope binding affinities (218) and the results were ultimately expressed as “Affinity Values” (Affinity Value = $1/IC_{50} \times 100$). Missense mutations were detected using tumor cDNA exome capture and Illumina deep sequencing as previously described in Chapter 3 of this dissertation.

Antibodies. Anti-H-2K^b (B8-24-3) and anti-H-2D^b (B22/249) mAbs were generously provided by Dr. Ted H. Hansen (Washington University School of Medicine). Anti-CD4 (GK1.5), anti-CD8 α (YTS169.4), anti-IFN- γ (H22) mAbs and control immunoglobulin (PIP, a mAb specific for bacterial glutathione S-transferase) were produced from hybridoma supernatants and purified in endotoxin-free form by Protein G affinity chromatography (Leinco Technologies, St. Louis, MO). Purified Rat IgG was purchased from Sigma (St. Louis, MO). CD45-FITC, CD45-PE, CD8-APC, and purified anti-CD16/32 were purchased from BioLegend (San Diego, CA). Anti-CTLA-4 (9H10), anti-PD-1 (RMP1-14), and anti-PD-L1 (10F.9G2) were purchased from BioLegend (San Diego, CA).

Establishment of CTL lines and clones. To generate the d42m1 escape variant specific CTL lines, wild type mice were injected with 1×10^6 d42m1-T3 or d42m1-es3 tumor cells and treated with antibodies against CTLA-4, PD-L1, or PD-1. Fourteen days after the tumor had been rejected, the spleen was harvested and a CTL line was established by stimulating 40×10^6 splenocytes with 2×10^6 d42m1-T3 or d42m1-es3 tumor cells pre-treated for 48 hr with 100 U/ml of recombinant murine IFN- γ and irradiated (100 Gy). CTL lines were purified using CD8⁺ magnetic-beads (Miltenyi Biotec).

Measurement of IFN- γ production. To generate target cells, tumor cells were treated with 100U/ml IFN- γ for 48 hrs and irradiated with 100 Gy prior to use. A CTL line was co-cultured at the indicated ratios with target tumor cells (10,000 or 5,000 cells) in 96-well round-bottomed plates overnight. IFN- γ in supernatants was quantified using an IFN- γ ELISA kit (eBioscience). For blocking assays, 10 μ g/ml of α -H-2K^b (B8-24-3) and/or α -H-2D^b (B22/249) were added to the cell culture of effector (CTL lines) and target cells (tumors).

Checkpoint blockade therapy. Mice transplanted with tumors were treated with 200 μ g of anti-CTLA-4, anti-PD-1, or anti-PD-L1 on day 3, 6, 9, 12, 15, and 18 post transplant.

Tumor and spleen harvest. After tumor cell transplantation, established tumors were excised from mice, minced and treated with 1 mg/ml type IA collagenase (Sigma) in HBSS (Hyclone) for 2 hrs at room temperature. The spleen was also harvested and crushed between two glass slides and vigorously resuspended to make single-cell suspensions.

Statistical Analysis. Samples were compared using an unpaired, two-tailed Student's *t* test.

RESULTS

d42m1 Variants Lacking Mutant Spectrin- β 2 Exhibit Residual Immunogenicity

To test whether d42m1 tumor variants that lack the major rejection antigen, mutant spectrin- β 2, would exhibit residual immunogenicity, we transplanted tumors into wild type mice and treated them with blocking antibodies specific for PD-1, PD-L1, or CTLA-4. When transplanted into wild type mice and treated with control Ig, both d42m1-T3 and d42m1-es3 grew progressively (Figure 1). In contrast, d42m1-T3 and d42m1-es3 were rejected in wild type mice that were treated with anti-PD-1, anti-PD-L1, or anti-CTLA-4 (Figure 1). These data reveal that antigen loss variants of the unedited sarcoma d42m1 manifest residual immunogenicity and are rejected in wild type mice when treated with antibodies specific for negative co-stimulatory molecules.

We next determined what are the critical immune components required to reject d42m1 antigen loss variants in this therapeutic setting. Specifically, neutralizing antibodies against IFN- γ , depleting antibodies against CD4⁺ T cells and CD8⁺ T cells, and gene-targeted *Rag2*^{-/-} mice that lack lymphocytes and *Batf3*^{-/-} mice that lack CD8 α ⁺ (38) and CD103⁺ (257) dendritic cells were used for the characterization of the immune response involved in the rejection of d42m1-T3 after anti-PD-L1 or anti-CTLA-4 treatment. As previously shown, d42m1-T3 grows progressively when transplanted into wild type mice but is rejected when mice are treated with anti-PD-L1. In contrast, d42m1-T3 grows progressively in wild type mice treated with anti-PD-L1 when mice are additionally treated with anti-CD4, anti-CD8, anti-CD4/CD8 combined, or anti-IFN- γ (Figure 2). Additionally, d42m1-T3 grows progressively when transplanted into *Rag2*^{-/-}

and *Batf3*^{-/-} mice treated with anti-PD-L1 (Figure 2). Thus, CD4⁺ and CD8⁺ T cells, IFN- γ and CD8 α ⁺/CD103⁺ dendritic cells are required to mediate d42m1-T3 rejection after PD-L1 blockade therapy. Similar findings were obtained with d42m1-T3 after anti-CTLA-4 treatment (Figure 2). Furthermore, a memory response develops against the tumors after rejection due to checkpoint blockade therapy. Specifically, mice that initially rejected d42m1-T3 or d42m1-es3 tumors with anti-PD-1 therapy are protected from secondary challenge with the same tumor several weeks later without any additional therapy (Figure 3). Secondary challenged mice reject d42m1-T3 or d42m1-es3 tumors rapidly with tumors reaching a smaller peak size, results that are consistent with a memory response (Figure 3). Taken together, all of these adaptive immune components are required to detect unveiled antigens and eliminate poorly immunogenic tumor cells after PD-L1 or CTLA-4 blockade and establish a memory response to the tumor antigens expressed in these antigen loss variants of d42m1.

Figure 1. d42m1-es3 and d42m1-T3 are rejected in mice treated with antibodies that block CTLA-4, PD-L1, or PD-1. Cohorts of five mice were transplanted with 1×10^6 d42m1-T3 (left) or d42m1-es3 (right) tumor cells and subsequently were treated with 200 μg of anti-CTLA-4 (9H10), anti-PD-1 (RMP1-14), anti-PD-L1 (10F.9G2) or control Ig (PIP) on day 3, 6, 9, 12, 15, and 18 post transplant.

d42m1-T3

d42m1-es3

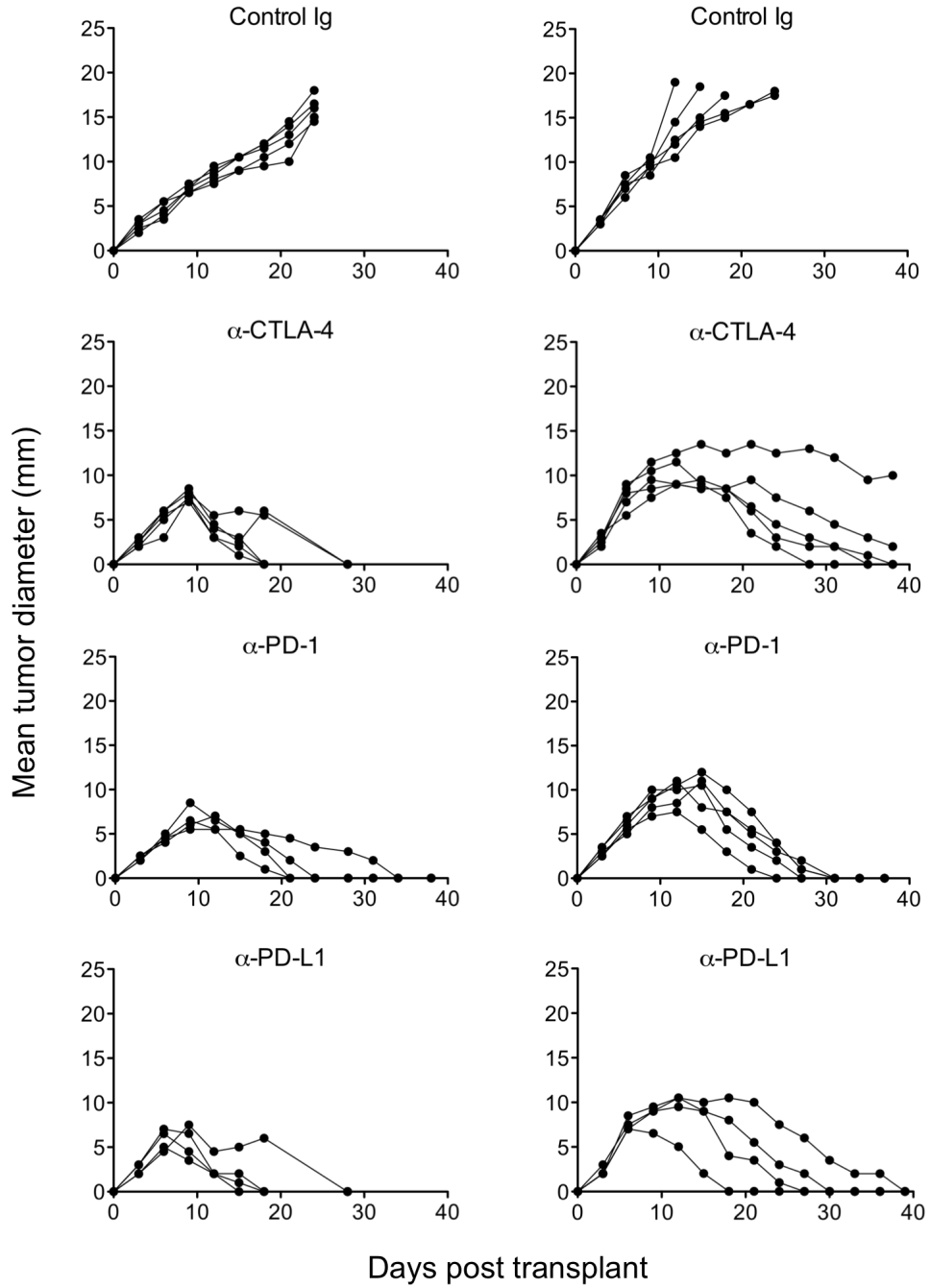


Figure 2. Adaptive immune components are required to mediate rejection of d42m1-T3 after checkpoint blockade therapy. Cohorts of *Rag2*^{-/-}, *Batf3*^{-/-}, or wild type mice treated with control Ig (PIP), anti-CD4, anti-CD8 α , or anti-IFN- γ mAbs were transplanted with 1×10^6 d42m1-T3 tumor cells and subsequently treated with 200 μ g of anti-CTLA-4 (9H10) (top) or anti-PD-L1 (10F.9G2) (bottom) on day 3, 6, 9, 12, 15, and 18 post transplant.

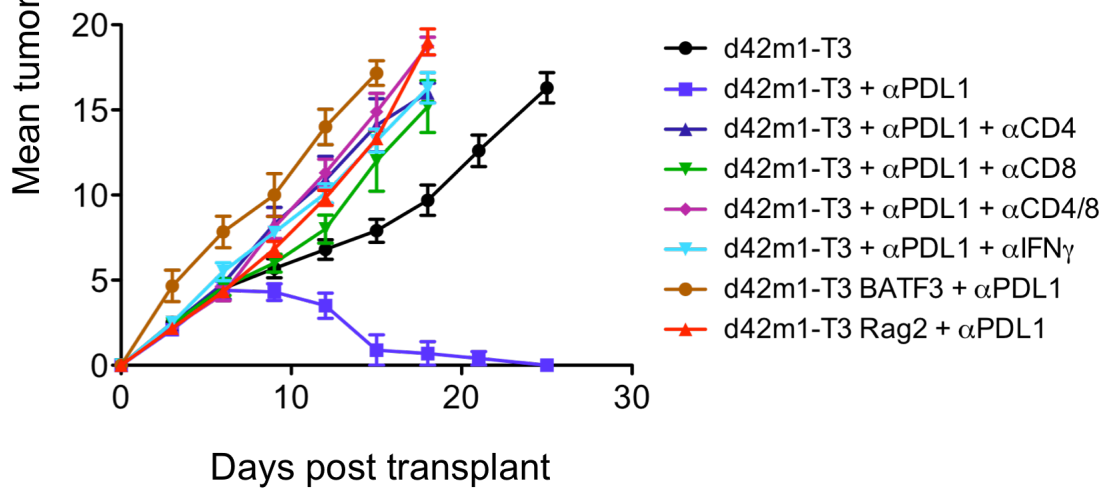
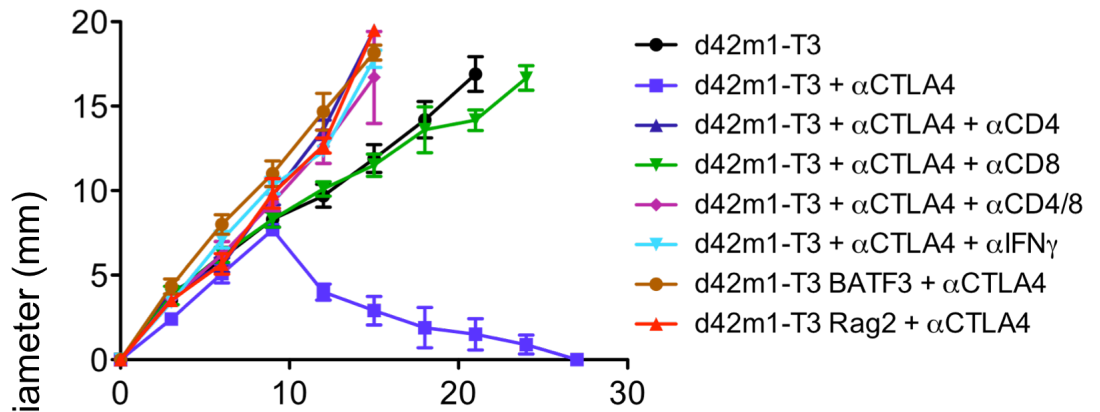
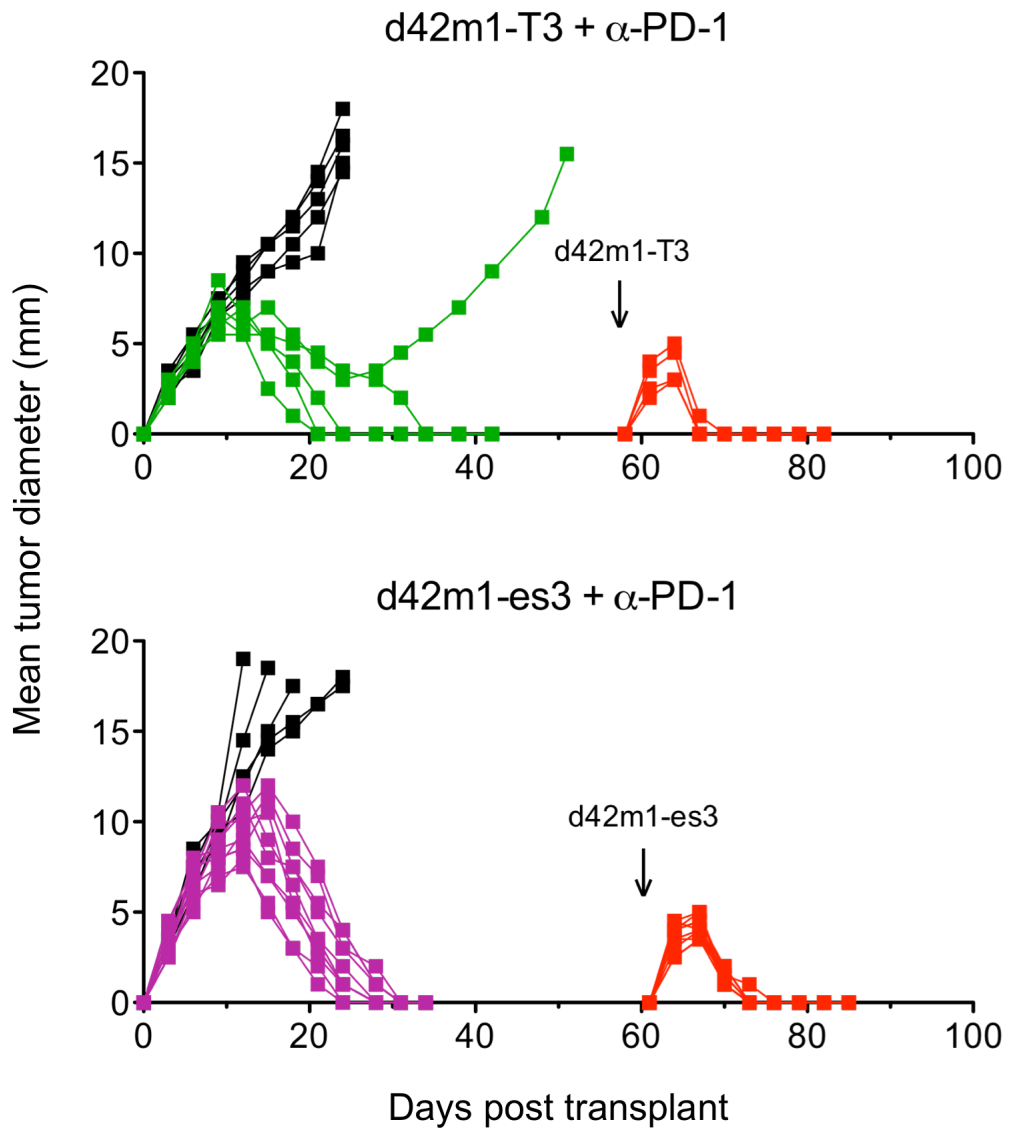


Figure 3. Checkpoint blockade therapy induces a memory immune response against d42m1 antigen loss variants. Cohorts of wild type mice were transplanted with 1×10^6 d42m1-T3 (top) or d42m1-es3 (bottom) tumor cells and treated with 200 μ g of control Ig (PIP) or anti-PD-1 (RPM1-14) mAbs on days 3, 6, 9, 12, 15, and 18 post transplant. Mice that rejected d42m1-T3 or d42m1-es3 tumors after anti-PD-1 therapy were subsequently challenged with same tumor (d42m1-T3 or d42m1-es3) several weeks later.



d42m1 Variants Confer Cross-protective Immunity to One Another

To test whether d42m1 tumor variants share similar antigens that may be cross protective, we first transplanted d42m1-T3 into wild type treated with antibodies specific for PD-1 to induce rejection. Several weeks later, these mice were challenged secondarily with d42m1-T3, d42m1-es3, or F244 (Figure 4). Mice initially challenged with d42m1-T3 tumor cells and treated with anti-PD-1 were able to reject d42m1-T3 and d42m1-es3 upon secondary challenge, suggesting that these tumors share antigens strong enough to mediate cross-protection (Figure 4). In contrast, secondary transplantation with F244 resulted in tumor outgrowth. The converse was also true, where d42m1-es3 was initially transplanted into wild type mice treated with anti-PD-1 and then the same mice were subsequently transplanted with d42m1-es3, d42m1-T3, and F244. Again, d42m1-es3 and d42m1-T3 cells were rejected, but F244 tumor cells grew in an unrestricted manner (Figure 4). Finally, when wild type mice that had spontaneously rejected parental d42m1 tumor cells were challenged with d42m1-T3, d42m1-es3, or F244 tumor cells, both d42m1-T3 and d42m1-es3, but not F244, were rejected. Importantly, when mice that had rejected F244 after treatment with anti-PD-1 were subsequently challenged with F244, d42m1-T3 or d42m1-es3, only F244 tumor cells were rejected, demonstrating that d42m1-T3 and d42m1-es3 do not share antigens with F244 (Figure 5). Taken together, these data show that the antigens expressed by d42m1-T3 and d42m1-es3 that may be the targets of checkpoint blockade are most likely shared.

We previously established through exome sequencing that all of the d42m1 variants including d42m1 parental, regressor d42m1 variants (d42m1-T1, d42m1-T2, and d42m1-T9), progressor d42m1 variants (d42m1-T3 and d42m1-10), and escape variants

(d42m1-es1, d42m1-es2, and d42m1-es3) share a high percentage of missense mutations with one another. Thus it is likely that these tumor variants may share similar antigens. In serial transplantation experiments, d42m1-T3, d42m1-es3 and parental d42m1 tumor cells appear to share antigens that can protect against secondary challenges. We sought to extend this analysis to one additional progressor d42m1 clone (d42m1-T10) and one additional d42m1 escape variant (d42m1-es2). Wild type mice initially transplanted with d42m1-es3 and treated with anti-CTLA-4 to induce the rejection of d42m1-es3 cells were subsequently challenged with d42m1-T10 or d42m1-es2 tumor cells. Both d42m1-T10 and d42m1-es2 tumor cells were rejected, suggesting that d42m1-es3 cells share common antigens with d42m1-T10 and d42m1-es2 cells that are strong enough to mediate rejection, even when challenged several months after the initial d42m1-es3 challenge (Figure 4). These results demonstrate the d42m1 variants express common antigens that are strong enough to mediate protection from secondary challenges of distinct, but related d42m1 variants.

Figure 4. Serial transplantation of related but distinct d42m1 tumor variants

results in cross-protective immunity. Cohorts of wild type mice were transplanted with 1×10^6 d42m1-T3 or d42m1-es3 tumor cells and treated with 200 μg of control Ig (PIP), anti-CTLA-4 (9H10) or anti-PD-1 (RPM1-14) mAbs on days 3, 6, 9, 12, 15, and 18 post transplant. Mice that rejected d42m1-T3 or d42m1-es3 tumors after anti-PD-1 or anti-CTLA-4 therapy were subsequently challenged with d42m1-T3, d42m1-T10, d42m1-es2, d42m1-es3, or F244 tumor cells several weeks later. Also, mice that rejected d42m1 parental tumors spontaneously were transplanted with d42m1-T3, d42m1-es3, or F244 tumor cells several weeks later.

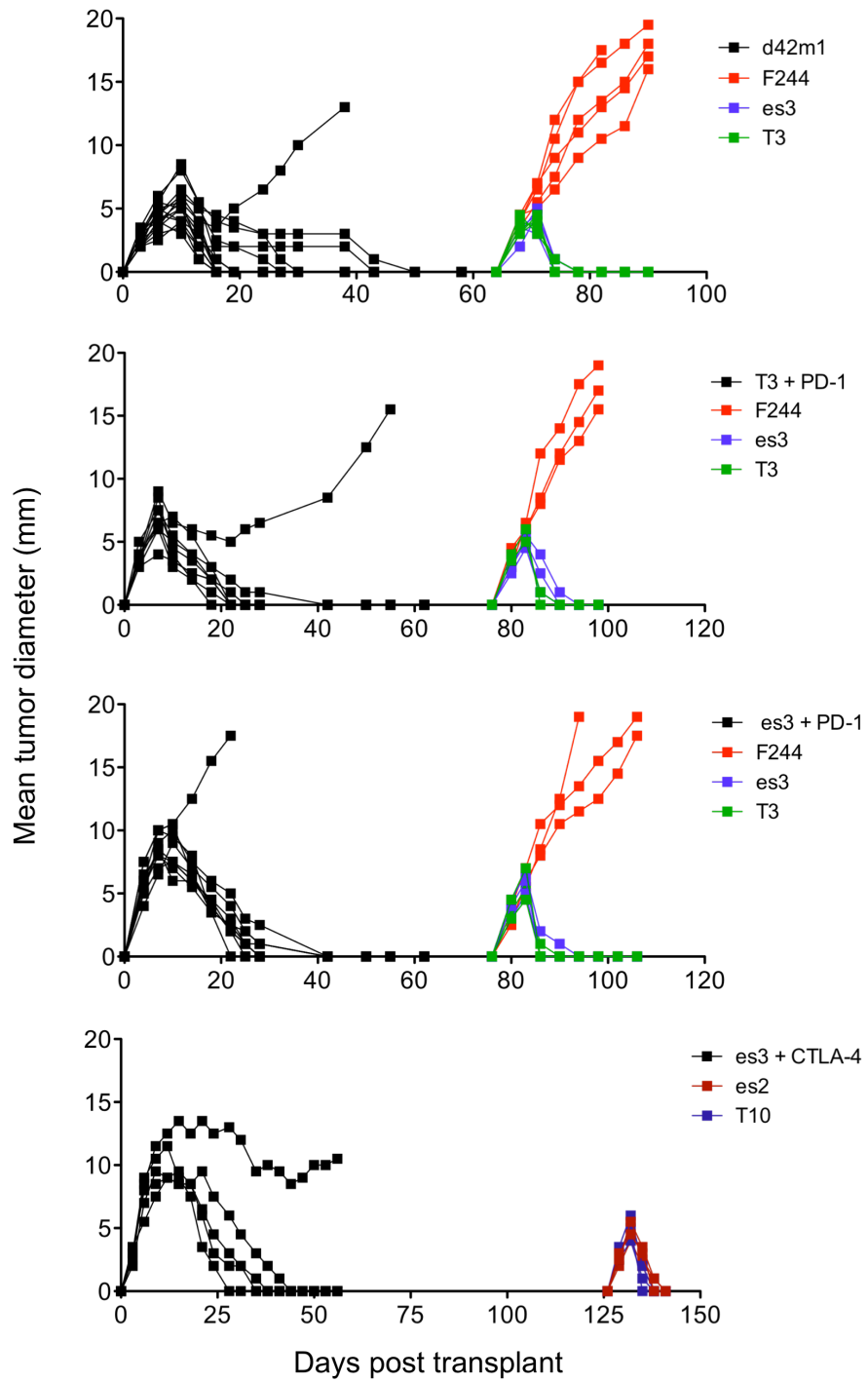
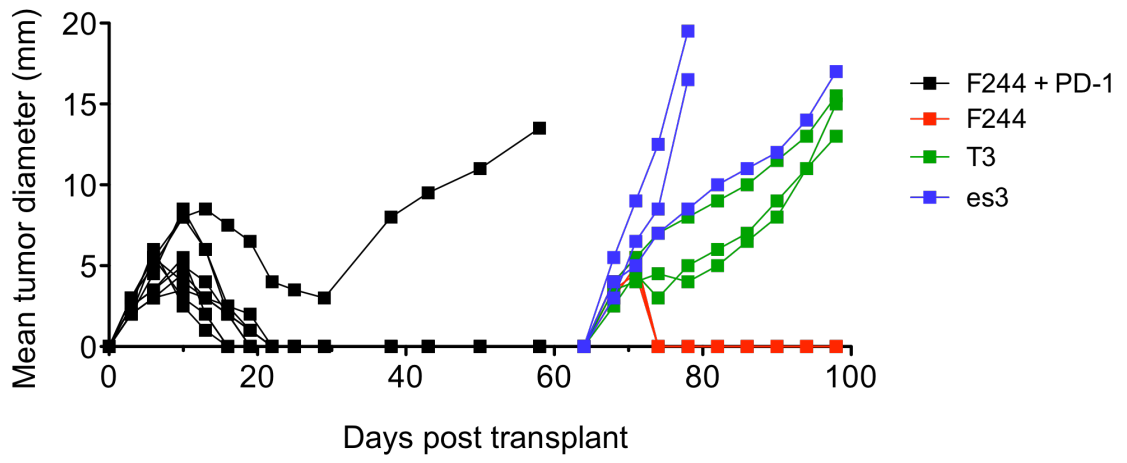


Figure 5. Serial transplantation of F244 does not cross-protect against d42m1 tumor variants. Cohorts of wild type mice were transplanted with 1×10^6 F244 tumor cells and treated with 200 μg of control Ig (PIP) or anti-PD-1 (RPM1-14) mAbs on days 3, 6, 9, 12, 15, and 18 post transplant. Mice that rejected F244 tumors after anti-PD-1 therapy were subsequently challenged with d42m1-T3, d42m1-es3, or F244 tumor cells several weeks later.



Exome Analysis Limits the Number of Candidate Antigens Targeted by Immunotherapy

Although we do not have direct evidence that the antigens targeted by checkpoint blockade therapy are expressed by all the d42m1 tumor variants, the ability of d42m1 tumor variants to protect against other d42m1 tumor variants upon secondary transplantation suggests that they do indeed share common antigens. Thus, we decided to mine our exome sequencing data we had recently generated that catalogues all the mutations expressed in the d42m1 tumor variants to see how many potential antigens were shared among all the variants. When we combined all the mutations sequenced that were found to be shared in all d42m1 variants (d42m1 parental, d42m1-T1, d42m1-T2, and d42m1-T9 regressor variants and well as d42m1-T3, d42m1-T10, d42m1-es1, d42m1-es2, and d42m1-es3 progressor variants), there were only a limited number of shared mutations that may be acting as the immunotherapeutic targets of progressor d42m1 variants (e.g., d42m1-T3 and d42m1-es3). When the shared missense mutations were submitted to the immunoepitope database (218), very few mutations resulted in potential H-2K^b epitopes and only three formed potential H-2D^b epitopes (Figure 6a). Since the most antigenic tumor-specific mutations most likely create peptides that bind with higher affinity to class I molecules than the wild type form of the peptide, we analyzed the potential antigen candidates for whether the mutant form of the peptide created a much stronger MHC class I binder than the wild type form (Affinity Value x Fold-Change). When this additional filter was used, only three mutations resulted in potential H-2K^b epitopes and none formed potential H-2D^b epitopes (Figure 6b). Thus,

these results would predict that if the antigenic target of checkpoint blockade were shared among all the d42m1 variants that it would be restricted to H-2K^b.

Next, we isolated and purified CD8⁺ T cells from the spleens of mice that had rejected d42m1-T3 after treatment with anti-CTLA-4/PD-L1 to generate CTL lines that recognize these tumor cells. To determine the MHC restriction of this bulk CTL line, we incubated the CD8⁺ T cells with irradiated d42m1-T3 tumor cells in the presence of antibodies that block H-2D^b or H-2K^b. IFN- γ production was detected when CD8⁺ T cells were co-cultured with d42m1, d42m1-T3, and d42m1-T3 in the presence of blocking H-2D^b antibodies. In contrast, IFN- γ production was blocked when antibodies specific for H-2K^b were added to the culture, indicating that the putative antigen expressed in d42m1-T3 tumor cells and recognized by this CTL line is presented on H-2K^b (Figure 7). This result corroborates the finding that shared antigens among the d42m1 tumor variants that were predicted by exome sequencing coupled to MHC class I epitope prediction algorithms would most likely be H-2K^b restricted.

Of the limited number of H-2K^b restricted peptides common to all the d42m1 tumor variants, the three peptides with the greatest change in H-2K^b binding represent the best candidate antigens targeted by checkpoint blockade (Figure 6b). Among the three (Gpr108 R101L; Olfr684 D72Y; Olfr1239 C177S), one mutant peptide (Gpr108 R101L, SVRSYRSL) is of particular interest due to its high affinity binding for H-2K^b (predicted IC₅₀ < 11 nM) and it resembles mutant spectrin- β 2 in that it has an R to L change at the anchoring residue (position 8 for H-2K^b) (Figure 7). Thus, we speculate that the CTL line generated against d42m1-T3 recognizes this R101L mutation in G-protein receptor 108 (Gpr108). Additional CTL lines have been generated against both d42m1-T3 and

d42m1-es3 using various combinations of checkpoint blockade antibodies that will be tested first, for their capacity to recognize d42m1 tumor variant cells and secondly, for their capacity to recognize synthesized mutant peptides (e.g. Gpr108 SVRSYRSL) presented on an unrelated H-2K^b expressing cells.

Although exome sequencing has provided a short list of putative epitopes that may be the targets of checkpoint blockade therapy for d42m1-T3 and d42m1-es3, more work is needed to identify the specific antigens and demonstrate its capacity to stimulate antigen-specific T cells *in vivo*. Nevertheless, the approach described herein that combines deep sequencing of tumor exomes and class I prediction algorithms with isolation of CD8⁺ T cells has greatly limited the number of potential antigens to three, and particularly one that most likely functions as the target of immune-mediated elimination of tumor cells by checkpoint blockade therapy.

Figure 6. Potential antigens shared among all the d42m1 tumor cell variants. a, Common missense mutations shared by all d42m1 tumor cell variants (d42m1 parental, d42m1-T1, d42m1-T2, d42m1-T3, d42m1-T9, d42m1-T10, d42m1-es1, d42m1-es2, and d42m1-es3) and detected using cDNA capture sequencing were analyzed for their capacity to form neoantigens to H-2D^b (top) or H-2K^b (bottom) using the class I prediction algorithm available from the Immune Epitope and Analysis Resource Center. Predicted IC₅₀ values were expressed as “Affinity Values” (Affinity Value = 1/IC₅₀ X 100). **b,** Affinity Values for H-2D^b (top) and H-2K^b (bottom) were multiplied by the fold-change of predicted IC₅₀ values from the wild type form to mutant form of the peptide.

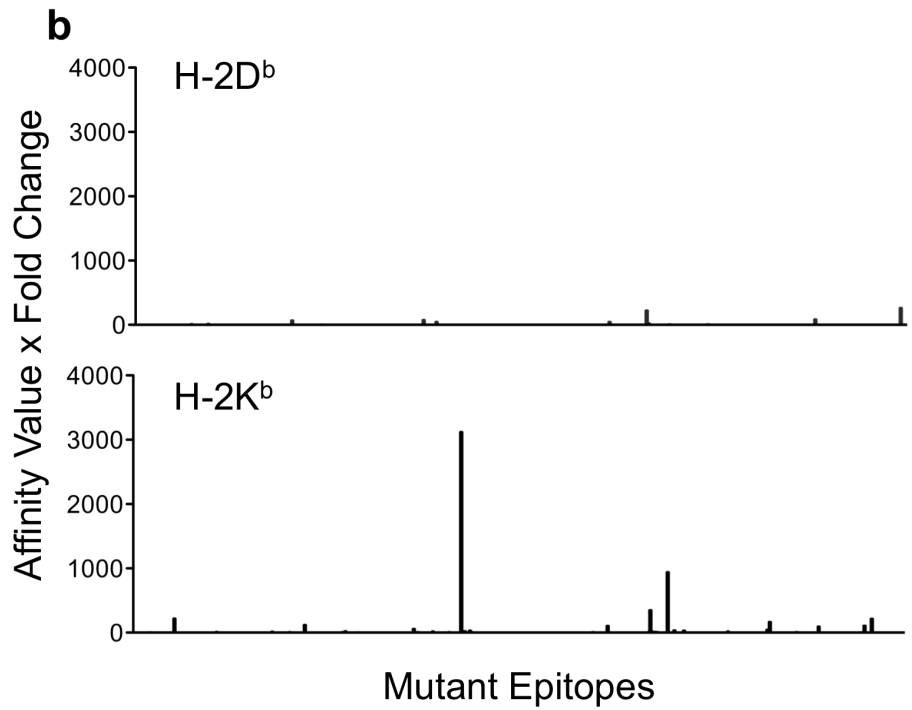
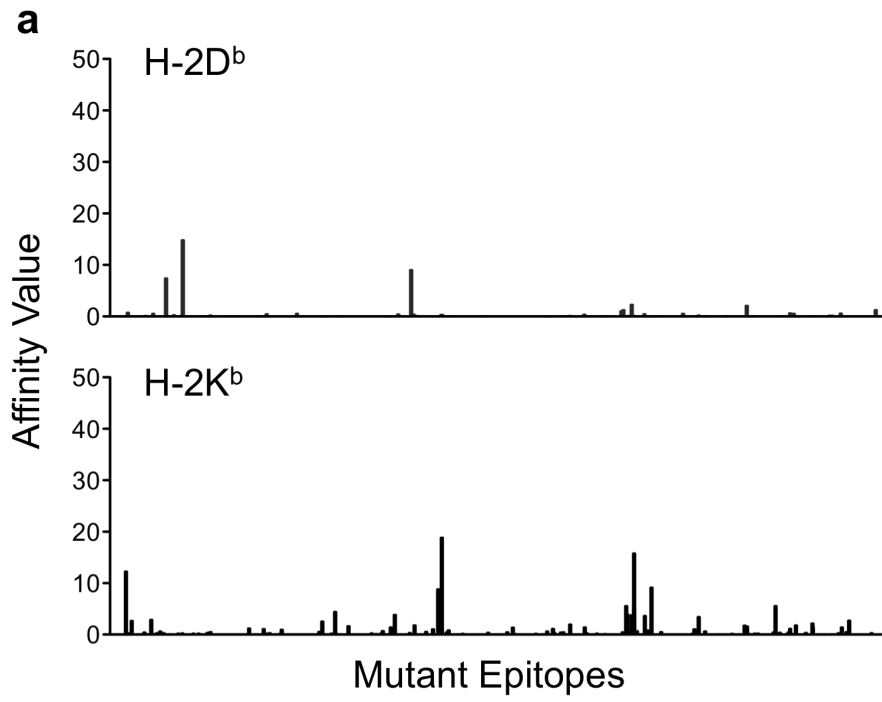


Figure 7. A CTL line generated against d42m1-T3 after checkpoint blockade therapy is restricted to H-2K^b. CD8⁺ T cells isolated from a mouse that had rejected d42m1-T3 after anti-CTLA-4 and anti-PD-L1 combination therapy were incubated with irradiated (100 Gy) d42m1 parental, d42m1-T3, or H31m1 tumor cells pre-treated with 100 U/ml IFN- γ for 48 hrs. CTLs and target tumor cells were co-cultured overnight in the presence or absence of antibodies that block H-2D^b or H-2K^b and supernatants were collected for IFN- γ ELISA assay.

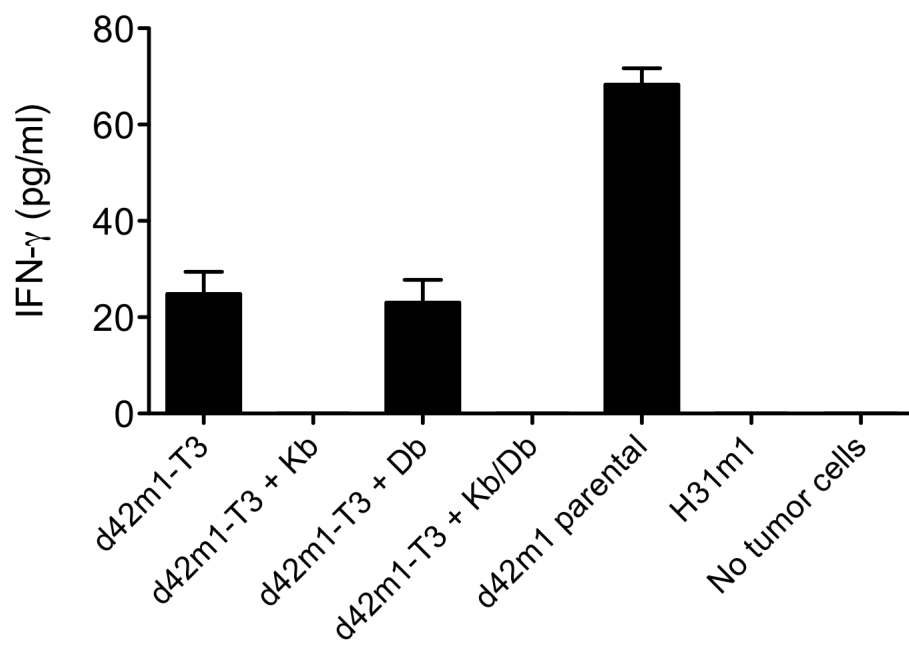


Figure 8. Best potential antigen candidates for H-2K^b shared among all the d42m1 tumor cell variants. The top three antigen candidates that are predicted to bind to H-2K^b with high affinity and are shared among all d42m1 tumor cell variants are listed. For comparison, the major rejection antigen of the regressor d42m1 variants, mutant spectrin- β 2, is listed.

Major rejection antigen – H-2D^b

Gene	Mutation	Sequence	Predicted IC ₅₀	Affinity Value	Fold Change	AV x Fold Change
Spnb2	R913L	WT VAVVNQIAR	5305			
		Mutant VAVVNQIAL	7	14.28	753	10757

Potential shared antigens – H-2K^b

Gene	Mutation	Sequence	Predicted IC ₅₀	Affinity Value	Fold Change	AV x Fold Change
Gpr108	R101L	WT SVRSYSRR	3985			
		Mutant SVRSYSRL	11	8.86	353	3127
Olfr684	D72Y	WT LSLLDIVL	6403			
		Mutant LSLLYIVL	27	3.70	256	945
Olfr1239	C177S	WT ICDMYPLL	2246			
		Mutant ISDMYPLL	25	3.98	90	356

DISCUSSION

These data reveal that antigen loss variants of the unedited sarcoma d42m1 manifest residual immunogenicity and are rejected in wild type mice when treated with antibodies specific for negative co-stimulatory molecules. The rejection of d42m1 tumors that lack mutant spectrin- β 2 by checkpoint blockade therapy requires CD4⁺ T cells, CD8⁺ T cells, IFN- γ , and CD8 α ⁺/CD103⁺ dendritic cells. Moreover, rejection of d42m1-T3 or d42m1-es3 by anti-PD-1 therapy establishes a long-lasting memory response to the original tumor such that secondary tumor challenge results in spontaneous rejection. Thus, edited d42m1 tumor cells retain expression of antigens that can serve as functional targets for immunotherapeutically induced anti-tumor immune responses.

We next established through serial transplantation experiments that d42m1 escape variants (d42m1-es2 and d42m1-es3), progressor d42m1 clones (d42m1-T3 and d42m1-T10) and parental d42m1 tumor cells confer cross-protective immunity for one another. Thus, both highly immunogenic d42m1 tumor cells and poorly immunogenic d42m1 tumor cells share common antigens strong enough to confer protective immunity. In contrast, cross-protective immunity was not observed between d42m1-related cells and an unrelated edited MCA sarcoma, F244, confirming previous exome sequencing analyses described in Chapter 3 that d42m1 tumor variants are a closely related, but heterogeneous group of tumor cell lines. Using the exome sequencing data, we found that only a limited number of antigens predicted to bind to H-2D^b or H-2K^b with high affinity are shared in the d42m1 tumor variants that induce cross-protective immunity. And when we apply an additional filter to identify those mutant proteins that form mutant

peptides with high binding affinity to class I molecules that have very low binding affinity as wild type peptides (i.e., neoantigens), we find only three potential antigens for H-2K^b and none for H-2D^b. In fact, a CTL line generated from a mouse that rejected d42m1-T3 when treated with anti-CTLA-4 and anti-PD-L1 combination therapy recognized d42m1 parental and d42m1-T3 tumor cells in a H-2K^b restricted manner. Thus, these results point to an H-2K^b restricted mutant peptide functioning as the antigenic target of checkpoint blockade therapy.

Among the three best antigen candidates restricted to H-2K^b, one seems very promising based on its high affinity for H-2K^b and its point mutation creates an amino acid substitution (R to L) at anchoring residue position 8 known to physiologically stabilize the peptide-MHC complex. Although exome sequencing has provided a short list of putative epitopes (one in particular – R101L mutation in G-protein receptor 108) that may be the targets of checkpoint blockade therapy for d42m1-T3 and d42m1-es3, more work is needed to identify the specific antigens and demonstrate its capacity to stimulate antigen-specific T cells *in vivo*. To this end, additional CTLs lines have been generated and the wild type and mutant forms of the peptides (Gpr108 R101L; Olfr684 D72Y; Olfr1239 C177S) have been synthesized. Specifically, three distinct CTL lines have been generated from mice that rejected d42m1-T3 or d42m1-es3 tumors in response to checkpoint blockade therapy. Next, the specificity of these CTL lines will be tested using the synthesized candidate peptides. If any mutant peptides presented on unrelated H-2K^b expressing cells activates one of these CTL lines, then these peptides will be used to assemble soluble H-2K^b MHC class I tetramers to track mutation-specific CD8⁺ T cells *in vivo*. Additionally, mutant peptides will be used to immunize wild type mice prior to

d42m1-T3 or d42m1-es3 tumor cell challenge to test if the peptide can be used to vaccinate against d42m1 antigen loss variants.

Although the identification of the antigens targeted by checkpoint blockade therapy are have yet to be identified, the approach described herein that combines deep sequencing of tumor exomes and class I prediction algorithms with isolation of CD8⁺ T cells has greatly limited the number of potential antigens to three, and particularly one that most likely functions as the target of immune-mediated elimination of tumor cells by checkpoint blockade therapy. We predict that the additional experiments outlined above will demonstrate which of the candidate antigens functions as the target for immunotherapeutically induced anti-tumor immune responses.

CHAPTER 5

Residual Immunogenicity of a Subset of Edited Sarcomas is Revealed by Checkpoint Blockade Therapy

INTRODUCTION

The findings from the previous chapter provide additional support to the growing body of evidence that checkpoint blockade can induce powerful anti-tumor responses in both mice and humans (206, 245, 258). Although the underlying mechanism of this type of immunotherapy remains to be elucidated, it seems likely that antigen expression by the tumor cells is critical to allow for tumor-targeted, immune-mediated destruction of transformed cells. In fact, human cancer patients that had the best clinical responses to anti-CTLA-4 therapy, had pre-existing immunity to the cancer-associated antigen, NY-ESO-1 (259, 260). Despite dramatic reductions in tumor-burden in some cancer patients treated with anti-CTLA-4, the majority of individuals treated do not respond to this form of therapy for reasons that remain unknown (206).

A number of pre-clinical studies have explored whether edited tumors that develop in immunocompetent mice can respond to checkpoint blockade therapy to determine the quality of the immune response necessary to induce tumor rejection. For example, one earlier study in mice demonstrated that a transplanted edited sarcoma could be successfully treated with antibodies specific for CTLA-4 (249), and laid the groundwork for future investigations into using these antibodies to boost anti-tumor immunity. Many poorly immunogenic tumors studied to date require additional therapies to be used in combination with checkpoint blockade therapy to induce host-protective immune responses. Specifically, the experimental tumor B16 melanoma does not respond to anti-CTLA-4 monotherapy or a GM-CSF expressing DC vaccine (GVAX) alone, but in combination this therapy causes tumor rejection that provides long-lasting

immunity against B16 melanoma cells (250). In this study, we sought to determine whether a large panel of edited, poorly immunogenic tumors derived from MCA-treated wild type mice also exhibit residual immunogenicity and respond to checkpoint blockade therapy.

In contrast to d42m1 antigen loss variants, where all tumor variants tested responded to checkpoint blockade therapy, only 70% (9/13) of edited MCA sarcomas were rejected from wild type mice treated with anti-CTLA-4 therapy. Moreover, the four different edited sarcomas that failed anti-CTLA-4 therapy also failed combinatorial therapies with anti-CTLA-4/PD-1 mAbs and thus, appear to be non-immunogenic. These results suggest tumor-specific mutations in edited tumors most likely arise stochastically and therefore express different levels of antigenicity/immunogenicity. To address this question, we used massively parallel sequencing of the exons from a panel of ten edited sarcomas, eight of which respond to anti-CTLA-4 therapy and two of which do not, and found that they display similar numbers of mutations. Current analysis is underway to determine whether there are differences in the number of potential neoantigens that form from these mutations between tumors that exhibit residual immunogenicity and tumors that appear to be non-immunogenic and fail checkpoint blockade therapy. Thus, it is tempting to speculate that the level of residual antigenicity remaining in a naturally immunoedited tumor and not the mutational load plays an important role in determining whether it can be effectively controlled by checkpoint blockade therapy.

MATERIALS AND METHODS

Mice. Wild type and *Rag2*^{-/-} mice were purchased from Taconic Farms. All mice were on a 129/Sv or C57BL/6 background and were housed in our specific pathogen-free animal facility. For all experiments, mice were 8-12 weeks of age and performed in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University in St. Louis.

Tumor transplantation. 3-methylcholanthrene (MCA) induced sarcomas used in this study were generated in 129/Sv or C57BL/6 strain wild type and banked as low passage tumor cells as previously described (19). Tumor cells derived from frozen stocks were propagated *in vitro* in RPMI media (Hyclone, Logan, UT) supplemented with 10% FCS (Hyclone) and injected subcutaneously in 150 μ l of endotoxin-free PBS into the flanks of recipient mice. Tumor cells were >90% viable at the time of injection as assessed by trypan blue exclusion and tumor size was quantified as the average of two perpendicular diameters.

Isolation of normal skin fibroblasts from wild type mice. Skin fibroblasts were isolated from three independent 129/Sv wild type pups by harvesting skin and incubating in 0.25% trypsin (Hyclone) at 37°C for 30 minutes prior to washing in DMEM media (Hyclone). After washing, chunks of skin were filtered to achieve single cell suspensions and cultured *in vitro* with DMEM media. After 3 passages, skin fibroblasts were harvested to isolate genomic DNA and total RNA.

Extraction of genomic or complementary DNA. Genomic DNA from sarcoma cells and normal skin fibroblasts was extracted using DNeasy Blood & Tissue Kit (Qiagen). For cDNA isolation, total RNA from sarcoma cells and normal skin fibroblasts was isolated using RNeasy Mini kit (Qiagen) and cDNA was synthesized using oligo (dT) primers and SuperScript III Reverse Transcriptase (Invitrogen).

cDNA capture, sequencing, and alignment (cDNA CapSeq) with Roche NimbleGen Exome Capture Array. cDNA samples from each tumor (100 ng) were constructed into Illumina libraries according to the manufacturer's protocol (Illumina Inc, San Diego, CA) with the following modifications: 1) cDNA was fragmented using Covaris S2 DNA Sonicator (Covaris, Inc. Woburn, MA) in 1X end-repair buffer followed by the direct addition of the enzyme repair cocktail (Lucigen, Madison, WI). Fragment sizes ranged between 100 and 500 bp. 2) Illumina adapter-ligated DNA was amplified in four 50 μ l PCRs for five cycles using 4 μ l adapter-ligated cDNA, 2X Phusion Master Mix and 250 nM forward and reverse primers, 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT CCGATC and 5' CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTC CGATC, respectively. 3) Solid Phase Reversible Immobilization (SPRI) bead cleanup was used to purify the PCR-amplified library and to select for 300-500 bp fragments. 500 ng of the size-fractionated Illumina library was hybridized with the Agilent mouse exome reagent. After hybridization at 65°C for 24 hrs, we added 50 μ l of DynaBeads M-270 Streptavidin-coated paramagnetic beads (10 mg/ml) to selectively remove the

biotinylated Agilent probes and hybridized cDNA library fragments. The beads were washed according to manufacturer's protocol (Roche NimbleGen) and the captured library fragments were released into solution using 50 μ l of 0.125 N NaOH and neutralized with an equal volume of neutralization buffer (Roche NimbleGen). The recovered fragments then were PCR amplified according to the manufacturer's protocol using 11 cycles in the PCR. Illumina library quantification was completed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA). The qPCR result was used to determine the quantity of library necessary to produce 180,000 clusters on a single lane of the Illumina GAIIx. One lane of 100 bp paired-end data was generated for each captured sample (since cDNA was used as the source for sequencing, we refer to this process as cDNA Capture Sequencing or CapSeq). Illumina reads were aligned to the NCBI build 37 (Mm9) mouse reference sequence using BWA (216) v0.5.5 (with $-q$ 5 soft trimming). Alignments from multiple lanes for the same sample were merged together using SAMtools r599, and duplicates were marked using Picard v1.29.

Mutation detection and annotation. Putative somatic mutations were identified using VarScan 2 (v2.2.4) (217) with the parameters "*--min-coverage 3 --min-var-freq 0.08 --p-value 0.10 --somatic-p-value 0.05 --strand-filter 1*" and specifying a minimum mapping quality of 10. Variants whose supporting reads exhibited read position bias (average read position <10 or >90), strand bias ($>99\%$ of reads on one strand), or mapping quality (score difference >30 , or mismatch quality sum difference >100) relative to reference supporting reads were removed as probable false positives. We also required that the variant allele be present in at least 10% of tumor reads and no more than 5% of normal

reads. The single nucleotide variants (SNVs) meeting these criteria were annotated using an internal database of Genbank/Ensembl transcripts (v58_73k). In the event that a variant was annotated using multiple transcripts, the annotation of most severe effect was used. Non-silent coding mutations (missense, nonsense/nonstop, or splice-site) were prioritized for downstream analysis.

Mutation rate and overlap comparisons. Mutation rates were estimated for each tumor sample using the number of putative “tier 1” SNVs (missense, nonsense/nonstop, splice site, silent, or noncoding RNA). To account for variability in coverage between samples, the SNV count for each tumor sample (S) was divided by a coverage factor (F), computed as the fraction of all tier 1 SNVs identified in any tumor sample ($n=16,991$) that were covered by at least 4 reads in a given sample. For example, in the d42m1 parental sample, 15,852 of 16,991 tier 1 SNV positions were covered, for a coverage factor of 93.30%. The number of coverage-adjusted mutations in each sample was divided by the total size of tier 1 space in the mouse genome (43.884 Mbp) to determine the number of coding mutations per megabase (R).

$$R = (S / F) / (43.884 \text{ Mbp})$$

MHC class I epitope prediction. All missense mutations for edited wild type sarcoma were analyzed for the potential to form MHC class I neoepitopes that bind to either H-2D^b or H-2K^b molecules. The artificial neural network (ANN) algorithm provided by the Immune Epitope Database and Analysis Resource (www.immuneepitope.org) was used to predict epitope binding affinities (218) and the results were ultimately expressed as

“Affinity Values” (Affinity Value = $1/IC_{50} \times 100$). An additional analysis using Affinity Value multiplied by the Fold Change (wild type IC_{50} /mutant IC_{50}) was also used.

Antibodies. Anti-CTLA-4 (9H10) was generously provided by Dr. James Allison, anti-PD-1 (RMP1-14) by Dr. Hideo Yagita, and anti-PD-L1 (10F.9G2) by Drs. Arlene Sharpe and Gordon Freeman. Subsequent quantities of these clones were purchased from BioLegend (San Diego, CA). Distinct clones of anti-CTLA-4 (9D9), anti-PD-1 (4H2), and anti-PD-L1 (14D8) were provided by Bristol Myers Squibb (New York, NY).

Checkpoint blockade therapy. Mice transplanted with tumors were treated with 200 μ g of anti-CTLA-4, anti-PD-1, or anti-PD-L1 on day 3, 6, 9, 12, 15, and 18 post transplant.

Statistical Analysis. Samples were compared using an unpaired, two-tailed Student's *t* test.

RESULTS

A Subset of Edited Sarcomas from Wild Type Mice Manifest Residual

Immunogenicity

As opposed to d42m1 antigen loss variants, where all progressor clones and escape variants are rejected with checkpoint blockade therapy, when a panel of edited MCA sarcomas derived from wild type mice were tested for anti-CTLA-4 sensitivity, only 70% (9/13) of sarcomas were rejected in wild type mice after transplantation of sarcoma cells and treatment with anti-CTLA-4 mAbs (Figure 1). Specifically, 10 edited sarcomas derived from 129/Sv strain mice (F279, F244, F236, H118m1, d4m3, d22m1, d22m2, c20m1, c1m2, and H128m8462) (19) and three edited sarcomas derived from C57BL/6 strain mice (9609, 9614, and 1956) (C.M. Koebel, J.D. Bui and R.D. Schreiber, unpublished results) were transplanted into syngeneic hosts and treated with anti-CTLA-4. Of the nine edited sarcomas that responded, eight were 129/Sv tumors (F244, F236, H118m1, d22m1, d22m2, c20m1, c1m2, and H128m8462) and one was a C57BL/6 tumor (1956), leaving two edited 129/Sv tumors (F279 and d4m3) and two edited C57BL/6 tumors (9609 and 9614) that did not respond to anti-CTLA-4 therapy (Figure 1). These results suggest that edited MCA sarcomas have varying levels of residual immunogenicity that can be revealed by checkpoint blockade therapy.

All edited sarcomas tested that are rejected with anti-CTLA-4 therapy are also rejected by either anti-PD-L1 mAb alone, anti-CTLA-4 mAb alone, or anti-PD-L1 and anti-CTLA-4 mAbs in combination. Thus, F244, F236, and 1956 edited sarcomas exhibit residual immunogenicity and are rejected when mice harboring these tumors are treated

with any checkpoint blockade antibodies (Figure 2). On the other hand, d4m3, F279, 9614, and 9609 appear to be non-immunogenic when assayed by this method since treatment with other checkpoint blockade antibodies did not restrain tumor growth in any manner, even when used in combination (Figure 3). Therefore, there is a subset of edited MCA sarcomas derived from wild type mice that appear to be non-immunogenic.

Exome Sequencing of Edited MCA Sarcomas

It is unknown whether the varying levels of residual immunogenicity remaining in naturally immunoedited tumors is determined by varying levels of antigenicity and thus, helps determine whether a tumor can be effectively controlled by checkpoint blockade therapy. Therefore, we performed exome sequencing on eight edited MCA sarcoma cell lines that respond to checkpoint blockade therapy (F244, F236, H118m1, d4m3, d22m1, d22m2, and H128m8462) and two edited MCA sarcoma cell lines that do not respond to checkpoint blockade therapy (F279 and d4m3) in order to characterize the mutations and identify the array of potential antigens expressed in edited sarcomas. These ten edited sarcomas were chosen to be sequenced among the panel of 13 edited sarcomas because they were all derived from wild type 129/Sv mice treated with 100 μ g of MCA, and thus are genetically- and carcinogen-load-matched. In addition, three normal skin fibroblast cell lines derived from wild type mice were generated and cDNA was isolated, exome captured and sequenced to serve as the reference sequence for the identification of non-synonymous mutations in the sarcoma cell lines. Specifically, cDNA was isolated from all 10 of these sarcoma cell lines and 3 normal fibroblast lines, constructed into Illumina

libraries, hybridized to mouse exome probes (Roche NimbleGen) and submitted for Illumina sequencing.

Exome sequencing revealed that edited sarcomas display a range of protein-coding mutations (tier 1 SNVs). The average number of non-synonymous mutations in all ten edited sarcomas was 2,267 with a range from 1,317 to 3,380 mutations for individual edited sarcomas (Figure 4). As a control, d42m1 parental cells, which are highly immunogenic and were derived in a *Rag2*^{-/-} immunodeficient mouse was included in this cDNA CapSeq experiment and found to express 2,707 non-synonymous mutations. Thus, there does not appear to be a significant difference in the number of mutations present in edited sarcomas when compared to those in unedited sarcomas. However, using two rounds of exome capture with Agilent mouse exome probes with d42m1 parental cell cDNA, we detected 3,737 non-synonymous mutations. Here we used a single round of exome capture with Roche NimbleGen mouse exome probes and detected nearly 1,000 fewer mutations. This discrepancy may be due in part to differences in the percentage of genes captured by the two different commercial exome mouse probe reagents (Agilent and Roche NimbleGen) as well as the differences in sequencing coverage that can be minimized by repeating cDNA CapSeq. Clearly, one approach is to perform cDNA CapSeq with the NimbleGen mouse exome probe reagent an additional time for parental d42m1 and edited sarcoma samples to enhance exome sequencing coverage and enhance mutation call accuracy.

Nevertheless, this preliminary sequencing data does provide some initial insight into the mutational landscape of edited sarcomas. First, it appears that there is no difference between edited and unedited MCA sarcomas, suggesting that the mutational

load present in these tumor cells is not greatly influenced by the host's immune status. Secondly, there is a very broad range of mutations detected in edited sarcomas with some tumors having nearly three times the number of mutations (Figure 4). For example, 1,317 mutations were detected in F279 tumor cells and 3,380 mutations were detected in c1m2 tumor cells. Interestingly, F279 tumors cannot be controlled in immunocompetent hosts treated with checkpoint blockade antibodies, while c1m2 tumors are rejected following anti-CTLA-4 treatment (Figure 1). However, the other edited sarcoma that fails checkpoint blockade, d4m3, has 2089 mutations which is comparable to the other sarcomas that do respond to anti-CTLA-4 therapy such as F236 which has 1,606 mutations. Thus, although there is a trend for edited sarcomas that fail anti-CTLA-4 therapy (F279 and d4m3) to have fewer mutations than those that do respond to immunotherapy, there is no statistical difference between the numbers of mutations in the two subsets of edited sarcomas (Figure 4). Current analysis is underway to determine if the number of potential antigens is distinct between sarcomas that respond and sarcomas that fail to respond to checkpoint blockade therapy by pipelining the exome sequencing data into MHC class I prediction algorithms.

Figure 1. A subset of edited MCA sarcomas derived from immunocompetent wild type mice respond to anti-CTLA-4 therapy. A panel of 13 edited MCA sarcomas were transplanted into wild type mice treated with 200 µg of control Ig (PIP) (black) or anti-CTLA-4 (9D9) (red) on days 3, 6, 9, 12, 15, and 18 post transplant. Ten of the 13 tumors are 129/Sv strain tumors (F279, F244, F236, H118m1, d4m3, d22m1, d22m2, c20m1, c1m2, and H128m8462) (19) and the remaining three edited sarcomas were derived from C57BL/6 strain mice (9609, 9614, and 1956) (C.M. Koebel, J.D. Bui and R.D. Schreiber, unpublished results).

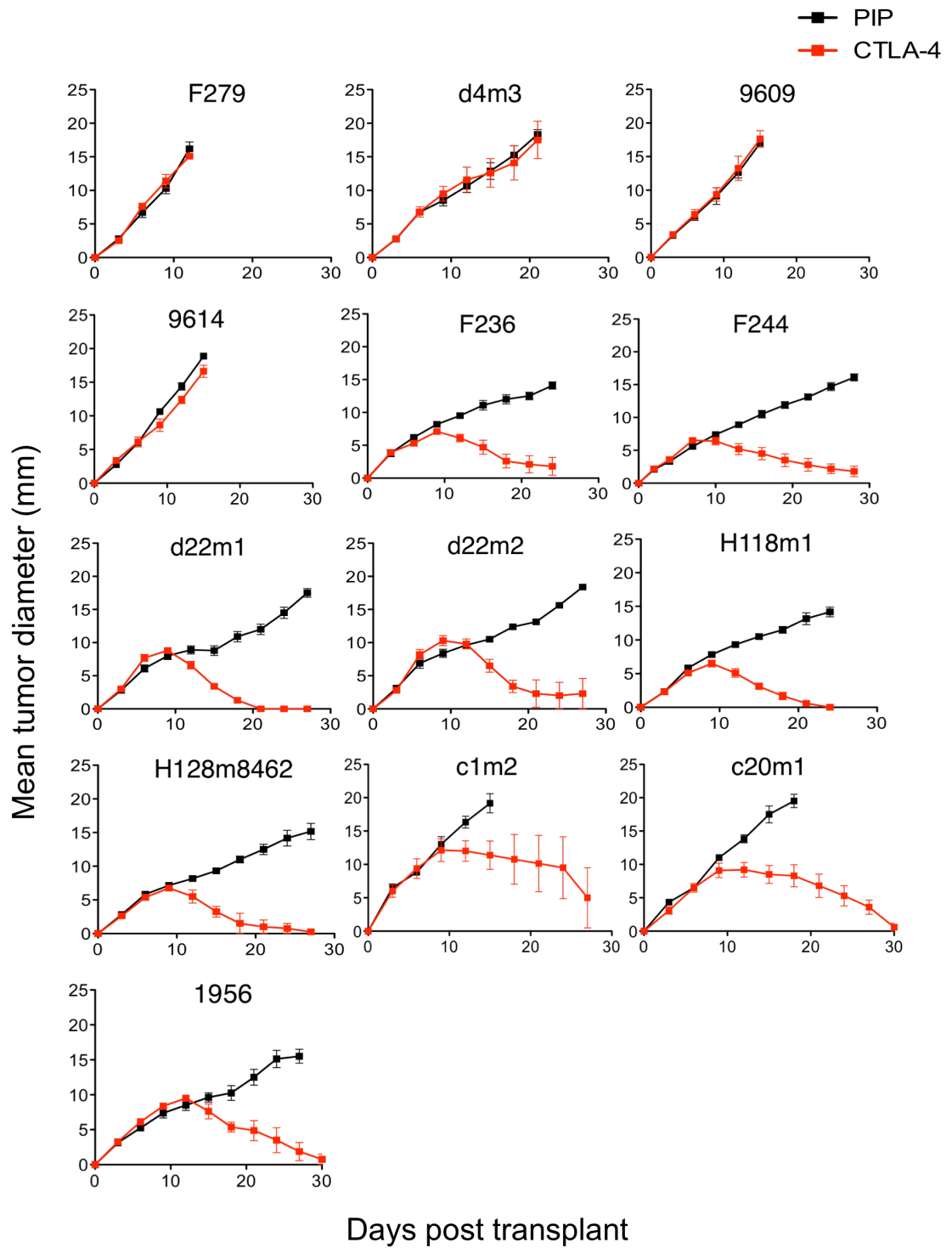


Figure 2. Edited MCA sarcomas that respond to anti-CTLA-4 therapy also respond to anti-PD-1 and anti-PD-L1 therapy. F244, F236, and 1956 were transplanted into wild type mice at a dose of 1×10^6 cells and treated with 200 μg of control Ig (PIP) (black), anti-CTLA-4 (9H10) (red), anti-PD-L1 (10F.9G2) (blue), anti-PD-1 (RMP1-14) (green) or combination of anti-CTLA-4/PD-L1 (purple) on days 3, 6, 9, 12, 15, and 18 post transplant.

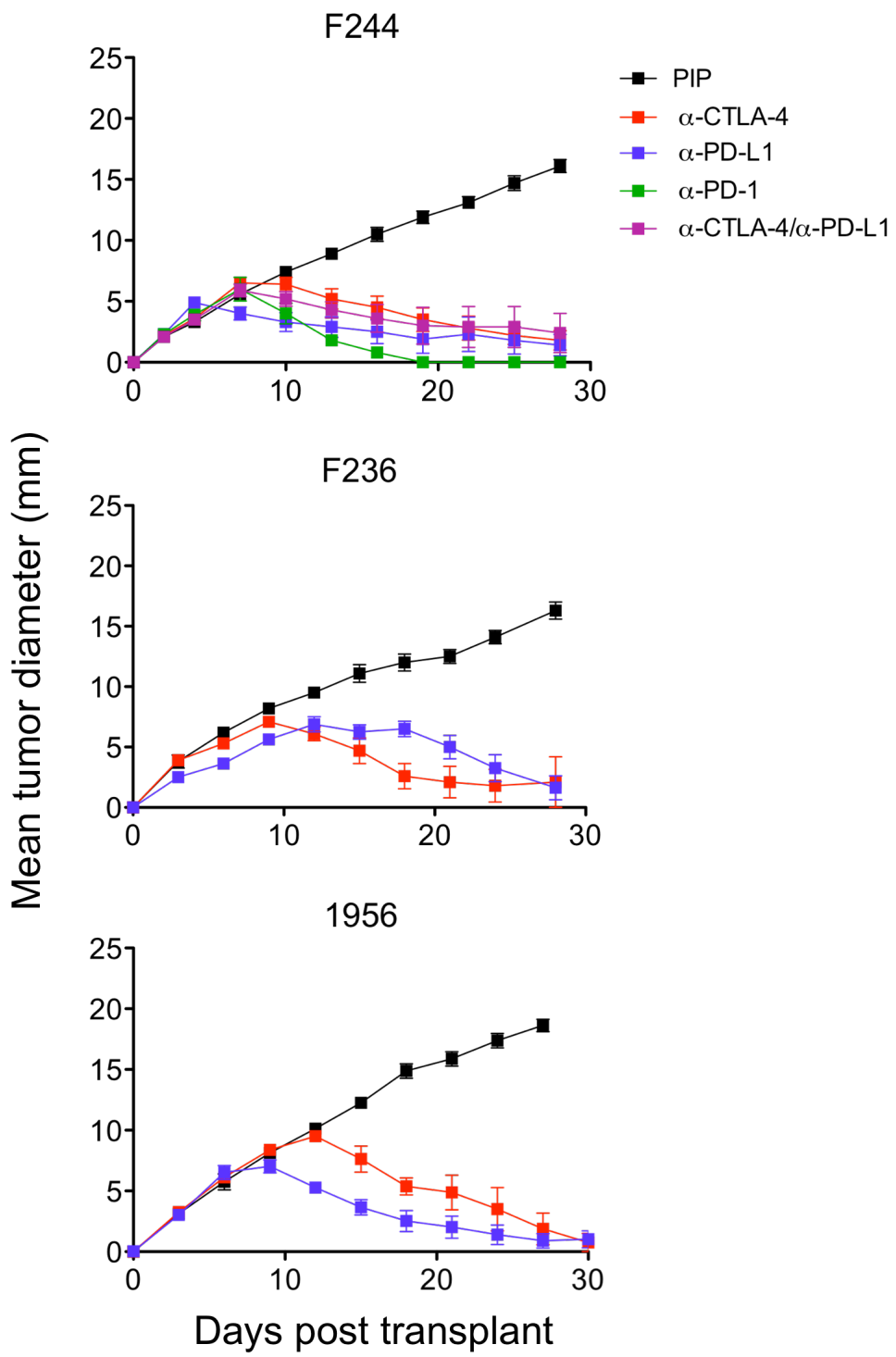


Figure 3. Edited MCA sarcomas that fail to respond to anti-CTLA-4 therapy also fail to respond to anti-PD-1 and anti-PD-L1 therapy or anti-CTLA-4/anti-PD-L1 combination therapy. F279, d4m3, 9606, and 9614 were transplanted into wild type mice at a dose of 1×10^6 cells and treated with 200 μg of control Ig (PIP) (black), anti-CTLA-4 (9H10) (red), anti-PD-L1 (10F.9G2) (blue) or combination of anti-CTLA-4/PD-L1 (green) on days 3, 6, 9, 12, 15, and 18 post transplant.

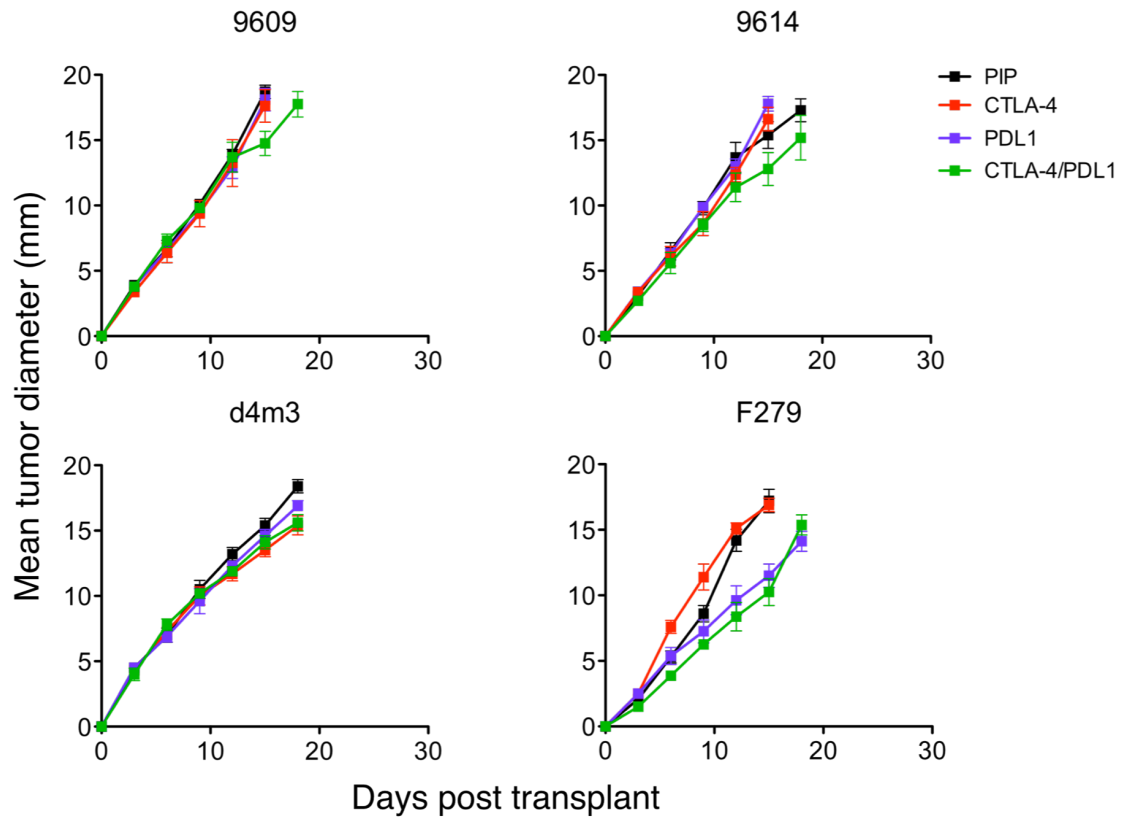
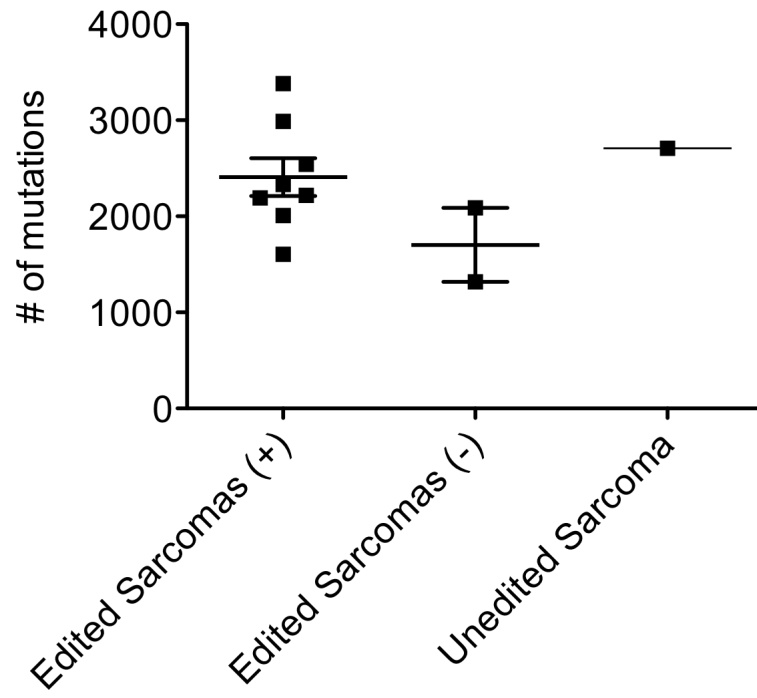


Figure 4. Number of mutations present in edited sarcomas as detected by exome sequencing. Number of mutations detected by exome sequencing on eight edited MCA sarcoma cell lines that respond to checkpoint blockade therapy (“Edited Sarcomas (+)” = F244, F236, H118m1, d4m3, d22m1, d22m2, and H128m8462) and two edited MCA sarcoma cell lines that do not respond to checkpoint blockade therapy (“Edited Sarcomas (+)” = F279 and d4m3). In addition, the unedited sarcoma d42m1 parental was included as a positive control for the NimbleGen mouse exome capture reagent (“Unedited Sarcoma”).



DISCUSSION

In this study, we have examined a set of edited sarcomas derived from MCA-treated immunocompetent wild type mice for residual immunogenicity by using checkpoint blockade therapy. When cohorts of wild type mice were transplanted with 13 different edited MCA sarcomas and subsequently treated with anti-CTLA-4 therapy, 9/13 (70%) sarcomas were rejected, suggesting that not all edited sarcomas exhibit residual immunogenicity. In addition, the four sarcomas that failed anti-CTLA-4 therapy also failed anti-PD-L1, anti-PD-1 therapy and anti-CTLA-4/PD-1 combination therapy. Similar to this panel of edited sarcomas, it is unclear why significant clinical responses to CTLA-4 blockade have been noted in some, but not all cancer patients (206). Perhaps the stochastic nature of the process that gives rise to antigens derived from tumor-specific mutations will produce edited tumors that express different levels of antigenicity/immunogenicity.

To address this issue, the exomes of MCA sarcomas derived from immunocompetent wild type mice were sequenced to first ask how many mutations are present in these tumors and second, whether the number of mutations differ between tumors generated in wild type mice (edited) versus tumors derived from *Rag2^{-/-}* mice (unedited)? First, there is a very broad range of mutations detected in edited sarcomas with some tumors have nearly three times the number of mutations. For example, 1,317 mutations were detected in F279 tumor cells and 3,380 mutations were detected in c1m2 tumor cells. Secondly, it appears that there is no difference between the number of mutations in edited and unedited MCA sarcomas, suggesting that the mutational load

present in these tumor cells is not greatly influenced by host's immune status.

Interestingly, there was a slight trend for edited sarcomas that fail anti-CTLA-4 therapy (F279 and d4m3) to have fewer mutations than those that do respond to immunotherapy, but there was no statistical difference between the numbers of mutations in the two subsets of edited sarcomas.

Pipelining the mutations identified by next-generation sequencing into MHC class I epitope prediction programs will be used to generate the antigenic profiles of tumors that have escaped immune control. It will be very interesting to use these antigenic profiles of edited tumors to correlate the quantity and quality of the potential antigens with response to immunotherapy. Current analyses are in progress to generate the antigenic profiles of all the edited sarcomas and results are pending. The ultimate test will be to use the antigenic profiles of experimental tumors to predict which respond to immunotherapy, which could have potential ramifications for human cancer immunotherapy.

In future studies, we will generate CD8⁺ CTL lines against edited sarcomas that respond to checkpoint blockade therapy, determine the MHC restriction of these CTL lines and synthesize peptide epitope candidates recognized by these CTL lines to identify the antigen(s) expressed in edited tumor cells that are putatively targeted by checkpoint blockade therapy. To confirm whether an identified antigen candidate is targeted by checkpoint blockade therapy *in vivo*, MHC class I tetramers specific for the mutant peptide recognized by the CTL lines will be generated to track endogenous mutant-specific CD8⁺ T cells. Ultimately, we will enforce expression of the mutant antigen from a susceptible tumor cell line in a tumor line that is not normally rejected by checkpoint

blockade therapy and will assess whether it now drives rejection following anti-CTLA-4 or anti-PD-1 therapy.

CHAPTER 6

Summary and Future Directions

SUMMARY AND CONCLUSIONS

The research presented in this dissertation represents a significant transition in the evolution of the cancer immunoediting concept. In the past, our efforts mostly centered on demonstrating that the process occurs, identifying the key players in it and attempting to define the positions that they play. We now enter a new phase in which we can begin to elucidate the molecular mechanisms that drive the process. We shifted our focus from the immune components that edit tumor immunogenicity, to the tumor cells themselves to ask whether the quality or quantity of tumor antigens expressed in nascent transformed cells determines immune-mediated elimination or sculpting. Thus, the overarching goal of this thesis was to understand what drives the editing of highly immunogenic tumor cells to those of reduced immunogenicity that grow in an immunologically unrestricted manner.

In the first study, we set out to further examine the editing of tumor immunogenicity by IFN- γ . Although other studies have shown that IFN- γ is critical to mediate immunosurveillance against primary tumors, these tumors lacked IFNGR1 and thus required additional manipulations to unmask their highly immunogenic character (16, 19). In fact, lymphomas that spontaneously form in gene-targeted mice deficient in IFN- γ do not reject when transplanted into wild type mice, suggesting that have been edited by non-IFN- γ mechanisms (22). To unequivocally demonstrate that IFN- γ plays a critical role in shaping tumor immunogenicity, we took the “gold standard” approach of generating primary MCA-induced sarcomas in an environment that lacks IFN- γ , harvested those tumors, generated cell lines, and transplanted them into wild type hosts to

see whether any unmanipulated sarcomas spontaneously reject in immunocompetent recipients.

Using a cohort of C57BL/6 mice exposed to MCA, we chronically treated these mice with neutralizing IFN- γ antibodies (H22) to generate sarcomas that developed in the absence of IFN- γ , but retained normal IFN- γ receptor signaling. These IFN- γ neutralized mice developed more MCA-induced sarcomas than their control counterparts, confirming previous reports that IFN- γ prevents development of primary tumors (16, 19, 55). In addition, 3/10 (30%) of MCA-induced sarcomas from IFN- γ neutralized mice spontaneously rejected when transplanted into naïve wild type hosts—a finding that is remarkably similar to *Rag2*^{-/-} mice and *Ifnar1*^{-/-} mice where 40% and 36% of the MCA sarcomas are highly immunogenic regressors, respectively (19, 24). These three highly immunogenic sarcomas, termed “H22-regressors” required CD4⁺ T cells, CD8⁺ T cells, and IFN- γ for their rejection, providing further evidence that they phenotypically resemble unedited tumors. Each of the three individual H22-regressors had different requirements for IFN- γ responsiveness at the level of the host and/or tumor to mediate tumor rejection, a result that is in contrast to *Rag2*^{-/-} MCA sarcomas generated on a 129/Sv background where either IFN- γ responsiveness at the level of the host or the tumor alone is sufficient to mediate tumor rejection (25) (C.M. Koebel and R.D. Schreiber, unpublished results). Taken together, these data suggest that IFN- γ sculpts tumor immunogenicity and, to date, is the best evidence that endogenous IFN- γ can alter tumor immunogenicity during primary tumor development. In addition, this is the first demonstration that unedited sarcomas can be generated using blocking monoclonal antibodies in wild type mice.

In the second study of this thesis, we show that cancer exome sequencing technology when combined with *in silico* epitope prediction algorithms can be used to identify expressed mutations in cancers that result in formation of tumor-specific antigens which function as targets for immune-mediated elimination. Initial work revealed that the highly immunogenic *Rag2*^{-/-} unedited tumors, H31m1 and d42m1, share a similar mutational landscape as carcinogen-induced human lung cancers. Although d42m1 and H31m1 display largely non-overlapping mutations that helps explain their distinct immunogenicities, they do share mutations in *Kras* (d42m1 *Kras* G12C; H31m1 *Kras* G12D) and in *Trp53* (d42m1: *Trp53* E295^{stop}; H31m1: *Trp53* S152R and S258I) that are frequently observed in human and mouse cancers (222-224). Next, we combined deep sequencing, algorithm analysis, and T cell epitope cloning to identify mutant spectrin- β 2 as a potential rejection antigen for d42m1 tumor cells. CD8⁺ T cells specific for mutant-spectrin- β 2 infiltrate d42m1 tumors just prior to rejection, but do not infiltrate d42m1 escape variants that lack mutant spectrin- β 2 expression. However, enforced expression of mutant spectrin- β 2 into these escape variants induces the infiltration of mutant-specific CD8⁺ T cells and tumor rejection, demonstrating that mutant spectrin- β 2 is the major rejection antigen of d42m1.

To our knowledge, this is the first study to identify an antigen from an unedited tumor (d42m1) that is responsible for tumor rejection. Moreover, we show that a T cell-dependent immunoselection process acting on an oligoclonal parental tumor cell population leads to the outgrowth of tumor cell variants that lack the immunodominant, tumor-specific rejection antigen—mutant spectrin- β 2. These results are consistent with the finding that both T cells and perforin are required for editing of primary MCA

sarcomas (87), primary lymphomas (22), and UV-induced tumors (212), although the targets of editing in these earlier studies were not defined. Thus, in the case of d42m1, the target of the immunoselection process has been clearly identified as the major rejection antigen. Certainly, similar mechanisms might also produce tumor variants with defects in MHC class I antigen processing and presentation or IFN- γ receptor signaling, which have been observed in clinically apparent human cancers (27, 97).

The apparent singular importance of mutant spectrin- β 2 in driving rejection of d42m1 in many ways resembles the known immunodominant behavior of certain viral antigens (234). However, preliminary analyses of other unedited MCA sarcomas (such as H31m1) reveal that some express multiple strong antigens. Thus, it is possible that the presence of one or more highly antigenic protein contributes to deciding whether a nascent tumor is eliminated or undergoes editing. Our results thus not only provide definitive evidence for at least one mechanism underlying the cancer immunoediting process, but also demonstrate the key role that tumor-specific mutations play in development of a tumor's immunogenic phenotype and subsequent fate.

In the third study, we examined whether d42m1 antigen loss variants exhibit residual immunogenicity. Since exome sequencing of d42m1 tumor variants detected thousands of mutations that result in protein-coding alterations and since one mutant protein (the R913L spectrin- β 2 mutant) was responsible for immune-mediated elimination of regressor d42m1 tumor cells, we asked whether these other mutations formed antigens that conferred residual immunogenicity. To test this, we used antibodies that block negative co-stimulatory molecules to boost anti-tumor immunity and overcome cancer-induced immunosuppression. Currently, this form of cancer immunotherapy,

termed “checkpoint blockade” therapy, has been the most successful form of immunotherapy to date (245). Specifically, anti-CTLA-4 therapy is quickly becoming a frontline therapeutic against malignant melanoma (206). When we transplanted d42m1-T3 and d42m1-es3 tumor cells into wild type mice and subsequently treated these mice with anti-CTLA-4, anti-PD-1, or anti-PD-L1 mAbs, both tumors were readily rejected. Rejection of d42m1-T3 and d42m1-es3 in the context of checkpoint blockade therapy required CD4⁺ T cells, CD8⁺ T cells, CD8 α ⁺/CD103⁺ DCs, and IFN- γ . Thus, antigen loss variants of d42m1, indeed, express antigens that confer residual immunogenicity and, presumably, these antigens are targeted by checkpoint blockade therapy. In addition, distinct d42m1 tumor variants could induce cross-protective immunity in serial transplantation experiments, suggesting that d42m1-related cells share common antigens. In contrast, d42m1 tumor variants could not protect against secondary challenge by an unrelated sarcoma, F244, indicating that there are unique antigens shared by the d42m1 variants capable of inducing protective immunity.

Based on these results, we mined our previous exome sequencing/*in silico* analysis for mutations that form potential antigens that are shared among the d42m1 variants. We found that there were only three potential antigens for H-2K^b and none for H-2D^b. In fact, a CTL line generated from a mouse that rejected d42m1-T3 when treated with anti-CTLA-4 and anti-PD-L1 combination therapy recognized d42m1 parental and d42m1-T3 tumor cells in a H-2K^b restricted manner. Thus, these results point to an H-2K^b restricted mutant peptide functioning as the antigenic target of checkpoint blockade therapy. Although exome sequencing has provided a short list of putative epitopes (one in particular – R101L mutation in G-protein receptor 108) that may be the targets of

checkpoint blockade therapy for d42m1-T3 and d42m1-es3, more work is needed to identify the specific antigens and demonstrate its capacity to stimulate antigen-specific T cells *in vivo*. To this end, additional CTLs lines have been generated and the wild type that rejected d42m1-T3 or d42m1-es3 tumors in response to checkpoint blockade therapy and mutant forms of the peptides (Gpr108 R101L; Olfr684 D72Y; Olfr1239 C177S) have been synthesized. We predict that the additional experiments outlined above will demonstrate which of the candidate antigens functions as the target for immunotherapeutically induced anti-tumor immune responses.

In the final study of this thesis, we explored whether edited MCA-induced sarcomas that developed in immunocompetent wild type mice also exhibited residual immunogenicity. Using the checkpoint blockade therapy, we found that 9/13 (70%) of edited sarcomas respond to anti-CTLA-4 therapy. Edited sarcomas that are rejected due to CTLA-4 therapy also rejected in mice treated with anti-PD-1 or anti-PD-L1 mAbs. In contrast, the four edited sarcomas that fail anti-CTLA-4 therapy (F279, d4m3, 9609, and 9614) do not respond to anti-PD-1 or anti-PD-L1 mAbs, even when used in combination with anti-CTLA-4 therapy. Thus, a subset of edited sarcomas respond to checkpoint blockade therapy and a subset of edited sarcomas fail to respond to checkpoint blockade therapy, a situation that resembles the clinical scenario. Currently, it is unknown why certain human cancer patients respond or fail to respond to anti-CTLA-4 therapy, but one study demonstrated that the patients that had the best clinical responses had pre-existing immunity to a cancer-associated antigen, NY-ESO-1 (259, 260). This result suggests that antigen expression by the tumor cells may be critical to allow for tumor-targeted, immune-mediated destruction of transformed cells by checkpoint blockade therapy.

To attempt to address this question, we sequenced the exons of ten edited wild type sarcomas, two of which fail to respond to anti-CTLA-4 therapy (F279 and d4m3). Although there was a trend for fewer numbers of mutations in F279 and d4m3 than the other eight edited sarcomas that do respond to anti-CTLA-4 therapy, it was not statistically significant. Moreover, the number of mutations in edited sarcomas was similar to the number detected in the highly immunogenic, unedited tumors (H31m1 and d42m1). These results suggest that the number of mutations present in MCA-induced sarcomas is due to the carcinogen-load, location, and tissue-type involved and not due to the immune status of the host. Thus, it is tempting to speculate that the level of residual antigenicity remaining in a naturally immunoedited tumor plays an important role in determining whether it can be effectively controlled by checkpoint blockade therapy. We are currently analyzing whether the mutations present in edited sarcomas form neoantigens using class I prediction algorithms.

Taking together, the data presented in this thesis has attempted to further our understanding of the factors that drive the cancer immunoediting process. We show that tumor-specific antigens of high affinity are targeted by the cancer immunosurveillance network to eliminate tumors cells and that antigen loss variants of reduced immunogenicity emerge due to a T-cell dependent immunoselection process. Furthermore, residual antigenicity is unmasked in antigen loss variants of d42m1 and edited wild type sarcomas using checkpoint blockade therapy. The approach of exome sequencing, *in silico* analysis, and CD8⁺ T cell cloning used in this thesis may be of beneficial use to both basic and clinical scientists. By defining the specific antigenic targets of immunotherapeutically-induced, immune-mediated tumor cell elimination, we

should obtain new levels of understanding of host responses to tumors during ongoing therapy that may facilitate the development of new therapeutic opportunities to direct the power and specificity of the immune system into controlling and/or destroying cancer. It may also be useful in identifying subsets of cancer patients whose tumors express antigens that can be most effectively targeted by checkpoint blockade immunotherapy and may provide a mechanism to longitudinally evaluate changes in a tumor's antigenic profile as a consequence of ongoing immunotherapy. Therefore, we predict that a genomic approach to tumor antigen identification such as the one reported here may, in the future, facilitate the development of individualized cancer immunotherapies directed at tumor-specific—rather than cancer-associated—antigens.

FUTURE DIRECTIONS

What are the host targets for IFN- γ mediated tumor rejection?

We now have extensive evidence that IFN- γ is critical for preventing primary tumor formation and for sculpting tumor immunogenicity (16, 19, 22, 55). In this thesis, we generated three unedited sarcomas from MCA-treated wild type mice chronically administered IFN- γ neutralizing antibodies (H22). All three of these tumors required IFN- γ for their rejection and for one tumor, H22-28027, IFN- γ responsiveness at the level of the tumor alone was sufficient to mediate rejection, confirming previous results from our laboratory (15). For H22-28032 tumors, IFN- γ responsiveness at the level of the host was sufficient to mediate tumor rejection, as H22-28032 tumor cells grew out in the majority of *Ifngr1*^{-/-} recipients. However, it has yet to be determined which host cells stimulated by IFN- γ are critical to mediate IFN- γ 's anti-tumor effects. Currently, a fellow graduate student in the Schreiber laboratory, Sang-hun Lee, recently generated conditional *Ifngr1* gene-targeted mice to selectively delete IFNGR1 in specific tissues to address this issue. Unedited tumors will be transplanted into tissue-specific *Ifngr1*^{-/-} mice to determine which IFN- γ responsive host cells are required for tumor rejection. The two unedited tumors he will use for this analysis will be H22-28032 that was generated as part of this thesis and 1969 that, in addition to requiring host cell IFN- γ responsiveness, also requires type I (IFN- α/β) interferon responsiveness at the level of CD11c⁺ cells to mediate tumor rejection (25).

Identifying the antigenic targets of checkpoint blockade therapy in antigen loss variants

Although exome sequencing and class I prediction algorithms has provided a short list of putative epitopes for d42m1-T3 and d42m1-es3, more work is needed to identify the specific antigens that are targeted by checkpoint blockade therapy. Specifically, CTL lines developed against d42m1-T3 and d42m1-es3 will be tested for their capacity to recognize candidate epitopes and if so, demonstrate that the mutant peptide stimulates antigen-specific T cells *in vivo*. To this end, additional CTLs lines have been generated from wild type mice that rejected d42m1-T3 or d42m1-es3 tumors in response to checkpoint blockade therapy and mutant forms of the peptides (Gpr108 R101L; Olfr684 D72Y; Olfr1239 C177S) have been synthesized. The synthesized candidate peptides are currently being tested against the CTL line that recognizes d42m1 and d42m1-T3 parental cells in an H-2K^b restricted manner. If any mutant peptides presented on unrelated H-2K^b expressing cells activates this CTL line or any of the other CTL lines to be tested, then these peptides will be used to assemble soluble H-2K^b MHC class I tetramers to track mutation-specific CD8⁺ T cells *in vivo*. Alternatively, mutant peptides will be used to immunize wild type mice prior to d42m1-T3 or d42m1-es3 tumor cell challenge to test if the peptide can be used to vaccinate against d42m1 antigen loss variants. We predict that the additional experiments outlined above will demonstrate which of the candidate antigens functions as the target for immunotherapeutically induced anti-tumor immune responses.

Do edited sarcomas that respond to anti-CTLA-4 therapy have more potential antigens than edited sarcomas that fail to respond to anti-CTLA-4 therapy?

Recently, we sequenced the exons of ten different edited sarcomas derived from immunocompetent 129/Sv wild type mice that were initially treated with 100 µg of the MCA carcinogen. Two (F279 and d4m3) of the ten edited sarcomas (20%) do not respond to checkpoint blockade immunotherapy (i.e., anti-CTLA-4, anti-PD-1, or anti-PD-L1) even when used in combination, suggesting that these edited sarcomas are non-immunogenic. Exome sequencing revealed that these tumors express slightly fewer mutations, but the mutational load is essentially the same between edited sarcomas that respond to anti-CTLA-4 therapy and those that do not respond to anti-CTLA-4 therapy. In fact, there appears to be no difference in the number of mutations between highly immunogenic, unedited sarcomas (d42m1 and H31m1) and edited sarcomas of reduced immunogenicity. Thus, for MCA-induced sarcomas, the level of residual immunogenicity cannot be inferred from the mutational load or the number of mutations they express. However, it is still possible that the potential antigenic array differs between unedited and edited sarcomas as well as between edited sarcomas that respond to anti-CTLA-4 therapy and those that fail anti-CTLA-4 therapy. Currently, we are in the process of submitting our exome sequencing data from edited sarcomas into class I prediction algorithms to generate antigenic profiles for each individual tumor. In order to correlate the quality and quantity of potential antigens of a tumor with sensitivity to checkpoint blockade immunotherapy, additional tumors may need to be sequenced to enhance the statistical power of the dataset.

Identifying the antigenic targets of checkpoint blockade therapy in edited tumors

One approach to identifying the antigens expressed in transplantable, edited tumors that respond to checkpoint blockade therapy is to use the cDNA CapSeq/*in silico* analysis described in this thesis. As detailed above, we have already sequenced the exons of a panel of edited sarcoma cell lines, some of which respond to anti-CTLA-4 therapy and some that do not. Thus, any potential antigens predicted by class I algorithms can be synthesized and tested for their capacity to stimulate CTL lines generated from mice that reject edited tumors after checkpoint blockade therapy. Any mutant epitope that positively stimulates a CTL could be used to generate soluble MHC class I tetramers to track endogenous antigen-specific CD8⁺ T cells *in vivo*.

The second and more rigorous approach is to perform a similar analysis on primary MCA sarcomas in wild type mice. Here, the same general principles apply in that tumor cDNA will be sequenced, potential antigens will be predicted, and tumor-specific CTLs will be generated but, the tumor tissue will come from developing primary tumors undergoing tumor progression or immunotherapeutically induced tumor regression. Specifically, tumor tissue will be harvested from at least 5 primary sarcomas that respond to checkpoint blockade therapy (as evidenced by tumor regression but before total elimination of the tumor) and at least 5 primary MCA sarcomas that do not respond to checkpoint blockade therapy (as evidenced by tumor progression), cDNA will be isolated, Illumina libraries will be generated, hybridized to mouse exome probes (Agilent) and submitted for sequencing. The study of primary MCA sarcomas as opposed to cell lines not only has the advantage of most closely resembling the clinical scenario of

anti-CTLA-4 therapy in terms of tumor development (autochthonous tumor model) and in terms of anti-CTLA-4 therapeutic responses, but also provides the opportunity to isolate normal tissue from the same individual mouse as the tumor for comparative genome analyses. By pipelining the exome sequencing data into MHC class I epitope prediction algorithms, we will generate antigenic profiles for each individual sarcoma and compare whether the sarcomas that respond to immunotherapy have a greater number of potential high- to medium-binding affinity antigens than sarcomas that fail to respond to immunotherapy. Finally, any mouse that rejects its primary MCA sarcoma during the course of checkpoint blockade therapy will be used to generate a CD8⁺ CTL line against the primary sarcoma cells in an attempt to identify the tumor-specific antigens targeted by this form of immunotherapy.

Potential clinical implications for cancer genome sequencing on tumor immunology

Cancer genome sequencing analyses has provided many important insights into the nature of mutations that facilitate transformation (261). Although, the primary focus of this field is to identify the “driver” mutations, it is becoming apparent that the vast majority of mutations present in cancer cells are “passenger” mutations that may function as targets for elimination by immunotherapy (213, 214). The large datasets of information from the many cancer genome initiatives could be of extreme value to tumor immunologists to define the “antigen landscape” as opposed to the “mutational landscape” of human cancers (262). One application of this approach is that it could be used to identify the subset of cancer patients whose tumors express antigens that can be

most effectively targeted by checkpoint blockade immunotherapy. In addition, this approach may provide a mechanism to longitudinally evaluate changes in a tumor's antigenic profile as a consequence of ongoing immunotherapy.

It is difficult to predict whether this type of analysis will yield prognostic value in the clinic, as genome analysis can be costly and requires streamlined computational analysis. Nevertheless, third-generation sequencing technologies are already commercially available and costs for cancer genome sequencing are predicted to fall sharply over the next decade (263), thus this type of analysis may be feasible to perform on a individual patient's cancer cells in the not-to-distant future. Whether it proves useful will require extensive studies performed initially in pre-clinical models like the ones used in this thesis as well as retrospective and longitudinal clinical studies.

REFERENCES

1. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW. 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445: 656-60
2. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88: 593-602
3. Danial NN, Korsmeyer SJ. 2004. Cell death: critical control points. *Cell* 116: 205-19
4. Peter ME, Krammer PH. 2003. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 10: 26-35
5. Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* 100: 57-70
6. Janes SM, Watt FM. 2006. New roles for integrins in squamous-cell carcinoma. *Nat Rev Cancer* 6: 175-83
7. Humbert P, Russell S, Richardson H. 2003. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *Bioessays* 25: 542-53
8. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3: 991-8
9. Zitvogel L, Tesniere A, Kroemer G. 2006. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 6: 715-27
10. Curiel TJ. 2007. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 117: 1167-74
11. Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. *Cell* 144: 646-74
12. Ehrlich P. 1909. Ueber den jetzigen stand der Karzinomforschung. *Ned. Tijdschr. Geneesk.* 5: 273-90
13. Burnet FM. 1957. Cancer - a biological approach. *Brit. Med. J.* 1: 841-7
14. Thomas L. 1959. *Cellular and Humoral Aspects of the Hypersensitive States*. New York: Hoeber-Harper
15. Dighe AS, Richards E, Old LJ, Schreiber RD. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* 1: 447-56

16. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* 95: 7556-61
17. Engel AM, Svane IM, Mouritsen S, Rygaard J, Clausen J, Werdelin O. 1996. Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *Apmis* 104: 629-39
18. Engel AM, Svane IM, Rygaard J, Werdelin O. 1997. MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. *Scand J Immunol* 45: 463-70
19. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410: 1107-11
20. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. 2000. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* 192: 755-60
21. Korner H, Cretney E, Wilhelm P, Kelly JM, Rollinghoff M, Sedgwick JD, Smyth MJ. 2000. Tumor necrosis factor sustains the generalized lymphoproliferative disorder (gld) phenotype. *J Exp Med* 191: 89-96
22. Street SE, Trapani JA, MacGregor D, Smyth MJ. 2002. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med* 196: 129-34
23. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC. 2001. Regulation of cutaneous malignancy by gammadelta T cells. *Science* 294: 605-9
24. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM, Schreiber RD. 2005. A critical function for type I interferons in cancer immunoediting. *Nat Immunol* 6: 722-9
25. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U, Murphy KM, Schreiber RD. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med* 208: 1989-2003
26. Dunn GP, Old LJ, Schreiber RD. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21: 137-48
27. Dunn GP, Old LJ, Schreiber RD. 2004. The three Es of cancer immunoediting. *Annu Rev Immunol* 22: 329-60
28. Dunn GP, Koebel CM, Schreiber RD. 2006. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 6: 836-48

29. Smyth MJ, Dunn GP, Schreiber RD. 2006. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol* 90: 1-50
30. Swann JB, Smyth MJ. 2007. Immune surveillance of tumors. *J Clin Invest* 117: 1137-46
31. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. 2011. Natural Innate and Adaptive Immunity to Cancer. *Annu Rev Immunol* 29: 235-71
32. Schreiber RD, Old LJ, Smyth MJ. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331: 1565-70
33. Smyth MJ, Crowe NY, Godfrey DI. 2001. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* 13: 459-63
34. Girardi M, Glusac E, Filler RB, Roberts SJ, Propperova I, Lewis J, Tigelaar RE, Hayday AC. 2003. The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer. *J Exp Med* 198: 747-55
35. Swann JB, Uldrich AP, van Dommelen S, Sharkey J, Murray WK, Godfrey DI, Smyth MJ. 2009. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood* 113: 6382-5
36. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 191: 661-8
37. Smyth MJ, Cretney E, Takeda K, Wiltout RH, Sedger LM, Kayagaki N, Yagita H, Okumura K. 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* 193: 661-70
38. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM. 2008. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097-100
39. Simson L, Ellyard JI, Dent LA, Matthaei KI, Rothenberg ME, Foster PS, Smyth MJ, Parish CR. 2007. Regulation of carcinogenesis by IL-5 and CCL11: a potential role for eosinophils in tumor immune surveillance. *J Immunol* 178: 4222-9
40. Street SE, Cretney E, Smyth MJ. 2001. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* 97: 192-7

41. Swann JB, Hayakawa Y, Zerafa N, Sheehan KC, Scott B, Schreiber RD, Hertzog P, Smyth MJ. 2007. Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. *J Immunol* 178: 7540-9
42. Swann JB, Vesely MD, Silva A, Sharkey J, Akira S, Schreiber RD, Smyth MJ. 2008. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* 105: 652-6
43. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. 2002. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* 168: 1356-61
44. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H, Okumura K. 2002. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* 195: 161-9
45. Smyth MJ, Taniguchi M, Street SE. 2000. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. *J Immunol* 165: 2665-70
46. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, Yasui T, Kikutani H, Shibuya K, Shibuya A. 2008. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med* 205: 2959-64
47. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. 2005. NKG2D function protects the host from tumor initiation. *J Exp Med* 202: 583-8
48. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, Knoblaugh S, Cado D, Greenberg NM, Raulet DH. 2008. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 28: 571-80
49. Elboim M, Gazit R, Gur C, Ghadially H, Betser-Cohen G, Mandelboim O. 2010. Tumor immunoediting by NKp46. *J Immunol* 184: 5637-44
50. Alimonti J, Zhang QJ, Gabathuler R, Reid G, Chen SS, Jefferies WA. 2000. TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat Biotechnol* 18: 515-20
51. Fallarino F, Gajewski TF. 1999. Cutting edge: differentiation of antitumor CTL in vivo requires host expression of Stat1. *J Immunol* 163: 4109-13
52. Zhang B, Karrison T, Rowley DA, Schreiber H. 2008. IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J Clin Invest* 118: 1398-404
53. Qin Z, Kim HJ, Hemme J, Blankenstein T. 2002. Inhibition of methylcholanthrene-induced carcinogenesis by an interferon gamma receptor-dependent foreign body reaction. *J Exp Med* 195: 1479-90

54. Liu J, Xiang Z, Ma X. 2004. Role of IFN regulatory factor-1 and IL-12 in immunological resistance to pathogenesis of N-methyl-N-nitrosourea-induced T lymphoma. *J Immunol* 173: 1184-93
55. Wakita D, Chamoto K, Ohkuri T, Narita Y, Ashino S, Sumida K, Nishikawa H, Shiku H, Togashi Y, Kitamura H, Nishimura T. 2009. IFN-gamma-dependent type 1 immunity is crucial for immunosurveillance against squamous cell carcinoma in a novel mouse carcinogenesis model. *Carcinogenesis* 30: 1408-15
56. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, Gajewski TF. 2011. Host type I IFN signals are required for antitumor CD8⁺ T cell responses through CD8⁺ dendritic cells. *J Exp Med* 208: 2005-16
57. Johansen C, Vestergaard C, Kragballe K, Kollias G, Gaestel M, Iversen L. 2009. MK2 regulates the early stages of skin tumor promotion. *Carcinogenesis* 30: 2100-8
58. Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, Holdsworth H, Turner L, Rollins B, Pasparakis M, Kollias G, Balkwill F. 1999. Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 5: 828-31
59. Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth PP, Hess J, Angel P. 2008. RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med* 205: 275-85
60. Muller AJ, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BA, 3rd, Kahler DJ, Pihkala J, Soler AP, Munn DH, Prendergast GC, Mellor AL. 2008. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. *Proc Natl Acad Sci U S A* 105: 17073-8
61. Yusuf N, Nasti TH, Katiyar SK, Jacobs MK, Seibert MD, Ginsburg AC, Timares L, Xu H, Elmets CA. 2008. Antagonistic roles of CD4⁺ and CD8⁺ T-cells in 7,12-dimethylbenz(a)anthracene cutaneous carcinogenesis. *Cancer Res* 68: 3924-30
62. Gasser S, Orsulic S, Brown EJ, Raulet DH. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436: 1186-90
63. Oppenheim DE, Roberts SJ, Clarke SL, Filler R, Lewis JM, Tigelaar RE, Girardi M, Hayday AC. 2005. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol* 6: 928-37

64. Strid J, Roberts SJ, Filler RB, Lewis JM, Kwong BY, Schpero W, Kaplan DH, Hayday AC, Girardi M. 2008. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat Immunol* 9: 146-54
65. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schutz G, Greiner EF, Kemp CJ, Walczak H. 2008. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. *J Clin Invest* 118: 100-10
66. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M. 2006. IL-23 promotes tumour incidence and growth. *Nature* 442: 461-5
67. Teng MW, Andrews DM, McLaughlin N, von Scheidt B, Ngiow SF, Moller A, Hill GR, Iwakura Y, Oft M, Smyth MJ. 2010. IL-23 suppresses innate immune response independently of IL-17A during carcinogenesis and metastasis. *Proc Natl Acad Sci U S A* 107: 8328-33
68. Xiao M, Wang C, Zhang J, Li Z, Zhao X, Qin Z. 2009. IFN γ promotes papilloma development by up-regulating Th17-associated inflammation. *Cancer Res* 69: 2010-7
69. Norbury KC, Kripke ML. 1978. Ultraviolet carcinogenesis in T-cell-depleted mice. *J Natl Cancer Inst* 61: 917-21
70. Ward PL, Koeppen HK, Hurteau T, Rowley DA, Schreiber H. 1990. Major histocompatibility complex class I and unique antigen expression by murine tumors that escaped from CD8⁺ T-cell-dependent surveillance. *Cancer Res* 50: 3851-8
71. Street SE, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, Diefenbach A, Yagita H, Godfrey DI, Smyth MJ. 2004. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J Exp Med* 199: 879-84
72. Zerafa N, Westwood JA, Cretney E, Mitchell S, Waring P, Iezzi M, Smyth MJ. 2005. Cutting edge: TRAIL deficiency accelerates hematological malignancies. *J Immunol* 175: 5586-90
73. Davidson WF, Giese T, Fredrickson TN. 1998. Spontaneous development of plasmacytoid tumors in mice with defective Fas-Fas ligand interactions. *J Exp Med* 187: 1825-38
74. Chia J, Yeo KP, Whisstock JC, Dunstone MA, Trapani JA, Voskoboinik I. 2009. Temperature sensitivity of human perforin mutants unmasks subtotal loss of

- cytotoxicity, delayed FHL, and a predisposition to cancer. *Proc Natl Acad Sci U S A* 106: 9809-14
75. Stutman O. 1975. Immunodepression and malignancy. *Adv Cancer Res* 22: 261-422
 76. Gershwin ME, Ohsugi Y, Castles JJ, Ikeda RM, Ruebner B. 1983. Anti-mu induces lymphoma in germfree congenitally athymic (nude) but not in heterozygous (nu/+) mice. *J Immunol* 131: 2069-73
 77. Hayashi T, Faustman DL. 2002. Development of spontaneous uterine tumors in low molecular mass polypeptide-2 knockout mice. *Cancer Res* 62: 24-7
 78. Airoidi I, Di Carlo E, Cocco C, Sorrentino C, Fais F, Cilli M, D'Antuono T, Colombo MP, Pistoia V. 2005. Lack of Il12rb2 signaling predisposes to spontaneous autoimmunity and malignancy. *Blood* 106: 3846-53
 79. Enzler T, Gillessen S, Manis JP, Ferguson D, Fleming J, Alt FW, Mihm M, Dranoff G. 2003. Deficiencies of GM-CSF and interferon gamma link inflammation and cancer. *J Exp Med* 197: 1213-9
 80. Coussens LM, Werb Z. 2002. Inflammation and cancer. *Nature* 420: 860-7
 81. Bolitho P, Street SE, Westwood JA, Edelmann W, Macgregor D, Waring P, Murray WK, Godfrey DI, Trapani JA, Johnstone RW, Smyth MJ. 2009. Perforin-mediated suppression of B-cell lymphoma. *Proc Natl Acad Sci U S A* 106: 2723-8
 82. Mitra-Kaushik S, Harding J, Hess J, Schreiber R, Ratner L. 2004. Enhanced tumorigenesis in HTLV-1 tax-transgenic mice deficient in interferon-gamma. *Blood* 104: 3305-11
 83. Finnberg N, Klein-Szanto AJ, El-Deiry WS. 2008. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. *J Clin Invest* 118: 111-23
 84. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, Freschi M, Casorati G, Dellabona P. 2010. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS One* 5: e8646
 85. Willimsky G, Blankenstein T. 2005. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature* 437: 141-6
 86. Aguirre-Ghiso JA. 2007. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer* 7: 834-46
 87. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. 2007. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450: 903-7

88. Farrar JD, Katz KH, Windsor J, Thrush G, Scheuermann RH, Uhr JW, Street NE. 1999. Cancer dormancy. VII. A regulatory role for CD8+ T cells and IFN-gamma in establishing and maintaining the tumor-dormant state. *J Immunol* 162: 2842-9
89. Saudemont A, Quesnel B. 2004. In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. *Blood* 104: 2124-33
90. Loeser S, Loser K, Bijker MS, Rangachari M, van der Burg SH, Wada T, Beissert S, Melief CJ, Penninger JM. 2007. Spontaneous tumor rejection by cbl-b-deficient CD8+ T cells. *J Exp Med* 204: 879-91
91. Schumacher K, Haensch W, Roefzaad C, Schlag PM. 2001. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. *Cancer Res* 61: 3932-6
92. Eyles J, Puaux AL, Wang X, Toh B, Prakash C, Hong M, Tan TG, Zheng L, Ong LC, Jin Y, Kato M, Prevost-Blondel A, Chow P, Yang H, Abastado JP. 2010. Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. *J Clin Invest* 120: 2030-9
93. Bromberg JF, Horvath CM, Wen Z, Schreiber RD, Darnell JE, Jr. 1996. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci U S A* 93: 7673-8
94. Mandal M, Bandyopadhyay D, Goepfert TM, Kumar R. 1998. Interferon-induces expression of cyclin-dependent kinase-inhibitors p21WAF1 and p27Kip1 that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK). *Oncogene* 16: 217-25
95. Muller-Hermelink N, Braumuller H, Pichler B, Wieder T, Mailhammer R, Schaak K, Ghoreschi K, Yazdi A, Haubner R, Sander CA, Mocikat R, Schwaiger M, Forster I, Huss R, Weber WA, Kneilling M, Rocken M. 2008. TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. *Cancer Cell* 13: 507-18
96. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA. 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314: 126-9
97. Khong HT, Restifo NP. 2002. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 3: 999-1005
98. Kyewski B, Klein L. 2006. A central role for central tolerance. *Annu Rev Immunol* 24: 571-606

99. Schell TD, Knowles BB, Tevethia SS. 2000. Sequential loss of cytotoxic T lymphocyte responses to simian virus 40 large T antigen epitopes in T antigen transgenic mice developing osteosarcomas. *Cancer Res* 60: 3002-12
100. Restifo NP, Marincola FM, Kawakami Y, Taubenberger J, Yannelli JR, Rosenberg SA. 1996. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst* 88: 100-8
101. Khong HT, Wang QJ, Rosenberg SA. 2004. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother* 27: 184-90
102. Jager E, Ringhoffer M, Karbach J, Arand M, Oesch F, Knuth A. 1996. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. *Int J Cancer* 66: 470-6
103. Dunn GP, Sheehan KC, Old LJ, Schreiber RD. 2005. IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. *Cancer Res* 65: 3447-53
104. Stern-Ginossar N, Gur C, Biton M, Horwitz E, Elboim M, Stanietsky N, Mandelboim M, Mandelboim O. 2008. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat Immunol* 9: 1065-73
105. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H. 2004. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 10: 48-54
106. Kataoka T, Schroter M, Hahne M, Schneider P, Irmeler M, Thome M, Froelich CJ, Tschopp J. 1998. FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. *J Immunol* 161: 3936-42
107. Hinz S, Trauzold A, Boenicke L, Sandberg C, Beckmann S, Bayer E, Walczak H, Kalthoff H, Ungefroren H. 2000. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene* 19: 5477-86
108. Shin MS, Kim HS, Lee SH, Park WS, Kim SY, Park JY, Lee JH, Lee SK, Lee SN, Jung SS, Han JY, Kim H, Lee JY, Yoo NJ. 2001. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. *Cancer Res* 61: 4942-6

109. Takahashi H, Feuerhake F, Kutok JL, Monti S, Dal Cin P, Neubergh D, Aster JC, Shipp MA. 2006. FAS death domain deletions and cellular FADD-like interleukin 1beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes. *Clin Cancer Res* 12: 3265-71
110. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8: 793-800
111. Tripathi P, Agrawal S. 2006. Non-classical HLA-G antigen and its role in the cancer progression. *Cancer Invest* 24: 178-86
112. Derre L, Corvaisier M, Charreau B, Moreau A, Godefroy E, Moreau-Aubry A, Jotereau F, Gervois N. 2006. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J Immunol* 177: 3100-7
113. Park JM, Terabe M, Sakai Y, Munasinghe J, Forni G, Morris JC, Berzofsky JA. 2005. Early role of CD4+ Th1 cells and antibodies in HER-2 adenovirus vaccine protection against autochthonous mammary carcinomas. *J Immunol* 174: 4228-36
114. Radoja S, Rao TD, Hillman D, Frey AB. 2000. Mice bearing late-stage tumors have normal functional systemic T cell responses in vitro and in vivo. *J Immunol* 164: 2619-28
115. Groh V, Wu J, Yee C, Spies T. 2002. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 419: 734-8
116. Villablanca EJ, Raccosta L, Zhou D, Fontana R, Maggioni D, Negro A, Sanvito F, Ponzoni M, Valentinis B, Bregni M, Prinetti A, Steffensen KR, Sonnino S, Gustafsson JA, Doglioni C, Bordignon C, Traversari C, Russo V. 2010. Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses. *Nat Med* 16: 98-105
117. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, Corzo A, Cho HI, Celis E, Lennox B, Knight SC, Padhya T, McCaffrey TV, McCaffrey JC, Antonia S, Fishman M, Ferris RL, Kagan VE, Gabilovich DI. 2010. Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med*
118. Gabilovich DI, Ishida T, Nadaf S, Ohm JE, Carbone DP. 1999. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. *Clin Cancer Res* 5: 2963-70

119. Wrzesinski SH, Wan YY, Flavell RA. 2007. Transforming growth factor-beta and the immune response: implications for anticancer therapy. *Clin Cancer Res* 13: 5262-70
120. Aruga A, Aruga E, Tanigawa K, Bishop DK, Sondak VK, Chang AE. 1997. Type 1 versus type 2 cytokine release by Vbeta T cell subpopulations determines in vivo antitumor reactivity: IL-10 mediates a suppressive role. *J Immunol* 159: 664-73
121. Fujii S, Shimizu K, Shimizu T, Lotze MT. 2001. Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. *Blood* 98: 2143-51
122. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, Mordoh J, Fainboim L, Podhajcer OL, Rabinovich GA. 2004. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell* 5: 241-51
123. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ. 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med* 9: 1269-74
124. Lob S, Konigsrainer A, Rammensee HG, Opelz G, Terness P. 2009. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? *Nat Rev Cancer* 9: 445-52
125. Terabe M, Berzofsky JA. 2004. Immunoregulatory T cells in tumor immunity. *Curr Opin Immunol* 16: 157-62
126. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. 2009. Regulatory T cells: how do they suppress immune responses? *Int Immunol* 21: 1105-11
127. Teng MW, Swann JB, von Scheidt B, Sharkey J, Zerafa N, McLaughlin N, Yamaguchi T, Sakaguchi S, Darcy PK, Smyth MJ. 2010. Multiple antitumor mechanisms downstream of prophylactic regulatory T-cell depletion. *Cancer Res* 70: 2665-74
128. DiLillo DJ, Matsushita T, Tedder TF. 2010. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Ann N Y Acad Sci* 1183: 38-57
129. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, Godfrey DI, Ostrand-Rosenberg S, Smyth MJ, Berzofsky JA. 2005. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* 202: 1627-33

130. Gabrilovich DI, Nagaraj S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 9: 162-74
131. Li H, Han Y, Guo Q, Zhang M, Cao X. 2009. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol* 182: 240-9
132. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. 2010. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res* 70: 68-77
133. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, Gabrilovich DI. 2007. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 13: 828-35
134. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. 2006. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 66: 1123-31
135. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA. 2008. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* 111: 4233-44
136. Wei S, Kryczek I, Zou L, Daniel B, Cheng P, Mottram P, Curiel T, Lange A, Zou W. 2005. Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. *Cancer Res* 65: 5020-6
137. Conejo-Garcia JR, Benencia F, Courreges MC, Kang E, Mohamed-Hadley A, Buckanovich RJ, Holtz DO, Jenkins A, Na H, Zhang L, Wagner DS, Katsaros D, Carroll R, Coukos G. 2004. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med* 10: 950-8
138. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA. 2010. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* 328: 749-52
139. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. 2008. Macrophage polarization in tumour progression. *Semin Cancer Biol* 18: 349-55
140. Zippelius A, Batard P, Rubio-Godoy V, Bioley G, Lienard D, Lejeune F, Rimoldi D, Guillaume P, Meidenbauer N, Mackensen A, Rufer N, Lubenow N, Speiser D, Cerottini JC, Romero P, Pittet MJ. 2004. Effector function of human tumor-

specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res* 64: 2865-73

141. Boshoff C, Weiss R. 2002. AIDS-related malignancies. *Nat Rev Cancer* 2: 373-82
142. Frisch M, Biggar RJ, Engels EA, Goedert JJ. 2001. Association of cancer with AIDS-related immunosuppression in adults. *JAMA* 285: 1736-45
143. Chaturvedi AK, Pfeiffer RM, Chang L, Goedert JJ, Biggar RJ, Engels EA. 2007. Elevated risk of lung cancer among people with AIDS. *AIDS* 21: 207-13
144. Kirk GD, Merlo C, P OD, Mehta SH, Galai N, Vlahov D, Samet J, Engels EA. 2007. HIV infection is associated with an increased risk for lung cancer, independent of smoking. *Clin Infect Dis* 45: 103-10
145. Vajdic CM, McDonald SP, McCredie MR, van Leeuwen MT, Stewart JH, Law M, Chapman JR, Webster AC, Kaldor JM, Grulich AE. 2006. Cancer incidence before and after kidney transplantation. *JAMA* 296: 2823-31
146. Moloney FJ, Comber H, O'Lorcain P, O'Kelly P, Conlon PJ, Murphy GM. 2006. A population-based study of skin cancer incidence and prevalence in renal transplant recipients. *Br J Dermatol* 154: 498-504
147. Penn I. 1996. Malignant melanoma in organ allograft recipients. *Transplantation* 61: 274-8
148. Rizzi R, Curci P, Delia M, Rinaldi E, Chiefa A, Specchia G, Liso V. 2009. Spontaneous remission of "methotrexate-associated lymphoproliferative disorders" after discontinuation of immunosuppressive treatment for autoimmune disease. Review of the literature. *Med Oncol* 26: 1-9
149. Carey TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ. 1976. Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proc Natl Acad Sci U S A* 73: 3278-82
150. Ueda R, Shiku H, Pfreundschuh M, Takahashi T, Li LT, Whitmore WF, Oettgen HF, Old LJ. 1979. Cell surface antigens of human renal cancer defined by autologous typing. *J Exp Med* 150: 564-79
151. Reuschenbach M, von Knebel Doeberitz M, Wentzensen N. 2009. A systematic review of humoral immune responses against tumor antigens. *Cancer Immunol Immunother* 58: 1535-44
152. Jager E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jager D, Arand M, Ritter G, Old LJ, Knuth A. 1999. Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 84: 506-10

153. Soussi T. 2000. p53 Antibodies in the sera of patients with various types of cancer: a review. *Cancer Res* 60: 1777-88
154. Ferradini L, Mackensen A, Genevee C, Bosq J, Duvillard P, Avril MF, Hercend T. 1993. Analysis of T cell receptor variability in tumor-infiltrating lymphocytes from a human regressive melanoma. Evidence for in situ T cell clonal expansion. *J Clin Invest* 91: 1183-90
155. Zorn E, Hercend T. 1999. A natural cytotoxic T cell response in a spontaneously regressing human melanoma targets a neoantigen resulting from a somatic point mutation. *Eur J Immunol* 29: 592-601
156. Knuth A, Danowski B, Oettgen HF, Old LJ. 1984. T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures. *Proc Natl Acad Sci U S A* 81: 3511-5
157. Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, Maki RG, Dupont B, Ritter G, Chen YT, Knuth A, Old LJ. 2003. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci U S A* 100: 8862-7
158. Jager E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, Dunbar PR, Lee SY, Jungbluth A, Jager D, Arand M, Ritter G, Cerundolo V, Dupont B, Chen YT, Old LJ, Knuth A. 2000. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci U S A* 97: 4760-5
159. Griffioen M, Borghi M, Schrier PI, Osanto S. 2001. Detection and quantification of CD8(+) T cells specific for HLA-A*0201-binding melanoma and viral peptides by the IFN-gamma-ELISPOT assay. *Int J Cancer* 93: 549-55
160. Pittet MJ, Valmori D, Dunbar PR, Speiser DE, Lienard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini JC, Romero P. 1999. High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J Exp Med* 190: 705-15
161. Albert ML, Darnell RB. 2004. Paraneoplastic neurological degenerations: keys to tumour immunity. *Nat Rev Cancer* 4: 36-44
162. Albert ML, Darnell JC, Bender A, Francisco LM, Bhardwaj N, Darnell RB. 1998. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med* 4: 1321-4
163. Mathew RM, Cohen AB, Galetta SL, Alavi A, Dalmau J. 2006. Paraneoplastic cerebellar degeneration: Yo-expressing tumor revealed after a 5-year follow-up with FDG-PET. *J Neurol Sci* 250: 153-5

164. Darnell RB, DeAngelis LM. 1993. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. *Lancet* 341: 21-2
165. Horino T, Takao T, Yamamoto M, Geshi T, Hashimoto K. 2006. Spontaneous remission of small cell lung cancer: a case report and review in the literature. *Lung Cancer* 53: 249-52
166. Clark WH, Jr., Elder DE, Guerry Dt, Braitman LE, Trock BJ, Schultz D, Synnestvedt M, Halpern AC. 1989. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst* 81: 1893-904
167. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, Cascinelli N. 1996. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 77: 1303-10
168. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. 2003. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 348: 203-13
169. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K. 2005. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A* 102: 18538-43
170. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. 1998. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 58: 3491-4
171. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J. 2005. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 353: 2654-66
172. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-4
173. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD, Bast MA, Rosenwald A, Muller-Hermelink HK, Rimsza LM, Campo E, Delabie J, Braziel RM, Cook JR, Tubbs RR, Jaffe ES, Lenz G, Connors JM, Staudt LM, Chan WC, Gascoyne RD. 2010. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* 362: 875-85

174. Scanlan MJ, Simpson AJ, Old LJ. 2004. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 4: 1
175. van Houdt IS, Sluijter BJ, Moesbergen LM, Vos WM, de Gruijl TD, Molenkamp BG, van den Eertwegh AJ, Hooijberg E, van Leeuwen PA, Meijer CJ, Oudejans JJ. 2008. Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression. *Int J Cancer* 123: 609-15
176. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10: 942-9
177. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. 2006. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 12: 5423-34
178. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. 2009. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 27: 186-92
179. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF, Tartour E. 2006. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 12: 465-72
180. Buckowitz A, Knaebel HP, Benner A, Blaker H, Gebert J, Kienle P, von Knebel Doeberitz M, Kloor M. 2005. Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases. *Br J Cancer* 92: 1746-53
181. Dolcetti R, Viel A, Doglioni C, Russo A, Guidoboni M, Capozzi E, Vecchiato N, Macri E, Fornasarig M, Boiocchi M. 1999. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 154: 1805-13
182. Nakata B, Wang YQ, Yashiro M, Nishioka N, Tanaka H, Ohira M, Ishikawa T, Nishino H, Hirakawa K. 2002. Prognostic value of microsatellite instability in resectable pancreatic cancer. *Clin Cancer Res* 8: 2536-40
183. Callaway MP, Briggs JC. 1989. The incidence of late recurrence (greater than 10 years); an analysis of 536 consecutive cases of cutaneous melanoma. *Br J Plast Surg* 42: 46-9

184. Weckermann D, Muller P, Wawroschek F, Harzmann R, Riethmuller G, Schlimok G. 2001. Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. *J Urol* 166: 699-703
185. Karrison TG, Ferguson DJ, Meier P. 1999. Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst* 91: 80-5
186. Sagalowsky AI, Molberg K. 1999. Solitary metastasis of renal cell carcinoma to the contralateral adrenal gland 22 years after nephrectomy. *Urology* 54: 162
187. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, Beitsch PD, Leitch M, Hoover S, Euhus D, Haley B, Morrison L, Fleming TP, Herlyn D, Terstappen LW, Fehm T, Tucker TF, Lane N, Wang J, Uhr JW. 2004. Circulating tumor cells in patients with breast cancer dormancy. *Clin Cancer Res* 10: 8152-62
188. Myron Kauffman H, McBride MA, Cherikh WS, Spain PC, Marks WH, Roza AM. 2002. Transplant tumor registry: donor related malignancies. *Transplantation* 74: 358-62
189. MacKie RM, Reid R, Junor B. 2003. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. *N Engl J Med* 348: 567-8
190. Dhodapkar MV. 2005. Immune response to premalignancy: insights from patients with monoclonal gammopathy. *Ann N Y Acad Sci* 1062: 22-8
191. Dhodapkar MV, Krasovsky J, Osman K, Geller MD. 2003. Vigorous premalignancy-specific effector T cell response in the bone marrow of patients with monoclonal gammopathy. *J Exp Med* 198: 1753-7
192. Davis TA, Maloney DG, Czerwinski DK, Liles TM, Levy R. 1998. Anti-idiotypic antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood* 92: 1184-90
193. Medzhitov R. 2008. Origin and physiological roles of inflammation. *Nature* 454: 428-35
194. Balkwill F, Mantovani A. 2001. Inflammation and cancer: back to Virchow? *Lancet* 357: 539-45
195. Bui JD, Schreiber RD. 2007. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Curr Opin Immunol* 19: 203-8
196. Krelin Y, Voronov E, Dotan S, Elkabets M, Reich E, Fogel M, Huszar M, Iwakura Y, Segal S, Dinarello CA, Apte RN. 2007. Interleukin-1beta-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. *Cancer Res* 67: 1062-71

197. Betts G, Twohig J, Van den Broek M, Sierro S, Godkin A, Gallimore A. 2007. The impact of regulatory T cells on carcinogen-induced sarcogenesis. *Br J Cancer* 96: 1849-54
198. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. 2007. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 317: 121-4
199. Rakoff-Nahoum S, Medzhitov R. 2007. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* 317: 124-7
200. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 13: 1050-9
201. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccocanti F, Piacentini M, Zitvogel L, Kroemer G. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 13: 54-61
202. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasmimir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L. 2009. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 15: 1170-8
203. Calzascia T, Pellegrini M, Hall H, Sabbagh L, Ono N, Elford AR, Mak TW, Ohashi PS. 2007. TNF-alpha is critical for antitumor but not antiviral T cell immunity in mice. *J Clin Invest* 117: 3833-45
204. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR. 2008. "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* 205: 1261-8
205. Hedvat M, Huszar D, Herrmann A, Gozgit JM, Schroeder A, Sheehy A, Buettner R, Proia D, Kowolik CM, Xin H, Armstrong B, Beberitz G, Weng S, Wang L, Ye M, McEachern K, Chen H, Morosini D, Bell K, Alimzhanov M, Ioannidis S, McCoon P, Cao ZA, Yu H, Jove R, Zinda M. 2009. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell* 16: 487-97
206. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh

- AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. 2010. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N Engl J Med*
207. Coughlin CM, Salhany KE, Gee MS, LaTemple DC, Kotenko S, Ma X, Gri G, Wysocka M, Kim JE, Liu L, Liao F, Farber JM, Pestka S, Trinchieri G, Lee WM. 1998. Tumor cell responses to IFN γ affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity* 9: 25-34
208. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M. 1993. Immune response in mice that lack the interferon-gamma receptor. *Science* 259: 1742-5
209. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254: 1643-7
210. Lurquin C, Van Pel A, Mariame B, De Plaen E, Szikora JP, Janssens C, Reddehase MJ, Lejeune J, Boon T. 1989. Structure of the gene of tumor-transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells. *Cell* 58: 293-303
211. Van den Eynde B, Lethe B, Van Pel A, De Plaen E, Boon T. 1991. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J Exp Med* 173: 1373-84
212. Urban JL, Holland JM, Kripke ML, Schreiber H. 1982. Immunoselection of tumor cell variants by mice suppressed with ultraviolet radiation. *J Exp Med* 156: 1025-41
213. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. 2006. The consensus coding sequences of human breast and colorectal cancers. *Science* 314: 268-74
214. Segal NH, Parsons DW, Peggs KS, Velculescu V, Kinzler KW, Vogelstein B, Allison JP. 2008. Epitope landscape in breast and colorectal cancer. *Cancer Res* 68: 889-92
215. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264: 1918-21

216. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25: 1754-60
217. Koboldt DC, Chen K, Wylie T, Larson DE, McLellan MD, Mardis ER, Weinstock GM, Wilson RK, Ding L. 2009. VarScan: variant detection in massively parallel sequencing of individual and pooled samples. *Bioinformatics* 25: 2283-5
218. Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O. 2003. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. *Protein Sci* 12: 1007-17
219. Ranganath S, Ouyang W, Bhattacharya D, Sha WC, Grupe A, Peltz G, Murphy KM. 1998. GATA-3-dependent enhancer activity in IL-4 gene regulation. *J Immunol* 161: 3822-6
220. Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, Rahman N, Stratton MR. 2004. A census of human cancer genes. *Nat Rev Cancer* 4: 177-83
221. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R. 2004. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 91: 355-8
222. Chen AC, Herschman HR. 1989. Tumorigenic methylcholanthrene transformants of C3H/10T1/2 cells have a common nucleotide alteration in the c-Ki-ras gene. *Proc Natl Acad Sci U S A* 86: 1608-11
223. Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, Hingorani SR, Zaks T, King C, Jacobetz MA, Wang L, Bronson RT, Orkin SH, DePinho RA, Jacks T. 2004. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* 5: 375-87
224. Kirsch DG, Dinulescu DM, Miller JB, Grimm J, Santiago PM, Young NP, Nielsen GP, Quade BJ, Chaber CJ, Schultz CP, Takeuchi O, Bronson RT, Crowley D, Korsmeyer SJ, Yoon SS, Hornicek FJ, Weissleder R, Jacks T. 2007. A spatially and temporally restricted mouse model of soft tissue sarcoma. *Nat Med* 13: 992-7
225. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, Dooling D, Dunford-Shore BH, McGrath S, Hickenbotham M, Cook L, Abbott R, Larson DE, Koboldt DC, Pohl C, Smith S, Hawkins A, Abbott S, Locke D, Hillier LW, Miner T, Fulton L, Magrini V, Wylie T, Glasscock J, Conyers J, Sander N, Shi X, Osborne JR, Minx P, Gordon D, Chinwalla A, Zhao Y, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson M, Baty J, Ivanovich J, Heath S, Shannon WD, Nagarajan R, Walter MJ, Link DC, Graubert TA, DiPersio JF, Wilson RK.

2008. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature* 456: 66-72

226. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, Fulton LA, Locke DP, Magrini VJ, Abbott RM, Vickery TL, Reed JS, Robinson JS, Wylie T, Smith SM, Carmichael L, Eldred JM, Harris CC, Walker J, Peck JB, Du F, Dukes AF, Sanderson GE, Brummett AM, Clark E, McMichael JF, Meyer RJ, Schindler JK, Pohl CS, Wallis JW, Shi X, Lin L, Schmidt H, Tang Y, Haipek C, Wiechert ME, Ivy JV, Kalicki J, Elliott G, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson MA, Baty J, Heath S, Shannon WD, Nagarajan R, Link DC, Walter MJ, Graubert TA, DiPersio JF, Wilson RK, Ley TJ. 2009. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 361: 1058-66
227. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL, Abbott RM, Hoog J, Dooling DJ, Koboldt DC, Schmidt H, Kalicki J, Zhang Q, Chen L, Lin L, Wendl MC, McMichael JF, Magrini VJ, Cook L, McGrath SD, Vickery TL, Appelbaum E, Deschryver K, Davies S, Guintoli T, Crowder R, Tao Y, Snider JE, Smith SM, Dukes AF, Sanderson GE, Pohl CS, Delehaunty KD, Fronick CC, Pape KA, Reed JS, Robinson JS, Hodges JS, Schierding W, Dees ND, Shen D, Locke DP, Wiechert ME, Eldred JM, Peck JB, Oberkfell BJ, Lolofie JT, Du F, Hawkins AE, O'Laughlin MD, Bernard KE, Cunningham M, Elliott G, Mason MD, Thompson DM, Jr., Ivanovich JL, Goodfellow PJ, Perou CM, Weinstock GM, Aft R, Watson M, Ley TJ, Wilson RK, Mardis ER. 2010. Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature* 464: 999-1005
228. Shah SP, Morin RD, Khattra J, Prentice L, Pugh T, Burleigh A, Delaney A, Gelmon K, Guliany R, Senz J, Steidl C, Holt RA, Jones S, Sun M, Leung G, Moore R, Severson T, Taylor GA, Teschendorff AE, Tse K, Turashvili G, Varhol R, Warren RL, Watson P, Zhao Y, Caldas C, Huntsman D, Hirst M, Marra MA, Aparicio S. 2009. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature* 461: 809-13
229. Lee W, Jiang Z, Liu J, Haverty PM, Guan Y, Stinson J, Yue P, Zhang Y, Pant KP, Bhatt D, Ha C, Johnson S, Kennemer MI, Mohan S, Nazarenko I, Watanabe C, Sparks AB, Shames DS, Gentleman R, de Sauvage FJ, Stern H, Pandita A, Ballinger DG, Drmanac R, Modrusan Z, Seshagiri S, Zhang Z. 2010. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. *Nature* 465: 473-7
230. Pleasance ED, Stephens PJ, O'Meara S, McBride DJ, Meynert A, Jones D, Lin ML, Beare D, Lau KW, Greenman C, Varela I, Nik-Zainal S, Davies HR, Ordóñez GR, Mudie LJ, Latimer C, Edkins S, Stebbings L, Chen L, Jia M, Leroy C, Marshall J, Menzies A, Butler A, Teague JW, Mangion J, Sun YA, McLaughlin SF, Peckham HE, Tsung EF, Costa GL, Lee CC, Minna JD, Gazdar A, Birney E, Rhodes MD, McKernan KJ, Stratton MR, Futreal PA, Campbell PJ.

2010. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature* 463: 184-90
231. Totoki Y, Tatsuno K, Yamamoto S, Arai Y, Hosoda F, Ishikawa S, Tsutsumi S, Sonoda K, Totsuka H, Shirakihara T, Sakamoto H, Wang L, Ojima H, Shimada K, Kosuge T, Okusaka T, Kato K, Kusuda J, Yoshida T, Aburatani H, Shibata T. 2011. High-resolution characterization of a hepatocellular carcinoma genome. *Nat Genet* 43: 464-9
232. Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, Escaramis G, Jares P, Bea S, Gonzalez-Diaz M, Bassaganyas L, Baumann T, Juan M, Lopez-Guerra M, Colomer D, Tubio JM, Lopez C, Navarro A, Tornador C, Aymerich M, Rozman M, Hernandez JM, Puente DA, Freije JM, Velasco G, Gutierrez-Fernandez A, Costa D, Carrio A, Guijarro S, Enjuanes A, Hernandez L, Yague J, Nicolas P, Romeo-Casabona CM, Himmelbauer H, Castillo E, Dohm JC, de Sanjose S, Piris MA, de Alava E, San Miguel J, Royo R, Gelpi JL, Torrents D, Orozco M, Pisano DG, Valencia A, Guigo R, Bayes M, Heath S, Gut M, Klatt P, Marshall J, Raine K, Stebbings LA, Futreal PA, Stratton MR, Campbell PJ, Gut I, Lopez-Guillermo A, Estivill X, Montserrat E, Lopez-Otin C, Campo E. 2011. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 475: 101-5
233. Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordonez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR. 2010. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* 463: 191-6
234. Yewdell JW. 2006. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity* 25: 533-43
235. Foley EJ. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res* 13: 835-7
236. Prehn RT, Main JM. 1957. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 18: 769-78
237. Old LJ, Boyse EA. 1964. Immunology of Experimental Tumors. *Annu Rev Med* 15: 167-86
238. Ikeda H, Ohta N, Furukawa K, Miyazaki H, Wang L, Kuribayashi K, Old LJ, Shiku H. 1997. Mutated mitogen-activated protein kinase: a tumor rejection antigen of mouse sarcoma. *Proc Natl Acad Sci U S A* 94: 6375-9

239. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med* 174: 561-9
240. Brunet JF, Denizot F, Luciani MF, Roux-Dosseto M, Suzan M, Mattei MG, Golstein P. 1987. A new member of the immunoglobulin superfamily--CTLA-4. *Nature* 328: 267-70
241. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctl4-4. *Science* 270: 985-8
242. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, Murphy TL, Murphy KM. 2010. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol* 11: 129-35
243. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-5
244. Egen JG, Kuhns MS, Allison JP. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* 3: 611-8
245. Peggs KS, Quezada SA, Allison JP. 2008. Cell intrinsic mechanisms of T-cell inhibition and application to cancer therapy. *Immunol Rev* 224: 141-65
246. Sotomayor EM, Borrello I, Tubb E, Allison JP, Levitsky HI. 1999. In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. *Proc Natl Acad Sci U S A* 96: 11476-81
247. Shrikant P, Khoruts A, Mescher MF. 1999. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity* 11: 483-93
248. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, Burg MB, Allison JP. 1997. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci U S A* 94: 8099-103
249. Leach DR, Krummel MF, Allison JP. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271: 1734-6
250. van Elsas A, Hurwitz AA, Allison JP. 1999. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines

- induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190: 355-66
251. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682-7
 252. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 99: 12293-7
 253. Strome SE, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, Salomao D, Cheville J, Hirano F, Lin W, Kasperbauer JL, Ballman KV, Chen L. 2003. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res* 63: 6501-5
 254. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, Rietz C, Flies DB, Lau JS, Zhu G, Tamada K, Chen L. 2005. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 65: 1089-96
 255. Curran MA, Montalvo W, Yagita H, Allison JP. 2010. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* 107: 4275-80
 256. Zang X, Allison JP. 2007. The B7 family and cancer therapy: costimulation and coinhibition. *Clin Cancer Res* 13: 5271-9
 257. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, Moon C, Albring JC, Ise W, Michael DG, Bhattacharya D, Stappenbeck TS, Holtzman MJ, Sung SS, Murphy TL, Hildner K, Murphy KM. 2010. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med* 207: 823-36
 258. Chambers CA, Kuhns MS, Egen JG, Allison JP. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* 19: 565-94
 259. Yuan J, Gnjatic S, Li H, Powel S, Gallardo HF, Ritter E, Ku GY, Jungbluth AA, Segal NH, Rasalan TS, Manukian G, Xu Y, Roman RA, Terzulli SL, Heywood M, Pogoriler E, Ritter G, Old LJ, Allison JP, Wolchok JD. 2008. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. *Proc Natl Acad Sci U S A* 105: 20410-5

260. Callahan MK, Wolchok JD, Allison JP. 2010. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol* 37: 473-84
261. Stratton MR. 2011. Exploring the genomes of cancer cells: progress and promise. *Science* 331: 1553-8
262. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, Vogelstein B. 2007. The genomic landscapes of human breast and colorectal cancers. *Science* 318: 1108-13
263. Stratton MR, Campbell PJ, Futreal PA. 2009. The cancer genome. *Nature* 458: 719-24