INVESTIGATING IMMUNE SURVEILLANCE, TOLERANCE, AND THERAPY IN CANCER

by

Ann F. Cheung

Sc.B. Biochemistry and Molecular Biology
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Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

Maximizing the potential of cancer immunotherapy requires model systems that closely recapitulate human disease to study T cell responses to tumor antigens and to test immune therapeutic strategies. Current model systems largely relied on chemically-induced and spontaneous tumors in immunodeficient mice or on transplanted tumors. Such systems are limited because they fail to reproduce the complex interactions that exist among an emerging tumor, its microenvironment and the multiple elements of an intact immune system. We created a new system that is compatible with Cre-loxP-regulatable mouse cancer models in which the defined antigen SIY is specifically over-expressed in tumors, mimicking clinically-relevant tumor-associated antigens. To demonstrate the utility of this system, we characterized SIY-reactive T cells in the context of lung adenocarcinoma, revealing multiple levels of antigen-specific T cell tolerance that serve to limit an effective anti-tumor response. Thymic deletion reduced the number of SIY-reactive T cells present in the animals. When potentially self-reactive T cells in the periphery were activated, they were efficiently eliminated. Inhibition of apoptosis resulted in more persistent self-reactive T cells, but these cells became anergic to antigen stimulation. Finally, in the presence of tumors over-expressing SIY, SIY-specific T cells required a higher level of costimulation to achieve functional activation.

Adoptive cell transfer (ACT) therapy for cancer has demonstrated tremendous efficacy in clinical trials, particularly for the treatment of metastatic melanoma. There is great potential in broadening the application of ACT to treat other cancer types, but the threat of severe autoimmunity may limit its use. Studies in other model systems have demonstrated successful induction of anti-tumor immunity against self-antigens without detrimental autoimmunity. This is possibly due to the preferential recognition of tumor over normal somatic tissue by activated T cells. In our system, SIY provides a means to achieve this bias because of its over-expression in tumors. Thus, we applied adoptive T cell transfer therapy combined with lymphodepleting pre-conditioning to treat autochthonous lung tumors over-expressing SIY self-antigen. With this treatment, we overcame peripheral tolerance, successfully inducing large number of functional anti-tumor T cells. These T cells are able to influence lung tumors over-expressing self-antigen. Importantly, despite large numbers of potentially self-reactive T cells, we did not observed overt autoimmunity.

Immune tolerance thwarts efforts to utilize immune therapy against cancer. We have discerned many mechanisms by which tolerance to cancer in potential achieved. Both $Foxp3^+$ T

regulatory cell and myeloid-derived suppressor cell populations are expanded in the presence of cancer in our mouse models. In addition, we identified LAG-3 as a potential factor that serves to limit anti-tumor T cell activity in the context of adoptive cell transfer therapy. Our new system represents a valuable tool in which to explore the mechanisms that contribute to T cell tolerance to cancer and to evaluate therapies aimed at overcoming this tolerance. In addition, our model provides a platform, on which more advanced mouse models of human cancer can be generated for cancer immunology.

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- Poster title: "*A model tumor antigen to activate immunity in a mouse model of lung adenocarcinoma*"

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CHAPTER 1:

Introduction

I. THE CANCER PARTS LIST

Cancer as a list of parts

Cancer is a collection of diseases characterized by aberrant cellular proliferation and altered tissue homeostasis. The general paradigm for cancer development and progression around the turn of the century was the accumulation of sequential genetic and epigenetic alterations that give cells a selective advantage. This perspective focuses on mutant genes and the resulting alterations in cell signaling pathways and epigenetic state. Indeed, Hanahan and Weinberg's seminal review on the "hallmarks of cancer" highlights genetic changes in cancer that lead to the acquisition of six essential traits: (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of programmed cell death, (4) limitless replicative capacity, (5) sustained ability to recruit blood vessels, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Although the latter two capacities involve interaction with non-mutant cells in the tumor microenvironment, the cause of these events was attributed to genetic alterations in the tumor cells. The presumption with this mutant cancer cell-centric view is that the key to cancer therapy lies in targeting signaling pathways and cellular processes within the cancer cells themselves. We know, however, that cancer is not simply a mass of mutant cells, but rather a heterogeneous tissue consisting of mutant tumor cells within a highly interactive microenvironment (Bissell and Radisky, 2001).

Evidence that both cell autonomous and non-cell autonomous elements contribute to cancer initiation and progression has been present for over a century (Mantovani et al., 2008). Hanahan and Weinberg did, in fact, state that the tumor microenvironment was sure to play a major role in tumorigenesis, noting in particular that tumors are composed of heterogeneous cell types (Hanahan and Weinberg, 2000). The study of non-cell autonomous modifiers of cancer,

however, has proven unwieldy compared to the study of cell-autonomous factors. The former requires the use complex model systems, such as three-dimensional cultures and animal models (Debnath and Brugge, 2005; Fischbach et al., 2007; Frese and Tuveson, 2007; Jonkers and Berns, 2002). The necessity of these higher order systems and other complications have caused the study of the tumor microenvironment to lag behind the study of cancer genetics and its cell autonomous consequences. Despite this, important insights into the tumor microenvironment and systemic host environment in cancer have emerged. These studies have opened interesting avenues for more fruitful research and more possibilities for the development of cancer therapies.

The tumor microenvironment consists of extracellular matrix (ECM), fibroblasts, immune cells, and vasculature. These components interact heavily with one another and certainly with the mutant tumor cells themselves. The tumor microenvironment supports tumor progression, but, in principle, each part can be altered to either render tumor cells vulnerable to suppression or to directly suppress tumor advancement. With this in mind, I propose an alternative framework with which to view cancer. Rather than focusing on acquired traits derived from genetic events, one can view cancer in terms of its parts list, and how these components influence cancer progression (Figure 1).

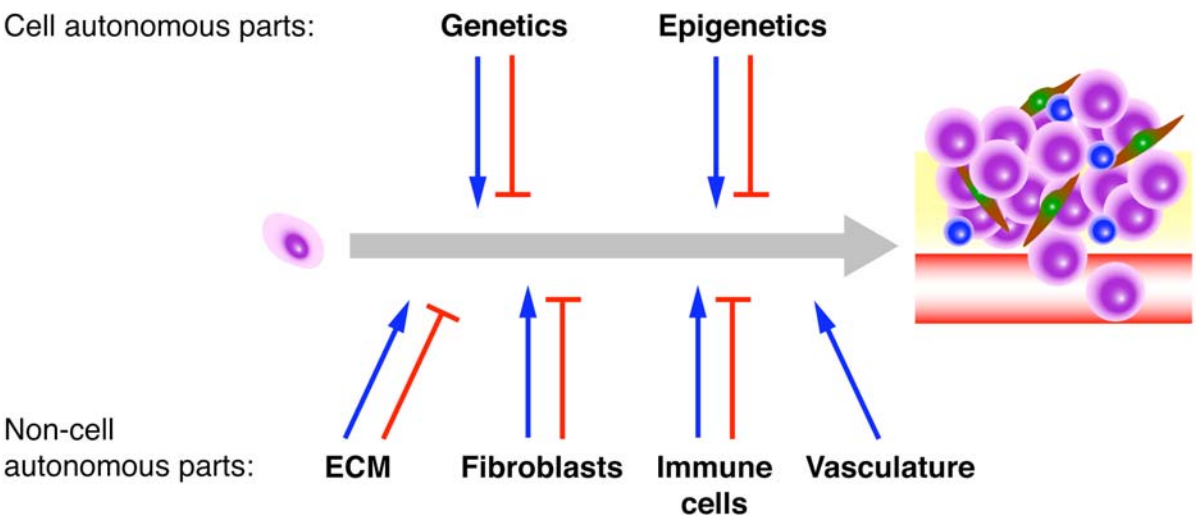

Figure 1. The cancer parts list. Cancer consists of many parts including the genetics and epigenetics of the tumors cells and elements in the tumor microenvironment. The non-cell autonomous parts that can influence cancer progression in positive and negative ways are the extracellular matrix (ECM), fibroblasts in the cancer stroma, the host's immune system, and blood vessels that are recruited to the lesion. All of parts of cancer can influence one another and interact cooperatively to promote malignant disease.

The cancer parts list consists of cell-autonomous parts and non-cell-autonomous parts and includes (1) the genetics and (2) the epigenetics of the mutant tumor cells, (3) the ECM, (4) fibroblasts, (5) immune cells, and (6) the vasculature (Figure 1). The genetics component includes mutations in oncogenes and tumor suppressor genes that drive cancer development (Futreal et al., 2004; Sherr, 2004). Furthermore, non-mutant genetic variants, or polymorphisms, are also important in modifying the phenotype of cancer (de la Chapelle, 2004; Pharoah et al., 2004). The epigenetics of cancer is influenced by the cell and tissue type of origin and the differentiation status, or potency, of particular cancer cells (Bernstein et al., 2007). In addition, genetic events can lead to alterations in epigenetic state, which can, in turn, affect the dynamics of additional genetic change (Jallepalli and Lengauer, 2001; Michor et al., 2004).

The non-cell autonomous parts of cancer, or the tumor microenvironment, can be recruited to the lesion or subverted by genetic and epigenetic changes in the mutant tumor cells, as previously mentioned (Hanahan and Weinberg, 2000). The microenvironment, however, can also feed back and influence genes and gene expression in the mutant tumor cells as well as affect other components of the tumor microenvironment. The ECM controls tissue architecture and regulates the availability of soluble factors, both of which can influence tumor cell signaling (Butcher et al., 2009; Erler and Weaver, 2009). Fibroblasts can express factors that directly promote tumor growth and metastasis. In addition, they also produce ECM, help to recruit blood vessels, secrete factors to employ immune cells to aid in cancer development, and contribute to immune suppression (Kalluri and Zeisberg, 2006). Blood vessels recruited to cancer provide tumor cells with oxygen and nutrients to promote growth as well as conduits through which tumor cells can metastasize (Bergers and Benjamin, 2003). Fortunately, the cancer vasculature also provides a means by which anti-tumor drugs can be delivered and immune cells can travel to

attack mutant tumor cells. Finally, the immune system consists of many cell types that collectively function to accomplish opposing roles in cancer (de Visser et al., 2006; Mantovani et al., 2008).

The immune system in cancer

The immune system can be divided into the innate and adaptive arms. The innate immune system represents the first line of defense against pathogens and functions independently of specific antigens. Innate immune cells recognize molecules associated with pathogens and pathogen infected cells, leading to engulfment and neutralization of pathogens. Innate immune responses also activate adaptive immunity. The innate immune system consists of myeloid cell types, such as macrophages, neutrophils, and dendritic cells, and lymphoid cells, such as natural killer (NK) cells. Macrophages and dendritic cells, specifically, can cross-present pathogen-associated antigens to the adaptive immune system and activate it. In contrast to the innate immune system, the adaptive immune system is antigen-dependent. The adaptive arm consists of T and B lymphocytes, which bear receptors that possess specificity for particular antigens. T cell receptors (TCRs), in association with co-receptors CD8 and CD4, recognize antigens associated with major histocompatibility (MHC) molecules, class I and class II, respectively. B cell receptors (BCRs), on the other hand, can recognize soluble antigens. BCRs can be surface-bound or secreted from B cells as antibodies. TCRs and BCRs both exhibit enormous diversity allowing the immune system the ability to specifically recognize and defend its host from generally any pathogen. Further general discussion of the immune system is beyond the scope of this thesis. More information can be found in the textbook Janeways' Immunobiology (Murphy et al., 2008).

The link between chronic inflammation caused by pathogens, independent of the role of oncogenic viruses, and cancer has been established (Mantovani et al., 2008). For example, infection of the bacterium *Helicobacter pylori* predisposes individuals to gastric cancer. Exactly how inflammation promotes tumor initiation and progression is still being explored, but innate immune cells have been described in this role. Macrophages, mast cells, and granulocytes are thought to generate free radicals that lead to genetic mutation and secrete cytokines that promote tumorigenesis via paracrine signaling in tumor cells (de Visser et al., 2006). In addition, macrophages can promote neo-angiogenesis and produce immunosuppressive cytokines that block anti-tumor immune responses (Murdoch et al., 2008). Furthermore, quite counter-intuitively, both B cells and T cells of the immune system have been described to promote cancer growth (de Visser et al., 2005; Prehn and Prehn, 2008). I will end the general discussion of how the components of cancer influence tumor progression here. I will dedicate the rest of this thesis to describing the immune system's role, and that of cytotoxic $CD8^+$ T cells in particular, in tumor suppression.

II. A HISTORICAL PERSPECTIVE ON CANCER IMMUNE SURVEILLANCE

History

The notion that a functional interaction exists between the immune system and cancer, be it supporting or suppressing, has subsisted for more than a century. In 1863, upon observing tumor-infiltrating lymphocytes, Rudolf Virchow speculated that inflammation was a predisposing element in tumorigenesis. In direct opposition to this idea, in 1909, Paul Ehrlich proposed that the immune system could be used therapeutically to treat cancer. Interestingly, despite preceding major conceptual advances in immunology, the suggestion of immune therapy

for cancer had already been put into practice by then. In the 1890s, William Coley used preparations of killed bacteria cultures to inoculate patients bearing sarcomas. This vaccination did, in fact, result in some cases of tumor regression (Visser, 2008).

Several decades later, after the discovery of allograft rejection, several researchers were able to establish the existence of tumor antigens. With the use of inbred mouse and rat strains, researchers vaccinated animals against syngeneic transplantation of tumors induced by carcinogens or transforming viruses (Foley, 1953; Klein, 1966; Old and Boyse, 1964). Although specific identification of these tumor antigens was not possible at this time, this substantiated the hypothesis that the immune system could indeed distinguish tumor from self. Soon afterwards, the conjecture that the immune system suppressed malignancies was formally articulated as the "cancer immunosurveillance" hypothesis by Sir Macfarlane Burnet and Lewis Thomas, in the 1960s (Dunn et al., 2002).

As the cancer immunosurveillance hypothesis was being formulated, experiments concerning the immune system's role in tumor suppression continued (Dunn et al., 2002). The basic premise of these studies was aimed at testing the prediction that immune deficiency in animal models would lead to a higher incidence of spontaneous and carcinogen-induced cancer. Initial experiments utilizing experimentally-induced methods of immune suppression, however, concluded in the late 1960s with ambiguous and contradictory results. The only firm conclusion that could be made was that the immune system suppressed malignancies associated with bacterial and viral infections. A few years later, following the discovery of the athymic nude mouse, additional studies were performed by Osias Stutman and others in these genetically immunodeficient animals. These extensive experiments demonstrated no difference in the incidence of chemically-induced or spontaneous cancer between wild-type and nude mice,

irrespective of age, dose of carcinogen, or observation time. Unfortunately, the nature of the immunodeficiency and compensatory immune mechanisms in nude mice were not well understood at that time, and, thus, caused misinterpretation of the data. While these studies did not completely extinguish the idea of cancer immunosurveillance, they undoubtedly dampened interest towards it for the next two decades.

Reviving history

In the 1990s and early 2000s, a collection of new experiments examining tumorigenesis in immunodeficient mice, either induced or genetically-defined, restored enthusiasm for the concept of immune-mediate suppression of cancer. Animals lacking the cytokine interferon-γ (IFN-γ) demonstrated an increased incidence of carcinogen-induced and spontaneous tumors (Street et al., 2001; Street et al., 2002). Similar results were observed for animals unable to respond to IFN-γ either because they lacked a subunit of the IFN-γ receptor IFNGRI or a component involved in signaling downstream of the receptor Stat-1 (Kaplan et al., 1998; Shankaran et al., 2001). Furthermore, enhanced susceptibility to carcinogen-induced and spontaneous tumors was also observed in mice deficient for the cytolytic effector molecule perforin (Smyth et al., 2000a; Smyth et al., 2000b; van den Broek et al., 1996). A comprehensive list of studies involving tumor incidence in immunodeficient animals can be found in (Dunn et al., 2006; Swann and Smyth, 2007).

These new studies were supported by a greater understanding of immunology, including knowledge of the caveats present in experiments involving nude mice. In addition, the new technological breakthrough of constructing gene-targeted mice made the use of animals with numerous researcher-defined immune deficiencies possible (Thompson et al., 1989). Work with these gene-targeted immunodeficient mice, although not without caveats themselves, has not

only led to a resurgence of activity in tumor immunology, but has laid the groundwork by identifying specific immune components involved in cancer immunosurveillance.

III. THE CURRENT PERSPECTIVE ON CANCER IMMUNE SURVEILLANCE

In the following section, I will discuss the types of tumor antigens that are recognized by cytotoxic T cells and what we know of how the adaptive immune system is activated in response to these antigens in cancer. Then, in the next section, I will describe how the anti-cancer response can be circumvented or blocked by tumor cells and by the host's own immune system resulting in tolerance to cancer. As there has been considerable progress in the area of tumor immunology over the years, these sections will not be an exhaustive review of the field's literature, but rather an account that highlights what I see to be the most salient features of the immune response to cancer.

Tumor antigens

As cancer develops, tumor cells display genetic instability and acquire numerous gene mutations (Leary et al., 2008; Sjoblom et al., 2006; Weir et al., 2007). These genetic events either directly or indirectly, through cell signaling modifications and epigenetic changes, result in gene expression alterations. Both the mutant gene products and the alternatively expressed genes can yield peptide antigens that the immune system can in turn utilize to recognize cancer (Finn, 2008; Novellino et al., 2005; Stevanovic, 2002b). I will start by mentioning some of the methodologies used to identify tumor antigens. Then, I will describe the types of tumor antigens that have been identified, and finish by discussing considerations when targeting each antigen type therapeutically.

Identification

The identification of immunogenic, non-viral neo-antigens and self-antigens in cancer patients, either endogenous or after vaccination, has provided concrete evidence in support of cancer immunosurveillance. The methodologies employed to identify these tumor antigens have evolved considerably over the last four decades (Stevanovic, 2002b). The earliest reliable assay for human tumor antigen identification utilized serological analysis for B cell antibody responses by mixed hemadsorption assay with established cancer cell lines (Carey et al., 1976). This technique, also called autologous typing, eliminated the confounding variable of reactivity to histocompatibility antigens and allowed determination of antigen specificity in tumor versus normal cells. This general approach was subsequently improved upon in the development of SEREX, a method utilizing cDNA libraries from fresh tumor specimens, which avoids artifacts produced in the *in vitro* propagation of tumor cells (Sahin et al., 1997). The libraries were generated in lambda-phage vectors and expressed in *E. coli*. The resulting recombinant protein was transferred onto nitrocellulose membranes and screened for cross-reactivity to antibodies in human sera. SEREX has resulted in the rapid discovery of numerous tumor antigens that elicit humoral, or B cell, immune responses in cancer patients.

While the immune system is known to integrate T cell and B cell responses, antibody cross-reactivity to tumor antigens was insufficient to prove the existence of cellular immune responses to these or associated antigens. Many technical advances in immunology have allowed the identification of tumor antigens presented by either MHC class I or MHC class II molecules that elicit T cell responses in cancer patients (Finn, 2008; Stevanovic, 2002a). The ability to propagate human T cells in culture for extended lengths of time was one of these advances (Morgan et al., 1976). This procedure facilitated the cloning of MAGE1, the first gene

discovered to encode a tumor antigen recognized by cytotoxic T lymphocytes (van der Bruggen et al., 1991). In addition, the establishment of *in vitro* growth conditions for dendritic cells has allowed the creation of cell lines that can recapitulate antigen cross-presentation in culture (Sallusto and Lanzavecchia, 1994). These dendritic cell lines, combined with proteomic methods, have been used to survey peptides eluted from MHC molecules for their ability to stimulate T cells in culture, providing the capacity to identify tumor antigens with high-throughput efficiency (Kao et al., 2001). Furthermore, the development of peptide-MHC tetramers has allowed the identification and characterization of antigen-specific T cells within a population containing diverse antigen specificity (Altman et al., 1996). Using peptide-MHC tetramers, researchers have been able to screen peripheral blood from cancer patients for T cells reactive to known or putative tumor antigens (Lee et al., 1999a).

Classification

Cancer antigens can be broadly classified as tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs) (Table 1). TSAs are derived from proteins to which the host immune system is naïve, and so, from an immunological perspective, are unique to tumor cells. TAAs, on the other hand, are derived from non-mutant proteins that are over-expressed, inappropriately expressed, or even expressed at normal levels in cancer. Therefore, TAAs represent self-antigens that the immune system recognizes to differentially target tumor cells and normal host tissues. By increasing avidity, or the concentration of possible interactions, antigen-MHC class I complexes with relatively low affinity for T cell receptors (TCRs) can be recognized more potently by the immune system; thus, the basis of immune reactivity to TAAs.

Class	Subclass	Description	Examples*
Tumor-specific antigen	Cancer-testis antigen	peptides shared between tumor and germ cells or placenta	MAGE1, TRAG3, NY-ESO-1
	Mutant peptide	unique peptides generated by point mutation, aberrant splicing, or fusion proteins	K-RAS, CDK-4, BCR-ABL
	Viral antigen	peptides derived from oncogenic viruses	HPV16-E6/E7, EBNA-1
Tumor-associated antigen	Differentiation antigen	peptides shared between tumor and the normal tissue from which the tumor derives	Tyrosinase, PSA, CEA
	Over-expression antigen	peptides expressed at high levels in tumor relative to normal tissue	hTERT, p53, CyclinB1

* See Novellino et al. (2005) for a more comprehensive listing of tumor antigens.

Table 1. Classes of antigens in cancer that are recognized by T cells. Cancer antigens can be broadly classified as tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs). TSAs are derived from proteins to which the host immune system is naïve, and so, from an immunological perspective, are unique to tumor cells. TAAs, on the other hand, are derived from non-mutant somatic proteins that are over-expressed, inappropriately expressed, or even expressed at normal levels in cancer. Therefore, TAAs represent self-antigens that the immune system recognizes to differentially target tumor cells and normal host tissues.

Both TSAs and TAAs can be further divided into subclasses (Table 1) (Novellino et al., 2005). Tumor-specific antigens include cancer-testis antigens, mutant peptides, and viral antigens. Cancer-testis antigens (sometimes referred to as germline antigens or oncofetal antigens) are peptides shared between tumor and germ cells or placenta. As these antigens, such as MAGE1, TRAG3, and NY-ESO-1, are normally only expressed in immune privileged sites, their mis-expression in tumors is considered foreign to the immune system. Mutant peptides can be generated by point mutation, aberrant splicing or gene fusion, and so are often thought of as altered-self antigens. Due to the genetic instability of tumor cells, mutant peptides as a class are common in cancer, but individual mutant peptides do not often overlap between distinct tumors or even discrete cells in a single tumor (Segal et al., 2008). Despite being rare, a handful of shared mutant peptides have been identified in cancer, deriving from obligatory mutant proteins, such as K-RAS and BCR-ABL (Gjertsen et al., 1997; Wagner et al., 2003). Finally, cancers with viral etiology express truly foreign viral antigens. This subclass includes peptides from the E6 and E7 proteins of human papillomavirus in cervical cancer and EBNA-1 from Epstein-Barr virus in Burkitt's lymphoma (Finn, 2008).

Tumor-associated antigens comprise differentiation antigens and over-expression antigens. Differentiation antigens define a specific cell lineage and are shared between tumor and the normal tissue from which the tumor derives. The majority of known differentiation antigens are melanocyte/melanoma antigens, such as Tyrosinase, GP100 and MART-1, but others have been identified in epithelial cancers, including PSA in prostate cancer and CEA in colon cancer (Novellino et al., 2005). Over-expression antigens are generally ubiquitously expressed across tissue types, but, as the name suggests, expressed at high levels in tumor relative to normal tissue. Many commonly over-expressed proteins in cancer have yielded

antigens that fall into this subclass, including hTERT, p53, Cyclin B1and ERBB2. Many of these are barely detectable in normal tissues and so provide great differential in immune reactivity (Kao et al., 2001).

Considerations for targeting tumor antigens with immune therapy

Experimental immune therapies against both tumor-specific antigens and tumor-associated antigens are being pursued in pre-clinical and clinical trials. While the specific strategies being employed will be discussed in a later section, I will discuss some of the advantages and disadvantages to targeting particular antigen classes or sub-classes here. The major considerations when choosing tumor antigens to pursue include: (1) the selectivity of the antigen to tumor compared to normal tissue, (2) the prevalence of the tumor antigen across cancer patients, (3) the dependency of cancer to the tumor antigen's expression, and (4) the extent to which the patient's immune system is tolerized or unresponsive to the antigen.

By virtue of being categorized as neo-antigens, tumor-specific antigens are not expressed in normal tissues surveyed by the immune system and thus offer absolute specificity for tumor cells. With this in mind, cancer-testis antigens and viral antigens would certainly be good targets when applicable, particularly because these antigens are also commonly found in certain cancers. Unfortunately, few cancer-testis antigens have been identified outside of melanoma and viral antigens are only relevant to a minority of cancer types (Novellino et al., 2005). Furthermore, as previously mentioned, most mutant proteins are limited to the individual tumors from which they were originally identified (Segal et al., 2008).

Fortunately, there are two scenarios in which mutant proteins would be appropriate to target. One is in the case of commonly found mutant proteins that are obligatory in cancer, such as peptides containing a codon 12 point mutation in K-RAS or the BCR-ABL fusion gene

product (Gjertsen et al., 1997; Wagner et al., 2003). In this situation, it would be important to consider the patients' MHC haplotypes as this is an important determinant of whether an antigen is presented and could considerably limit the applicability of a specific therapy. The second circumstance involves the use of individualized therapy, such as when a patient's own cancer is used to induce immunity in himself/herself (Gruijl et al., 2008). Individualized therapy will be described further later, but it should be noted here that mutant peptides are not the only subclass of tumor antigen involved with this therapy.

Concerning selectivity for cancer and prevalence across cancer types, tumor-associated antigens are characterized contrary to mutant peptides. TAAs represent the majority of known tumor antigens and are common among cancer types and tumors of the same variety. Thus, therapies devised against specific TAAs are likely to be widely applicable. Unfortunately, though, as TAAs are self-antigens, the risk of autoimmunity is clearly present when targeting this class of antigens. In fact, autoimmunity has been observed after immune therapy against differentiation antigens in melanoma both in the clinic and in experimental model systems (Dudley et al., 2002; Overwijk et al., 2003; Yee et al., 2000). Targeting TSAs, however, does not necessary escape this risk. Inducing immune reactivity to a specific antigen may activate additional reactivity to unspecified antigens linked in expression through the occurrence of epitope, or determinant, spreading (Anderson et al., 2000; Pilon et al., 2003). Despite this, in some cases, researchers have reported successful anti-tumor immunity, even when targeting TAAs, in the absence of overt autoimmunity in model systems (Eck and Turka, 2001; Morgan et al., 1998; Vierboom et al., 1997). Considering the model systems used, however, these conclusions need to be examined more rigorously.

Tumor cells can evade immune responses directed at a particular antigen by decreasing expression of that antigen. This phenomenon has been observed in experimental model systems when the expression of antigens under immune pressure was dispensable for cancer progression (Khong and Restifo, 2002). Thus, to circumvent this potential resistance mechanism, tumor antigens targeted for therapy should be evaluated on the basis of the relative survival benefit they confer on cancer. Research regarding various cancers' dependency on specific mutant gene products and signaling pathways is ongoing (Ding et al., 2008; Jones et al., 2008; Leary et al., 2008; Wood et al., 2007). Some insights into this dependency have, however, been elucidated, such as the requirement for activated K-ras in non-small cell lung cancer (Fisher, 2001). This strategy of targeting antigens to which tumors are dependent is consistent with the general tactic of exploiting cancer's vulnerabilities (Luo et al., 2009).

The consideration of established tolerance to tumor antigens is very important in immune therapy, but not one that can be strictly applied based on currently available data. Before cancer initiation, the host may be considered tolerant to tumor-associated antigens, as these self-antigens exist in the absence of immune reactivity. On the other hand, the host is naïve to tumor-specific antigens at this time, so this group of antigens is likely to be appropriate for prophylactic immune therapy. After cancer establishment, however, immune reactivity to both TAAs and TSAs is suppressed. The specific mechanisms governing immune tolerance to cancer have been described and will be discussed later in the chapter, in addition to the potential for breaking tolerance to cancer. In brief, immune tolerance is still being activity investigated and, at present, it is unclear how best to overcome these mechanisms.

Activating adaptive immunity against cancer

An effective adaptive immune response to cancer necessitates cross-presentation of tumor antigens by mature dendritic cells to $CD8^+$ T cells to promote the generation of cytotoxic function. This activity is supported by $CD4^+$ helper cells, which are also stimulated in response to tumor antigen cross-presentation. Here, I will describe how antigen-presenting cells (APCs), dendritic cells in particular, and $CD4^+$ T helper cells contribute to $CD8^+$ T cell activation in the context of cancer (Table 2). Activating anti-tumor immunity, however transient its function may be, serves as a guide in the development of immune therapies against cancer.

Dendritic cell activation

Studies of how dendritic cells (DCs), the link between innate and adaptive immunity, are activated in response to damaged and dying cells have been important in supporting the idea of cancer immunosurveillance. Cell death is a prominent feature of mutant tumor cells and can result from detrimental gene mutations, nutrient deprivation, or successful anti-cancer therapy. Dendritic cells phagocytose dying tumor cells and cross-present tumor antigens to lymphocytes. The activation state of DCs during cross-presentation determines whether productive anti-tumor immunity or immune tolerance will be generated. Activated dendritic cells are characterized by expression of the co-stimulatory molecules B7-1 and B7-2, increased MHC class II antigen presentation and the generation of immune-stimulatory cytokines (Murphy et al., 2008).

	Viruses	Cancer
Cause of cell death	viral lysis, apoptosis	apoptosis, necrosis, cytotoxic agents
Toll-like receptors (TLRs) activated	TLR-3, -7, (-8 in humans), -9	TLR-2 and -4
TLR ligands	dsRNA, ssRNA, unmethylated DNA	HMGB1, heat shock proteins, uric acid crystals, products of the ECM
Source of TLR ligands	viral genomes (exogenous)	dying or distressed cells (endogenous)
Source of antigens	viral proteins	mutant proteins, self proteins, viral proteins

Table 2. Comparison of the elements involved in immune activation to viruses and cancer. The immunogenicity of cancer parallels that of viral infection. Cancer cells can die via natural death pathways or be killed by cytotoxic agents, such as chemotherapy and radiation. Cancer cell death causes release or exposure of various ligands that stimulate pattern recognition receptors, such as toll-like receptors. This results in immune activation against tumor antigens which are cross-presented by antigen presenting cells that engulf dying or distress cancer cells.

The study of pattern-recognition receptors (PRRs), like toll-like receptors (TLRs), and their associated ligands, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), has been an intense and rapidly growing in the field of immunology over the last decade and a half (Kono and Rock, 2008; Medzhitov and Janeway, 2000; Tesniere et al., 2008). Associated with this area's rapid growth came much controversy, particularly concerning the source and nature of the ligands that activate adaptive immunity as well as the relative importance of different PRRs in this process. It suffices to say, though, that it is now established that dying somatic cells can induce immune reactivity leading to adaptive immunity in the absence of foreign microbes. In addition, it was long thought that programmed cell death, or apoptosis, was a sterile death that did not result in immune activation. This idea has been re-evaluated, leading to the notation of two forms of apoptosis, non-immunogenic and immunogenic, both of which are still being defined on the molecular level. Because the type(s) of cell death that lead to immune activation are still under contention, I will use general terms, such as "dying cell" and "cell death," to avoid confusion.

The realization that antigens from dying cells in the absence of foreign microbial adjuvants could indeed be recognized by the immune system came with the discovery of "eat me" signals – ligands on dying cells that could interact with receptors on phagocytic cells to encourage engulfment. One of these ligands phosphatidylserine (PS) is distributed asymmetrically on the inner-leaflet of cell membranes in healthy cells. PS becomes exposed on the outer surface of dying cells and its interaction with PS receptors on phagocytic cells appears critical for clearance of dying cells (Fadok, 2001; Hoffmann, 2001). A second ligand calreticulin is upregulated on cell surfaces during stress and damage and found to interact with the LDL-receptor-related protein LRP on DCs to promote phagocytosis (Gardai et al., 2005). In addition,

calreticulin exposure was found to govern the immunogenity of cancer cell death in response to chemotherapy (Obeid et al., 2007). Because calreticulin is also on the surface of healthy cells, it was discovered that integrin-associated protein CD47 on viable cells interacts with SIRPα on DCs and acts dominant to calreticulin-LRP engagement as a "don't eat me" signal (Gardai et al., 2005). Thus, phagocytosis of dying tumors cells involves both exposure of "eat me" signals and abrogation of "don't eat me" signals and can be enhanced by chemotherapy to support immune activation.

The discovery of damage-associated molecular patterns or "danger" signals that promoted dendritic cell maturation and antigen cross-presentation after phagocytosis further supported the notion that tumor cells could induce a productive adaptive immune response. Even prior to the identification of these signals, it was already observed that dying tumor cells and cell debris could induce DC maturation and the generation of cytotoxic T lymphocytes (Gallucci et al., 1999; Shi et al., 2000). Since then, a number of "danger" signals that stimulate pattern-recognition receptors, including Toll-like receptors (TLRs)-2 and -4, have been found to be derived from dying and distressed cells (Kono and Rock, 2008; Tesniere et al., 2008). The first of these molecules identified that could function in DC activation were heat shock proteins, either surface-bound or released from dying normal and tumor cells (Basu et al., 2000; Binder et al., 2000; Feng et al., 2003). Subsequently, a chromatin-associated protein HMGB1, uric acid crystals, and other "danger" signals have been recognize to support DC maturation and antigen cross-presentation in response to dying cells (Apetoh et al., 2007; Scaffidi et al., 2002; Shi et al., 2003; Tesniere et al., 2008). Studies of these "danger" signals and the response to them through pattern-recognition receptors are helping researchers to gain insight into the immune-stimulatory

component of chemotherapy and radiotherapy treatment for cancer and the potential to activate adaptive immunity through PRR stimulation (Table 2).

$CD4^+$ T cell help

Whether or not $CD4^+$ T cells are essential for cellular immunity is being debated, but, regardless, T cell help is certainly highly significant to $CD8^+$ T cell activation and function (Bevan, 2004; Kennedy and Celis, 2008; Knutson and Disis, 2005). Naïve $CD4^+$ T lymphocytes are activated by T cell receptor (TCR) engagement with cognate antigens presented by MHC class II molecules on dendritic cells and associated stimulatory signals. When these T cells are activated, they differentiate to assume one of many effector cell phenotypes that can greatly influence the strength and direction of an immune response. The developmental path taken by $CD4^+$ T cells depends on the microenvironment, including the co-stimulatory signals and the cytokine milieu present, in which these cells are activated. The details of these signals and the fate of $CD4^+$ T cell development are beyond the scope of this chapter; instead, we will focus here specifically on the T helper 1 (Th1) cell subset and its role in supporting cytotoxic T cell (CTL) activity against cancer. Consistent with Th1 cells' role in cancer is the fact that many of known tumor antigens are presented by MHC class II molecules and are encoded by genes that also produce MHC class I presented tumor antigens (Novellino et al., 2005).

T helper 1 cells are thought to assist cellular immune responses in four ways: (1) activation of APCs, (2) provision of cytokines that directly support CTL survival and function, (3) maintenance of $CD8^+$ T cell memory and (4) support of CTL effector function through direct contact. As previously mentioned, the state of dendritic cell activation when cross-presenting antigens to naïve $CD8^+$ T cells determines if immune activation or tolerance is produced. In addition to pattern-recognition receptor signaling described above, $CD4^+$ T cells can directly

activate dendritic cells that also cross-present antigen to $CD8^+$ T cells to induce cytotoxic T cell generation (Bennett et al., 1997; Cassell and Forman, 1988). Signaling via CD40 on DCs by interaction with CD40L on activated $CD4^+$ T cells directly mediates DC stimulation. In fact, dendritic cell activation followed by CTL priming can be reproduced with cross-linking antibodies to CD40 and abrogated by blocking antibodies to CD40L (Bennett et al., 1998b; Ridge et al., 1998; Schoenberger et al., 1998). Moreover, agonistic CD40 antibodies are being investigated as an immune therapy against cancer in animal models and in pre-clinical/clinical trials (van Mierlo et al., 2004; van Mierlo et al., 2002; Vonderheide et al., 2007).

Aside from DC licensing, T helper 1 cells produce cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ), that effect cytotoxic T cell function. IL-2 directly supports proliferation of CTLs after initial priming and is required for expansion of $CD8^+$ memory cells (Chen et al., 1990; Gao et al., 2002; Williams et al., 2006). In addition, IFN-γ has a potent effect on enhancing peptide processing and MHC class I antigen presentation (Früh and Yang, 1999; Yang et al., 1992). Through this function, IFN-γ can augment cross-presentation by dendritic cells and boost recognition of target tumor cells by CTLs (Lugade et al., 2008). Furthermore, direct contact between Th1 cells and CTLs via co-stimulatory molecules has been shown to promote CTL proliferation, survival and cytolytic function (Giuntoli et al., 2002; Kennedy and Celis, 2008). The *in vivo* details of this phenomenon are still being examined. As $CD4^+$ T cell help has been shown in many contexts to enhance cytotoxic T cell activity against tumors, the mechanisms employed by Th1 cells are currently being investigated for cancer immune therapy (Kennedy and Celis, 2008).

IV. IMMUNE TOLERANCE TO CANCER

Despite evidence for immune surveillance of cancer by T lymphocytes, the fact that tumors develop, progress, and persist is indicative of ineffective immunity. Immune evasion by tumors has been hypothesized to explain why the immune system is not adequate to eliminate cancer (Table 3). Alternatively, tolerance to cancer can occur due to immune regulatory mechanisms, which normally function to protect against autoimmunity and/or collateral damage during pathogenic responses (Table 3). Strategies used for immune suppression are mediated by cell surface receptors, soluble factors and immune regulatory cells. I will describe some examples of each of these in the following section.

Immune suppression mediated by direct contact

Evading recognition

One approach tumors use to avert immune attack is to prevent immune recognition. Since cytotoxic T cells recognize their targets via tumor antigens presented on MHC class I molecules, downregulation of antigen expression or presentation would block detection (Chang and Ferrone, 2006; Khong and Restifo, 2002). Consistent with this notion, was the observation that expression of genes encoding differentiation antigens correlated inversely with histological grade of melanoma (Hofbauer et al., 1998a; Hofbauer et al., 1998b). Furthermore, an inverse correlation was also noted between the expression of melanocyte differentiation antigens and spontaneous cytotoxic T cell reactivity (Jager et al., 1996). In line with these correlations are experiments in mouse models that result in loss of tumor antigens either spontaneously or after immune therapy (Khong and Restifo, 2002).

Mechanism	Description	Examples
Evading recognition	Cytotoxic T cells recognize cancer via specific antigens presented by MHC class I molecules on the surface of tumor cells and, thus, require sustained expression and presentation of tumor antigens.	~ downregulation/loss of antigen expression ~ reduction/loss/mutation of MHC class I ~ alteration of antigen processing machinery
Avoiding death	Cytotoxic T cells attack cancer via perforin and granzymes or death receptors, both of which cause apoptosis of tumor cells.	~ upregulation of anti-apoptotic proteins ~ downregulation of death receptors ~ neutralization of granzymes
Direct inhibition by cancer	Tumors can express or secrete molecules that can act directly on cytotoxic T cells to cause inhibition or death.	~ upregulation of inhibitory ligands ~ secretion of immunosuppressive cytokines ~ activation of death receptors on T cells
Indirect inhibition by cancer	Tumor cells can promote the expansion and recruitment of regulatory immune cells or alter the cancer microenvironment to be immune suppressive.	~ recruitment of immunosuppressive stroma ~ secretion of immunosuppressive cytokines ~ generation of physical barriers
Host tolerance	The immune system has many mechanisms by which it negatively regulates itself. Thus, tolerance to cancer may occur due to mechanisms that are in place to prevent autoimmunity or to reduce collateral damage after pathogen infection.	~ expansion of regulatory immune cells ~ upregulation of inhibitiory molecules on T cells ~ generation of toleragenic APCs

Table 3. Methods and examples of immune evasion and tolerance. The observation of cancer indicates that immunity against cancer is insufficient. Multiple mechanisms by which this insufficiency occurs has been described. There is indication that some of these sources of immune tolerance are coordinated.

Reduced levels of MHC class I antigen presentation have been found in many human cancers, as well (Cabrera et al., 1996; Korkolopoulou et al., 1996). Low levels of antigen presentation are also associated with increased malignancy (Vitale et al., 1998). The molecular basis of reduced antigen presentation was elucidated to be due either to decreased expression of MHC class I molecules or to defects in the antigen presentation machinery (APM). Loss of function mutations in β2-microglobulin, which makes up half of the MHC class I complex, have been identified in human cell lines (Hicklin et al., 1998; Restifo et al., 1996). In addition, reduced expression of APM proteins, such as transporter associated with antigen processing 1 (TAP1) and TAP2, has also been observed (Restifo et al., 1993; Sanda et al., 1995; Vitale et al., 1998). In many circumstances, however, exposure of tumor cell lines to IFN-γ re-established levels of antigen presentation. Moreover, exogenous expression of TAP1 in tumor cells, despite having reduced levels of other APM components, reinstated recognition by T cells, as well (Alimonti et al., 2000). Interestingly, ectopic TAP1 expression has also been shown to restore immunogenicity to IFN-γ-insensitive murine sarcoma cell lines (Shankaran et al., 2001).

Instead of thwarting immune recognition by altering antigen presentation on tumor cells, the complementary TCR-CD8 complex on cytotoxic T cells can be modified. T cell receptors associated with the co-receptor CD8 can bind to cognate peptide-MHC class I complexes with much greater affinity than in the absence of CD8 (Holler and Kranz, 2003). Furthermore, TCR co-signaling with p56-Lck, the intracellular kinase linked to CD8, enhances T cell activation (Abraham et al., 1991; Xu and Littman, 1993). Dissociation of TCR and CD8 was observed in tumor-infiltrating lymphocytes (TILs) with reduced effector function from patient samples, despite maintaining normal levels of TCR and CD8 expression (Demotte et al., 2008). Analysis of T cells with reduced function after prolonged antigen stimulation revealed that the TCRs,

instead, co-localized with galectin-3. Extracellular galectin-3 is thought to form a lattice around the TCR, blocking its association with CD8. Consistently, treatment with N-acteyllactosamine (LacNAc), a competitive inhibitor of galectin-3, restored $CD8^+$ T cell effector function (Demotte et al., 2008). Thus, galectin-3, besides having many other functions, can alter recognition of tumor cells by T cells and effect T cell function (Dumic et al., 2006). Galectin-3 is secreted by activated T cells themselves, a self-regulatory mechanism, and by tumor cells, and is found in high concentration in sera from cancer patients (Joo et al., 2001; Liu and Rabinovich, 2005).

Death receptors

Cytotoxic T lymphocytes can kill tumors by expressing ligands that interact with death receptors on target cells (Russell and Ley, 2002). When engaged, death receptors, such as Fas (Apo-1/CD95) and tumor necrosis factor-related apoptosis inducing ligand-receptors (TRAIL-R), signal downstream with the help of adaptor molecules to activate a series of proteases, called caspases, that lead to apoptosis of the targeted cell (Danial and Korsmeyer, 2004). Tumors can circumvent this death signal by decreasing expression or mutational inactivation of death receptors. In fact, somatic mutations in Fas, TRAIL-R1, and TRAIL-R2 have been identified in a number of human cancers of non-hematopoietic origin (Lee et al., 1999b; Lee et al., 1999c; Park et al., 2001; Shin et al., 1999). In addition, alterations in components downstream of death receptors have also been discovered in human cancer. Chromosomal loss of genomic regions containing initiator caspases in the extrinsic death pathway have been identified, as well as, inactivating mutations (Park et al., 2002). High expression of anti-apoptotic protein cFLIP, which functions proximal to cell surface death receptors, has also been demonstrated in human cancer (Irmler et al., 1997). Furthermore, in animal models, tumor cell lines transduced to over-express cFLIP escape T cell immunity *in vivo* (Medema et al., 1999). Lastly, somatic mutations

in death receptors and downstream components have been found associated with advanced and metastatic disease (Shin et al., 2002).

Induced apoptosis via death ligands and receptors have also been discovered to work in reverse; tumor can commandeer this system to induce death of attacking lymphocytes. Activated lymphocytes normally elevate expression of Fas and reduce expression of intracellular inhibitors of apoptosis when they become activated as a means to quell their own immune response after clearance of pathogens (Krammer et al., 2007). Thus, they are primed to respond to death ligands. Indeed, when this mechanisms is altered, such as by somatic mutations of death receptors, lymphoma can occur (Grønbæk et al., 2003; Lee et al., 2001). Expression of FasL, the ligand for Fas death receptor, has been observed in non-hematopoietic human cancers (Hahne et al., 1996; Niehans et al., 1997; O'Connell et al., 1998). In addition, tumor areas with higher FasL expression were associated with reduced numbers of tumor-infiltrating lymphocytes and increased death of these lymphocytes compared to areas with low FasL (Bennett et al., 1998a). Furthermore, FasL-expressing human cell lines can kill a T lymphocyte cell line *in vitro*, in a manner that can be blocked by soluble Fas receptor or anti-sense knock-down of Fas in the T cells (Niehans et al., 1997; O'Connell et al., 1996).

The expression of death ligands by cancer as a counter-attack to cytotoxic T cells, however, has been contested based on criticism of detection methods and assays used in the discovery of FasL expression on non-hematopoietic tumors (Restifo, 2000). With the use of intron-spanning primers for reverse transcriptase-polymerase chain reaction (RT-PCR) and short-term cultures of surgical specimens to eliminate contaminating lymphocytes, researchers found no expression of FasL in melanoma, directly refuting previous data (Chappell et al., 1999). Furthermore, to make sense of previous *in vitro* T cell killing data, melanoma cells not

expressing FasL were shown to cause antigen-specific T cell death upon TCR engagement (Zaks et al., 1999). This apoptosis, called activation-induced cell death (AICD), also functions through FasL engagement of Fas, but with both ligand and receptor originating from the T cells. T cell apoptosis then occurs through Fas ligation by FasL on the same cell, suicide, or by a neighboring T cell, fratricide (Krammer et al., 2007).

Co-signaling molecules

The B7 family of co-signaling molecules is important in regulating T cell activation and tolerance (Greenwald et al., 2005). The prototypical members, B7-1 (CD80) and B7-2 (CD86), are primarily expressed on hematopoietic cells and bind both CD28 and CTLA-4 receptors on T cells to stimulate or inhibit T cell activity, respectively. CTLA-4 is currently a major target in immunotherapy trials against cancer and will be discussed further in section V of this chapter. More recently discovered B7 molecules include PD-L1 (B7-H1), PD-L2 (B7-DC), ICOSL (B7-H2), B7-H3, and B7x (B7-H4) and have wider expression patterns, including expression on non-immune cells. In particular, immunostaining of patient samples has revealed higher expression of PD-L1, B7-H3, and B7x in tumors than in surrounding normal tissue (Boorjian et al., 2008; Dong et al., 2002; Gao et al., 2009; Zou and Chen, 2008). Moreover, elevated PD-L1 staining was specifically associated with advanced grade and poor prognosis. I will describe PD-L1 and its corresponding receptor PD-1 further as these have received the most attention of the recently identified B7 molecules. It is clear, though, that the other B7 molecules also have roles in generating and maintaining tolerance to cancer (reviewed in (Seliger et al., 2008; Zou and Chen, 2008)).

Mice lacking either PD-1 or PD-L1 exhibit T cell hyperactivity and autoimmune disease, underscoring the importance of these molecules in controlling immune reactivity (Latchman et

al., 2004; Nishimura et al., 1999). Engagement of PD-1 on T cells by PD-L1 results in inhibition of T cell proliferation and cytokine production (Freeman et al., 2000). PD-L1 over-expression on tumors also causes apoptosis of T cells *in vitro* and promotes the growth of tumors in mice (Dong et al., 2002; Iwai et al., 2002). Conversely, blocking antibodies to PD-L1 result in T cell-mediated immune rejection of tumors in animal models (Strome et al., 2003; Wei et al., 2008). Moreover, PD-L1 is upregulated on tumor-associated myeloid cells, blockade of which also promotes T cell activation (Curiel et al., 2003). Consistent with these data, PD-1 deficient transgenic T cells can reject transplanted tumors in mice under conditions which PD-1 proficient T cells are unable to do so (Blank et al., 2004). To make things more complicated, both PD-L1 and PD-1 can interact with additional molecules. Specifically, like PD-L1, PD-L2 also engages PD-1 to regulate T cell responsiveness (Latchman et al., 2001). Furthermore, B7-1 has been shown to interact with PD-L1 on T cells to suppress function (Butte et al., 2007). Thus, PD-L1 not only acts as a ligand, but as a receptor, mediating signals downstream of itself (Keir et al., 2008).

Soluble factors involved in immune suppression

Transforming growth factor-β

Transforming growth factor-β (TGF-β), a prototypical family of molecules that possess pleiotropic function, has been studied extensively in various contexts (reviewed in (Li et al., 2006; Massagué, 2008)). In cancer, TGF-β has roles in tumor suppression, angiogenesis, metastasis and, most relevant here, immune suppression. Concerning this latter function, mice deficient for TGF-β1, the predominant isoform expressed by immune cells, exhibit multi-focal inflammatory disease and tissue necrosis resembling autoimmune disease (Kulkarni et al., 1993;

Shull et al., 1992). This mutant phenotype was ameliorated in animals with additional deficiency for either MHC class I or class II (Kobayashi et al., 1999; Letterio et al., 1996). Specifically, TGF-β1's role in suppressing effector T cell responses was significant as *in vivo* antibody depletion of $CD8^+$ or $CD4^+$ T cells also suppressed autoimmunity in TGF-β1 null mice.

Tumor cells and stromal cells in the tumor microenvironment can secrete TGF-β to mediate immune suppression. In fact, a highly immunogenic tumor cell line over-expressing TGF-β1 failed to instigate CTL cytolytic activity *in vitro* and *in vivo* despite maintaining MHC class I expression (Torre-Amione et al., 1990). Conversely, $CD8^+$ T cells insensitive to TGF-β1, as a result of expressing a dominant-negative TGF-β receptor dnTGF-β-RII, expanded to greater numbers *in vivo* and eradicated transplanted tumors that that produced TGF-β (Gorelik and Flavell, 2001). Specifically, TGF-β can influence $CD8^+$ T cell effector function by repressing transcription of enzymes involved in cytolysis and cytokines. Neutralization of TGF-β by ectopic expression of a soluble TGF-β-RII trap in transplanted tumors restored effector gene expression in tumor antigen-specific cytotoxic T cells in mice (Thomas and Massagué, 2005). In addition, cytokine secretion from $CD8^+$ melanoma-infiltrating lymphocytes derived from patients was also blocked by TGF-β treatment (Ahmadzadeh and Rosenberg, 2005).

In addition to production by tumors, TGF-β secreted by the immune system also plays an important role in immune tolerance to cancer. TGF-β mediated the ability of T regulatory cells (or Tregs, described further later) to suppress $CD8^+$ T cell cytolytic activity *in vivo* using a transplant tumor model. Transfer of Tregs along with tumor-specific cytotoxic T cells abrogated tumor rejection, but expression of dnTGF-β-RII in these CTLs yielded tumor clearance despite the presence of Tregs (Chen et al., 2005).

Signal transducer and activator of transcription-3

Like TGF-β, signal transducer and activator of transcription 3 (Stat-3) also has diverse roles in cancer. Stat-3 is activated by cytokines and growth factors receptors, many of which are aberrantly activated or mutated in tumors. Not only can Stat-3 promote tumorigenesis in a cell autonomous fashion, it can mediate immune evasion by inhibiting immune responses to cancer (reviewed in (Yu et al., 2007)). Constitutive Stat-3 activation in tumor cells results in transcriptional repression of pro-inflammatory cytokines and chemokines (e.g. IFN-γ, IL-12, CXCL10) and induction of immunosuppressive factors (e.g. IL-10, VEGF, IL-6). Blocking Stat-3 signaling in murine tumor cells, either with dominant-negative Stat-3 or with anti-sense oligonucleotides, results in derepression of pro-inflammatory molecules and chemo-attractants and subsequent immune cell infiltration into transplanted tumors (Burdelya et al., 2005; Wang et al., 2004). Furthermore, in human melanoma cells bearing mutant B-RAF^{V600E}, blocking oncogenic mitogen-activated protein kinase (MAPK) signaling via drug inhibition or RNAi, resulted in reduced production of immunosuppressive cytokines. Although Stat-3 RNAi did not produce an additive effect, MAPK inhibition did not always result in reduced Stat-3 activation, suggesting that secretion of immunosuppressive cytokines may be regulated by many oncogenic pathways (Sumimoto, 2006).

Stat-3 signaling in tumors produces a cascade of Stat-3 activation through the immune system. In fact, tumor-infiltrating leukocytes often exhibit constitutive Stat-3 signaling themselves. Specifically, immunosuppressive molecules secreted by tumors activate Stat-3 in dendritic cells to block maturation (Wang et al., 2004). Hematopoietic cells in which Stat-3 has been genetically ablated display greater activation and tumor reactivity. Moreover, transplanted tumors are rejected in these mice, although the tumor themselves are proficient for Stat-3

(Kortylewski et al., 2005). Furthermore, T regulatory cells were under-represented in tumor-infiltrating lymphocytes of animals with Stat-3 deficient hematopoietic cells, suggesting a potential role for Stat-3 in Treg maintenance or function. Interestingly, $CD4^+$ T cells have been shown to produce IL-10 and TGF-β when deficient for suppressor of cytokine signaling 3 (SOCS3), a negative regulator of Stat-3 (Kinjyo, 2006).

Other soluble immune suppressive factors

There are other soluble factors that regulate anti-tumor T cell function. Enzymes that produce or degrade these molecules are often upregulated in the tumor microenvironment. Indoleamine 2,3-dioxygenase (IDO), which catabolizes the essential amino acid tryptophan, is expressed by tumor cells, surrounding stroma cells, and cancer-associated dendritic cells (reviewed in (Katz et al., 2008; Prendergast, 2008)). The potent role of IDO in effector T cell suppression was first shown by treating pregnant mice with an IDO inhibitor, which resulted in T cell-dependent immune rejection of their fetuses (Munn, 1998). In addition to tryptophan degradation, enzymes that catabolize (L)-arginine, such as arginase and nitric-oxide synthase (NOS), inhibit T cell proliferation and function (reviewed in (Bronte and Zanovello, 2005; Rodríguez and Ochoa, 2008)). Arginase and NOS are expressed by tumor cells, tumor-associated macrophages, and myeloid-derived suppressor cells (described further later). In addition, cyclooxygenase 2 (COX2) and its downstream product prostaglandin E2 (PGE2) are produced in cancer and promote the expression of IDO and arginase (Braun, 2005; Rodriguez, 2005). Furthermore, PGE2 induces the generation of both myeloid-derived suppressor cells and T regulatory cells and inhibition of COX2 promotes anti-tumor immunity (Baratelli et al., 2005; Sharma et al., 2005; Sinha et al., 2007; Stolina et al., 2000). Moreover, adenosine is expressed

by tumors under hypoxic conditions and produced by T regulatory cells to suppress T cell signaling and effector activity (reviewed in (Sitkovsky et al., 2008)).

Cellular mediators of immune suppression

The cells of the immune system influence each other greatly both through indirect soluble factors and through direct interaction of cell surface receptors and ligands. As described above, tumor cells can harness these communication lines to influence immune reactivity itself. That said, the soluble and surface-bound molecules normally function within the immune system, as it regulates itself. Here, I will describe two of many cellular regulators of the immune system, T regulatory cells and myeloid suppressor cells. These and other immune cells can restrict anti-tumor cytotoxic T cell function through some of the mechanisms already mentioned.

T regulatory cells

In section III, I described the role of $CD4^+$ T cells in supporting adaptive immunity, but $CD4^+$ T cells can have immunosuppressive roles, as well. The identities and functions of many of these and other lymphocytic immune suppressive populations are still being elucidated. Here, I will focus specifically on $CD4^+CD25^+$ T regulatory cells (Tregs), now well known to be a central regulator of autoimmunity and peripheral tolerance (reviewed in Sakaguchi et al., 2008).

Tregs are specifically identified by expression of the transcription factor Foxp3, which represses expression of genes directly involved in T cell activity (Marson et al., 2007; Schubert et al., 2001; Zheng et al., 2007). Spontaneous mutation of Foxp3 was found to be the cause of neonatal autoimmune syndromes *scurfy* and *IPEX*, in mouse and humans, respectively (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). In addition, targeted deletion of Foxp3 in mice also resulted in autoimmune disease and early lethality, confirming the critical role of

Foxp3 in immune regulation (Fontenot et al., 2003). Furthermore, adoptive transfer of either $CD4^+CD25^+$ Tregs or $CD4^+CD25^-$ T cells transduced to express Foxp3 rescued animals from disease. Moreover, specific deletion of Foxp3-expressing Tregs in adult mice also led to autoimmunity, supporting a essential role for T regulatory cells throughout life (Kim et al., 2007; Lahl et al., 2007). Based on the requirement for Tregs in maintaining peripheral tolerance, it is not surprising that there is now ample evidence for T regulatory cell participation in tolerance to cancer.

Expanded populations of T regulatory cells have been found within tumors, in tumor-draining lymph nodes, and in peripheral blood of patients with various types of cancer (Ichihara et al., 2003; Liyanage et al., 2002; Ormandy et al., 2005; Viguier et al., 2004; Woo et al., 2001). Tregs from these patients were found to constitutively express the inhibitory co-signaling molecule CTLA-4 and produce immunosuppressive cytokines TGF-β and IL-10. When isolated from patient tumors, Tregs inhibited proliferation and cytokine secretion of autologous $CD4^+$ and $CD8^+$ T lymphocytes *in vitro*, demonstrating their ability to suppress adaptive immune effectors (Ormandy et al., 2005; Viguier et al., 2004; Woo et al., 2002). Furthermore, high numbers of tumor-infiltrating $Foxp3^+$ T regulatory cells in patients with ovarian cancer predicted reduced survival compared to patients with low Treg numbers (Curiel et al., 2004). Conversely, in breast cancer, complete responders after neoadjuvant chemotherapy exhibited a reduced number of tumor-infiltrating Tregs and increased cytotoxic T cell responses in tumors (Ladoire et al., 2008).

In animal models, the functional relationship between T regulatory cells and suppressed anti-tumor immunity has been further investigated. The first demonstration of this association was through the use of monoclonal antibodies against the IL-2 receptor α-chain CD25, which depletes Tregs based on their relatively high expression of CD25. Depletion in mice before or a

day after tumor cell inoculation led to tumor rejection that was dependent on either $CD8^+$ T cells or NK cells (Onizuka et al., 1999; Shimizu et al., 1999). Since then, Treg depletion via CD25-specific antibodies has been found to similarly enhanced anti-tumor immunity in other transplant tumor models (Zou, 2006). Furthermore, denileukin diftitox, or ONTAK, a fusion protein of IL-2 and active domains of diphtheria toxin, has been used with the aim of depleting Tregs in patients with solid tumors. The limited number of clinical trials with ONTAK, however, has shown mixed results in patients (Attia et al., 2005; Barnett et al., 2005; Dannull, 2005).

Other methods to affect Tregs, that exploit cell-surface receptors besides CD25, have also been used in mice with potential for clinical application. These strategies include anti-CTLA-4 antibody blockade and agonistic antibodies against the glucocorticoid-induced tumor necrosis factor receptor (TNF-R) family-related protein GITR and OX40 of the TNF-R superfamily. These therapies have all demonstrated some ability to block suppressive Treg activity and promote anti-tumor CTL activity in various mouse models (Ko et al., 2005; Shimizu et al., 2002; Sutmuller et al., 2001; Takahashi et al., 2000; Vu et al., 2007). The functional purpose of these treatments, though, remain under question as each of the antibodies can directly promote T cell effector function in addition to limiting T regulatory cell function. Low-dose cyclophosphamide has also been used to reduce Treg-mediated immune suppression (Ercolini, 2005; Turk, 2004). It is, however, yet another example of a treatment regimen with multi-functional mechanisms of action (Loeffler et al., 2005).

T regulatory cell accumulation in and around tumor sites can occur due to trafficking of natural (thymus-derived) Tregs or conversion of non-Treg cells into Tregs in the periphery. Involvement of chemokine ligands and receptors has been demonstrated in both mice and humans (Curiel et al., 2004; Tan et al., 2009). Thus, an alternative or additive strategy to

depleting or blocking Tregs would be to inhibit migration of Tregs into tumor sites. In addition, immunosuppressive cytokines have been shown to support the generation of Tregs, offering another avenue for intervention (Sakaguchi et al., 2008; Zou, 2006). In fact, TGF-β produced by existing Tregs can support the conversion of non-Treg $CD4^+$ T cells into new Tregs (Andersson et al., 2008).

T regulatory cells have been suggested to exert immune suppressive function in three general ways. First, because Tregs express high levels of the CD25, direct competition for IL-2 can limit anti-tumor effector T cell survival and proliferation, particularly in the tumor microenvironment (Antony et al., 2006; Zhang et al., 2005). Second, Tregs may directly kill cross-presenting antigen-presenting cells or effector T cells via granzymes and/or perforin, although more studies are needed to understand this further (Cao et al., 2007; Gondek et al., 2005; Grossman et al., 2004; Zhao, 2006). Finally, described through a number of means and involving both direct contact and cytokines, Tregs can suppress T cell activity or induce myeloid cells to become suppressive of T cell activity (Chen et al., 2005; Sakaguchi et al., 2008; Zou, 2006). The relative importance of these immunosuppressive mechanisms to Treg function and the contexts in which each become relevant are still under investigation.

Myeloid-derived suppressor cells

In addition to immune suppressive cells of T cell origin, there are many immune regulatory cells of myeloid origin. Included in this group are immature antigen-presenting cells since antigen presentation without proper co-stimulatory and inflammatory signals can lead to tolerance to the presented antigens. Many of these myeloid/dendritic cell-like immune suppressors are still poorly defined, including IDO^+ DCs, $B7x^+$ DCs, $CD11b^+CD11c^+$ myeloid cells, and plasmacytoid DCs(Rabinovich et al., 2007; Zou, 2005). Myeloid-derived suppressor

cells (MDSCs, also called myeloid suppressor cells MSCs), however, have been better described, at least in mice.

MDSCs are a heterogenous population of myeloid cells that are generally defined as $CD11b^{+}Gr1^{+}$ in mice. In humans, there are no specific markers for MDSCs, but cells with similar characteristics and the ability to negatively effect effector T cell function have been described (Filipazzi et al., 2007; Rodriguez et al., 2009; Schmielau and Finn, 2001). Like T regulatory cells, myeloid-derived suppressor cells can inhibit anti-tumor T cell responses in many ways (Rodríguez and Ochoa, 2008). First, MDSCs express arginase and NOS (particularly the inducible form NOS2, or iNOS), which catabolize L-arginine, as previously mentioned (Bronte et al., 2003). Arginase, in particular, along with the cationic amino acid transporter 2B, limits the extracellular concentration of L-arginine in the tumor microenvironment. L-arginine starvation, in turn, reduces the expression of TCR-associated CD3ζ chain, altering the ability of effector T cells to interact with their target cells (Rodriguez et al., 2003). Decreased CD3ζ chain expression in tumor-infiltrating lymphocytes is also associated with MDSCs in cancer patients (Kuang et al., 2008; Zea et al., 2005). In addition, MDSCs suppress T cell proliferation as reduced levels of L-arginine affect cell cycle progression (Rodriguez et al., 2007).

Second, elevated levels of arginase and iNOS are found in human cancer patients and are associated with increased levels of nitrotyrosine (Bronte and Zanovello, 2005; Ekmekcioglu et al., 2000). Under low L-arginine due to high arginase, iNOS mediates production of reactive oxygen species and peroxynitrite resulting in tyrosine nitration. This can suppress anti-tumor T cell activity presumably by inhibiting T cell activation, which requires protein phosphorylation. In a three-dimensional human prostate cancer culture system, tumor-infiltrating lymphocytes had reduced level of nitrotyrosine and could respond to stimulation only after treatment with arginase

and NOS inhibitors. Similar results were observed in a transgenic mouse model of prostate cancer after arginase and NOS inhibition (Bronte, 2005). In addition to blocking signaling, tyrosine nitration can alter the ability of T cells to recognize their targets. Tolerized $CD8^+$ T cells were shown to have tyrosine nitration of their TCR-CD8 complex after contact with MDSCs in a process dependent on reactive oxygen species (Nagaraj et al., 2007). Consistent with this, MDSCs have been described to induce $CD8^+$ T cell tolerance in a manner dependent on MHC class I presentation of T cell-specific antigen (Kusmartsev et al., 2005).

Finally, subpopulations of $CD11b^+Gr1^+$ MDSCs are capable of inducing expansion of natural T regulatory cells and generation of Tregs by conversion of non-Treg cells in mice (Huang, 2006; Serafini et al., 2008). Many different immune regulatory cell populations are usually found together in the tumor microenvironment. IL-1β and PGE2 are thought to produce inflammation around tumors that promote the recruitment of immunosuppressive immune cells. Thus, inhibiting inflammation by IL-1R genetic ablation or COX2 inhibition in mice reduces the accumulation of MDSC and Tregs in the tumor microenvironment (Bunt et al., 2007; Sharma et al., 2005; Sinha et al., 2007). Moreover, surgical resection of tumors in mice decreases MDSC numbers, restoring them to normal levels, and promotes anti-tumor immunity (Salvadori et al., 2000). Besides recruitment of MDSCs, IL-13, produced predominantly by natural killer T cells and functioning through IL-4Rα and Stat-6, has been found to polarize macrophages to a tumor-promoting phenotype characteristic of MDSCs (Sinha, 2005). Furthermore, likely as a means of auto-regulation, IFN-γ produced by effector T cells can activate myeloid-derived suppressor cells (Gallina et al., 2006).

V. IMMUNE THERAPY AGAINST CANCER

Immunological methods for treating cancers hold great promise, yet most immunotherapeutic strategies have yet to demonstrate great efficacy. This is likely a result of our limited understanding of the mechanisms controlling immune tolerance to cancer. Despite this weakness, strategies to activate immunity towards cancer are continuing to be employed and, from these attempts, we are gaining knowledge of how to improve cancer immune therapies.

Vaccines against cancer

Vaccines used against cancer include both prophylactic vaccines and therapeutic vaccines (Lollini et al., 2006; Purcell et al., 2007). Prophylactic vaccines used to protect against cancer have generally been limited to diseases with viral etiology. Because the prevalence of oncogenic viruses in healthy individuals is relatively low, there are only few examples of preventative vaccines against cancer. These include formulations to block Hepatitis B and human papillomavisus (HPV) infections, which have been successful in reducing the risk of liver cancer and cervical cancer, respectively (Chang et al., 1997; Garland et al., 2007). It is important to mention, however, that both these vaccines likely induce B cell antibody responses to antigens on the surface of viral particles, rather than robust T cell responses to virus infected cells. In fact, in individuals with pre-existing HPV infection, the same vaccine that protects against the infection is unable to significantly eliminate HPV-infected cells or reduce viral load (Hildesheim et al., 2007). This information should not discourage the use of therapeutic vaccines against cancer, though, as there are likely context-dependent factors involved in this outcome.

Therapeutic vaccines come in many forms and have evolved tremendously over the years. In general, these vaccines have two components: tumor antigen(s) and an immune-stimulating component. Tumor antigens can be defined, through the use of peptides or genes

encoding the antigens, or unknown, such as in the use of damaged cancer cells. As I have described in section III, many different types of antigens have been identified in cancer and considerations must be made when selecting these for use in cancer immune therapy. The immune-stimulating component can include adjuvants, cross-linking antibodies, cytokines, and viruses with the goal of activating dendritic cells that will present the associated tumor antigens to the immune system. Adjuvants stimulate pattern-recognition receptors (PRRs) on antigen-presenting cells, as previously mentioned, and have included heat shock proteins and other toll-like receptor agonists (Belli, 2002; Ishii and Akira, 2007; Janetzki et al., 2000). In addition, to simulate $CD4^+$ T cell help, both recombinant CD40L and an agonistic antibody against CD40 have been used in clinical trials (Vonderheide et al., 2007; Vonderheide et al., 2001). Cytokines used in vaccination, include IL-12, IL-21, among others, and serve to promote anti-tumor immunity by multi-faceted means (Cebon et al., 2003; Skak et al., 2008). Moreover, due to the natural immunogenicity of viral pathogens, recombinant viruses expressing tumor antigen genes have been exploited as cancer vaccines (Eder et al., 2000; Harrop et al., 2006; Marshall et al., 2000; Rosenberg et al., 2003). Furthermore, researchers and clinicians have also employed combinations of these immunostimulatory agents (Ahonen, 2004; Arlen, 2006). Despite observing changes in anti-tumor immunity with these vaccines, however, all attempts have resulted in little or no clinical objective response in patients (Rosenberg et al., 2004). That said, many clinical trials involve patients that have failed to respond to other therapies and so likely bear highly aggressive cancer.

In addition to single agent vaccines, whole cell vaccines are also being applied in pre-clinical and clinical trials (Gruijl et al., 2008). Expression of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) is currently used together with irradiated

tumor cells in a formulation called GVAX (Eager and Nemunaitis, 2005). The exact functions of GM-CSF are still being elucidated, but this cytokine has been found to potently recruit antigen-presenting cells and promote anti-tumor immunity in mouse model systems (Dranoff, 2002; Jinushi et al., 2008). Irradiated autologous cancer cells biopsied from patients are transduced to express GM-CSF and used to induce immunity in that same patient. This personalized treatment has been used against a number of cancer types (Salgia, 2003; Soiffer, 2003; Soiffer et al., 1998). Furthermore, as antigens from these whole tumor cell vaccines were found to be cross-presented by host dendritic cells, researchers and clinicians have moved on to use allogenic cell lines, bearing potentially unmatched MHC molecules, that can be made to more efficiently express transgenes. These allogenic cancer cell lines are also irradiated and transduced to express GM-CSF and can be produced in bulk to provide a standard treatment for many patients (Emens et al., 2004; Higano et al., 2008; Small et al., 2007). Irradiated autologous tumor cells have also been mixed with irradiated cell lines expressing GM-CSF, even with additional expression of CD40L, to achieve cross-presentation of patient-specific tumor antigens (Dessureault et al., 2007; Nemunaitis et al., 2006).

Finally, mature dendritic cells, the most potent antigen-presenting immune cells, bearing tumor antigens themselves have been utilized as vaccines (Koski et al., 2008; Melief, 2008). Peptide-pulsed DCs induced stronger immune responses in cancer patients than peptides alone and viruses expressing the tumor antigen (Connerotte et al., 2008). DCs have been derived from both monocytes and $CD34^+$ progenitor cells for vaccine trials, although $CD34^+$ cell-derived DCs have been shown to be more effective at inducing T cell responses (Banchereau et al., 2001; Ferlazzo et al., 1999; Schuler-Thurner, 2002). Recently, a phase III clinical trial utilizing autologous DCs expressing GM-CSF and tumor antigen for prostate cancer was completed with

some success (Small, 2006). Despite this, FDA approval for this vaccine was denied in part owing to the inability to standardize autologous vaccine formulations (Gruijl et al., 2008). More standard DC vaccines have been used in the form of allogeneic cells, despite not having the capacity to prime T cells via syngeneic MHC molecules. In theory, this could strongly stimulate $CD4^+$ T cell help via allo-reactivity to potentially support anti-tumor cytotoxic T cell responses. Allogeneic DC vaccines, however, have failed to perform better than autologous DCs. Nonetheless, an interesting approach to achieve both cross-presentation of MHC class I tumor antigen presentation and strong $CD4^+$ T helper cell induction via allogeneic MHC reactions has been with the use of fused hybrid cells between autologous tumor cells and allogeneic DCs (Trefzer et al., 2004; Trefzer and Walden, 2003).

Adoptive T cell therapy

Cancer vaccines must compete with established immune suppressive factors to overcome tolerance in cancer patients, which explains why it has not been easy to develop vaccines for cancer. Furthermore, many endogenous tumor reactive T cells may be anergic or deleted and so less able to response to immune activating signals. Thus, rather than inducing an immune response, passive immune therapy involves the transfer of already activated immune cells or molecules into cancer patient for therapy. Transferred cells can be effector $CD4^+$ or $CD8^+$ T lymphocytes and transferred molecules are generally B cell antibodies. I will mention therapeutic antibody administration later when I describe combination cancer therapies. Here, I will describe the current use and future potential of adoptive T cell transfer therapy.

Adoptive cell transfer (ACT) therapy involves the *ex vivo* culture and expansion of tumor-infiltrating lymphocytes (TILs) derived from a patient and transfer back into the same patient for therapy. ACT was made possible by the use of high levels of IL-2, a T cell growth

factor, to culture human TILs. This culture method allowed clinicians to greatly expand TILs while maintaining the lymphocytes' ability to lyse autologous target cells in an antigen-specific manner (Dudley et al., 2003; Muul et al., 1987). Initial randomized trials using ACT therapy in combination with IL-2 resulted in no significant difference in efficacy compared to IL-2 treatment alone in either melanoma or renal cancer (Dréno et al., 2002; Figlin et al., 1999). A similar clinical trial with gastric cancer, however, did result in prolonged survival with ACT (Kono et al., 2002). Interestingly, these patients were treated with a low-dose chemotherapy regimen in addition to ACT compared to chemotherapy alone.

A breakthrough in ACT therapy came with the use of non-myeloablative, lymphodepleting chemotherapy. Patients receiving a regimen consisting of cyclophosphamide and fludarabine for 7 days prior to T cell transfer and IL-2 supplementation demonstrated *in vivo* expansion and persistence of antigen-specific T cells and resulted in cancer regression and autoimmunity (Dudley et al., 2002). Use of this pre-conditioning regimen with ACT has since resulted in 51% of patients with metastatic melanoma demonstrating objective clinical response in therapy (Dudley et al., 2005). This strategy was preceded by studies in mice where cyclophosphamide treatment increased the effectiveness of adoptive anti-tumor T cell transfer presumably by removing immunosuppressive T lymphocytes (Berendt and North, 1980; North, 1982). Recently, a multi-faceted rationale for lymphodepletion has been elucidated in mouse models, although other factors could be at play, as well. First, lymphodepletion reduces immune suppressive T regulatory cells, which are often expanded in the presence of cancer (Antony et al., 2005). Second, lymphodepletion allows for homeostatic proliferation of transferred T cells and eliminates lymphocytes that might compete with anti-tumor T cells for important cytokines (Dummer, 2002; Gattinoni, 2005). Finally, damaged cells and structures that block microbial

infection serve as natural adjuvants to boost immunity (Paulos et al., 2007). More effective immune depletion strategies that result in myeloablation are currently being investigated (Dudley et al., 2008).

The efficacy of ACT therapy with associated pre-conditioning in clinical trials is ample evidence of its potency as a cancer treatment (June, 2007; Rosenberg et al., 2008). Its success, however, has been largely limited to metastatic melanoma, thus far. Nevertheless, it is hopeful that the effectiveness of ACT will translate to other cancer types, particularly with research on T cell modifications that can improve efficacy. One limitation of the current approach for ACT is the ability to isolate tumor-infiltrating lymphocytes for *ex vivo* expansion. Thus, an alternative is gene modification of autologous peripheral blood lymphocytes (PBLs) to express T cell receptors specific for target tumor antigens. Success with this procedure has been demonstrated in both mouse models and human patients (Clay et al., 1999; Morgan et al., 2006; Weinhold et al., 2007). Furthermore, researchers are making efforts to eliminate the need for MHC-dependent recognition of target antigens by using chimeric monoclonal antibody single-chain variable fragments (scFvs) fused to the intracellular signaling domains of the TCR complex (Gross et al., 1989). In addition to modification of T cell-target cell recognition, T cells are being modified to improve their effector activity, survival, proliferation, and tumor-homing capacity (Kershaw et al., 2005). For example, human T cells transduced to express co-stimulatory ligands that are normally expressed on APCs can stimulate themselves and surrounding T lymphocytes to achieve a more potent anti-tumor response (Stephan et al., 2007). Furthermore, small interfering RNAs (siRNAs) targeting Fas have been used to improve the survival of human CTLs (Dotti et al., 2005). The use of engineered T cells will surely expand the use of adoptive cell transfer therapy.

In addition to autologous ACT therapy for metastatic melanoma, allogeneic ACT has also proven efficacious under some circumstances. As minor histocompatibility antigens can elicit potent T cell responses, allogeneic donor lymphocytes have demonstrated, although initially inadvertently, the ability to facilitate remission of cancer in some patients with leukemia and lymphoma. Allogeneic ACT has since been used intentionally with the realization that donor lymphocytes could function in the capacity of graft-versus-host (GVH) to eliminate malignant cells (Kolb, 2008).

Non-specific immune modulation

A number of immune therapy treatments assume the existence of endogenous anti-tumor responses and so seek to boost these non-specifically or to block inhibition of these responses. Current strategies include the use of pro-immunity cytokines (in the absence of gene or peptide vaccination), such as IL-2. Also being investigated are methods of blocking immune suppressive factors, such as TGF-β, and inhibitory molecules, such as PD-1. As there are numerous formulations for non-specific immune modulation, I will limit the following description to the blockade of the inhibitory CTLA-4 co-signaling molecule.

As I mentioned previously, CD28 and CTLA-4 on T cells both bind co-signaling ligands B7-1 and B7-2, generally provided by APCs. Signaling through CD28 promotes T cells activation, while CTLA-4 ligation results in inhibitory signaling. CTLA-4 has much greater affinity than CD28 for co-signaling ligands and so CTLA-4 also functions to compete with co-stimulation (Collins et al., 2002). Furthermore, CTLA-4 blocks the formation of lipid-rafts and microclusters resulting in impaired TCR signaling (Schneider et al., 2008). Because of CTLA-4's negative effect on T cells activation, blocking CTLA-4 with monoclonal antibodies was perceived to be a viable therapeutic strategy for cancer patients. CTLA-4 blockade has

demonstrated efficacy in improving anti-tumor T cell responses and facilitating tumor rejection in mouse cancer models (Kwon et al., 1997; Leach et al., 1996; Shrikant et al., 1999). The effectiveness of this treatment, however, depended on tumor stage, as anti-tumor effects were not enhanced with CTLA-4 blockade in mice with advanced lesions (Sotomayor et al., 1999; Yang et al., 1997). Furthermore, since enhanced immunity relies on a pre-existing anti-tumor response, the natural immunogenicity of tumors also posed a challenge. Thus, CTLA-4 blockade has since been combined with GM-CSF producing cancer cell vaccines with improved results in mouse models (Hurwitz et al., 2000; van Elsas et al., 1999). Furthermore, this combination mediates increased anti-tumor immunity in patients with a reduction in adverse side effects (Hodi et al., 2008; Melero et al., 2009). Clinical trials of CTLA-4 blockade via two different humanized antibodies are currently underway against a variety of cancers (Peggs et al., 2008; Weber, 2007).

As T regulatory cells constitutively express CTLA-4, it is unclear if CTLA-4 antibody blockade also affects this immunosuppressive population. Antibody blockade of CTLA-4 on Tregs has yielded abrogated suppressive activity towards T cells, but this result has been debated (Quezada, 2006; Takahashi et al., 2000). More convincingly, Tregs were found to require CTLA-4 to maintain peripheral tolerance as evidenced by fatal autoimmune disease in animals with Treg-specific CTLA-4 deficiency (Wing et al., 2008). Additional CTLA-4 deficiency in other lymphocytes enhanced the disease, however, indicating that CTLA-4 functions on many cell types to regulate immunity.

Combination therapy

As I have mentioned at the beginning of this chapter, there are multiple components to cancer that have coordinated functions in facilitating malignant progression. Based on this, the use of single agents or combination therapies aimed at altering multiple components of cancer,

rather than a single part, is likely to be advantageous in cancer therapy. In fact, many treatments intended to target cancer cells have important immune components that add to their effectiveness, including chemotherapies, radiation therapy, and targeted therapies based on antibodies.

The primary goal of conventional anti-cancer therapies, such as chemotherapies and radiation therapy, is to directly kill tumor cells that exist in the presence of many sources of cellular stress (Luo et al., 2009). Many of these agents, however, also promote anti-tumor immunity (Zitvogel et al., 2008b). For example, chemotherapy-induced death of tumor cells results in calreticulin exposure and release of HMGB1 promoting dendritic cell activation and tumor antigen cross-presentation (Apetoh et al., 2007; Obeid et al., 2007). Furthermore, ionizing radiation enhances MHC class I antigen presentation and, thus, improved CTL recognition of target tumor cells (Garnett et al., 2004; Reits et al., 2006). More directly, low-dose cyclophosphamide treatment reduces T regulatory cell-mediated immune suppression (Ercolini, 2005; Turk, 2004). In addition, the anti-metabolite gemcitabine decreases the number of myeloid-derived suppressor cells (Ko et al., 2007). Furthermore, as previously mentioned, immune depletion with chemotherapy and radiation augments the efficacy of adoptive T cell transfer therapy (Dudley et al., 2008). Efforts to further understand these pleiotropic effects and capitalize on synergistic interactions are in progress (Zitvogel et al., 2008a).

A number of monoclonal antibodies have been approved for cancer therapy. These include Herceptin, which targets the HER2 oncogene found predominantly in breast cancer, and Erbitux, which binds the epidermal growth factor receptor (EGFR) in a number of malignancies (Carter, 2001). Binding of target proteins by these antibodies can block oncogenic signaling or mediate cell death by antibody cross-linking. In addition, tumor cells bound by antibodies can

be killed via immune mechanisms, antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). ADCC relies on recognition of the antibodies bound to tumor cells by Fc receptors on innate immune cells, such as natural killer cells, macrophages, neutrophils, and eosinophils. Tumor cells are then killed via perforin/granzymes or other lytic enzymes depending on the immune cell type. CDC involves recognition of antibodies by complement proteins that initiate a biochemical cascade resulting in osmotic lysis of tumor cells (Murphy et al., 2008). The immune system's role in the effectiveness of targeted monoclonal antibodies has been demonstrated in model systems and its role in the clinic is being investigated (Carter, 2001)

VI. MOUSE MODELS OF CANCER FOR TUMOR IMMUNOLOGY

Model antigens

Many model T cell antigens have been used in cancer immunology studies, as well as, more general immunological research (Table 4). Some of these antigens are derived from human or mouse tumors, whereas others come from foreign sources, ranging from chickens to viruses, or are synthetically produced. The attraction of utilizing these defined antigens come from the various reagents that are available with which to investigate antigen-specific immune responses. As the identities of these antigens are known, peptides can be synthesized for use in stimulating antigen-specific T cells, detecting endogenous T cell responses via tetramer/Dimer staining, or vaccinating animals. Additional reagents, such as viruses or cell lines, expressing these antigens are often available, as well. Furthermore, T cell clones that specifically recognize these antigens presented by particular MHC molecules have also been developed. In many cases, the T cell receptors (TCR) of these T cell clones have been used to make TCR transgenic mice that

produce large numbers of antigen-specific T cells.

Antigen	AA sequence	Source	H-2	TCR transgenic mice
SIY	SIYRYYGL	synthetic	K^b	2C
GP33 (33-41)	KAVYNFATM	LCMV	D^b	P14
HA (512-520)	IYSTVASSL	Influenza virus	K^d	clone 4
TAg (560-568)	SEFLLEKRI	SV40	K^k	TCR-I
OVA (257-264)	SIINFEKL	chicken	K^b	OT-I
GP100 (25-33)	EGSRNQDWL	mouse melanocytes	D^b	Pmel-1
P1A (35-43)	LPYLGWLVF	mouse	L^d	TCRP1A

Table 4. Examples of model antigens used in immunological research. Defined peptides (amino acid sequences shown) are derived from various sources and presented via different MHC class I molecules. T cell receptor transgenic mice from antigen-reactive T cell clones have been generated that specifically recognize peptides presented by their respective MHC molecules. Only $CD8^+$ T cell clones and antigens are shown, but $CD4^+$ T cell clones, transgenic mice, and defined antigens are also available.

Mouse cancer models

Model antigens have been engineered into mouse tumor cell lines, if not already naturally expressed, in order to study immune reactivity and therapy to tumors. These lines vary in the type of cancer they represent, such as melanoma (B16), colon cancer (CT26), and breast cancer (4T1). These cell lines are either transplanted subcutaneously or orthotopically into syngeneic mice to generate *in vivo* cancer. Although widely used, such tumor models present a number of complications. Tumor cell injection results in tissue damage, death to transplanted cells, and an acute burden of tumor antigens that is likely to be highly immunogenic. In addition, transplanted cells are commonly injected under the skin, an organ that is surveyed by Langerhans cells, a dendritic cell subset described to be relatively potent at activating immune responses (Merad et al., 2008). Moreover, transplanted tumors are unlikely to possess a realistic tissue microenvironment due to their ectopic location and rapid growth rate, which can influence immune therapy and facilitate tumor rejection. Thus, to better understand the role of the immune system in cancer development, many groups have developed and/or used spontaneous cancer models in the mouse for the study of cancer immunology.

Spontaneous cancer models used in cancer immunology have generally been animals that are germline deficient for tumor suppressor genes and/or that express oncogenes in tissue-restricted patterns (Gendler and Mukherjee, 2001). In addition, model tumor antigens are then expressed via (another) transgene in a tissue-restricted pattern or derived from the oncogenic transgene. For example, two human tumor-associated antigens, CEA and MUC1, have been engineered to be expressed in transgenic mice (Clarke et al., 1998; Eades-Perner et al., 1994; Rowse et al., 1998). Anti-tumor immunity and immune therapy in CEA transgenics have been studied in various contexts, including spontaneous breast cancer, colon cancer, and lung cancer

(Greiner et al., 2002; Thompson et al., 1997; Zeytin et al., 2004). Tumor immunology in the MUC1 transgenic mouse has been examined in the context of pancreatic cancer and mammary cancer (Chen et al., 2003; Mukherjee et al., 2003). Since the defined tumor antigens in these transgenic mice are expressed across many tissues, these models represent cancer bearing tumor-associated antigens that do not offer a wide differential specificity between tumor and normal tissue.

Two commonly used spontaneous cancer models are the RIP-Tag2 and TRAMP mice, both of which expressed SV40 large T antigen. RIP-Tag2 express T antigen via the rat insulin promoter and develop insulinomas, tumors of the pancreatic β-islet cells (Hanahan, 1985). TRAMP mice, on the other hand, utilize the rat probasin promoter and develop prostate cancer (Greenberg et al., 1995). Various defined antigens have been studied in the context of these spontaneous tumor models, including epitopes of T antigens and of Influenza's hemagglutinin (HA) engineered to be expressed similarly to T antigen (Anderson et al., 2007; Drake et al., 2005; Lyman et al., 2004). Since both oncogenic T antigen and these cancers' associated tumor antigens are expressed in a tissue-restricted manner, the level of antigen expression is likely to be similar between tumor and normal tissue. Thus, these models most closely represent cancer bearing differentiation antigens, particularly because antigen expression likely precedes tumor formation.

In addition to spontaneous mouse cancer models, a few efforts have been made to generate conditional cancer models with associated tumor antigens that are regulated in a Cre-loxP-dependent fashion. The first of these utilized a ubiquitous promoter regulated by a floxed attenuator element to regulate expression of SV40 small and large T antigens (Willimsky and Blankenstein, 2005). Cre delivered via Adenovirus through the tail vein to the liver induced

liver cancer with high efficiency. In addition, through aberrant splicing or deletion of the attenuator, animals spontaneously developed focal tumors in various tissues. In these animals, prophylactic vaccination against T antigen protected animals from both spontaneous and Cre-induced tumor development. This result is similar to what one might expect of a tumor-specific antigen in which neither central nor peripheral tolerance in the absence of cancer restricts antigen-specific T cells. The utility of this model, however, is not only limited by the formation of spontaneous tumors, but also the dependence on SV40 T antigens as oncogenes.

A second conditional cancer model in mice also aims to generate tumors bearing tumor-specific expression of a defined antigen (Huijbers et al., 2006). In this model, the cancer-testis antigen P1A is expressed in melanoma driven by coordinated expression of oncogenic H-ras and loss of tumor suppressors $p16^{Ink4a}$ and $p19^{Arf}$. The group has yet to examine immune reactivity to these tumors due to incompatibility between the tumor antigen and the MHC haplotype expressed in the animals generated. The use of genetic mutations relevant to human cancer in this model, however, is a step forward in the faithful recapitulation human disease. Knowledge of the mutations that promote the development of different types of cancer in humans combined with the ability to specifically alter gene function in mice has allowed many laboratories to develop mouse models of various tumor types (Frese and Tuveson, 2007). A new allele that achieves conditional expression of a model antigen has recently been developed to be compatible with various Cre-loxP-dependent mouse cancer models (Cheung et al., 2008). This new model of an over-expressed self-antigen in cancer is described in Chapter 2.

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CHAPTER 2

Regulated expression of a tumor-associated antigen reveals multiple levels of T cell tolerance in a mouse model of lung cancer

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The author generated *R26*$^{LSL\text{-}LSIY}$ and *R26*$^{1Lox\text{-}LSIY}$ mice and performed all of the experiments described. Michel DuPage derived DC2.4.LSIY dendritic cell lines. Katie Dong, an undergraduate student supervised by the author, assisted in the quantification of tumor burden in Figures 3D and 4D. All experiments were performed in the laboratory of Tyler Jacks.

ABSTRACT

Maximizing the potential of cancer immunotherapy requires model systems that closely recapitulate human disease to study T cell responses to tumor antigens and to test immunotherapeutic strategies. We have created a new system that is compatible with Cre-loxP-regulatable mouse cancer models in which the SIY antigen is specifically over-expressed in tumors, mimicking clinically-relevant tumor-associated antigens. To demonstrate the utility of this system, we have characterized SIY-reactive T cells in the context of lung adenocarcinoma, revealing multiple levels of antigen-specific T cell tolerance that serve to limit an effective anti-tumor response. Thymic deletion reduced the number of SIY-reactive T cells present in the animals. When potentially self-reactive T cells in the periphery were activated, they were efficiently eliminated. Inhibition of apoptosis resulted in more persistent self-reactive T cells, but these cells became anergic to antigen stimulation. Finally, in the presence of tumors over-expressing SIY, SIY-specific T cells required a higher level of costimulation to achieve functional activation. This system represents a valuable tool in which to explore sources contributing to T cell tolerance of cancer and to test therapies aimed at overcoming this tolerance.

INTRODUCTION

Mouse models have been a mainstay of cancer immunology research. The mouse has been used to probe the function of cells and molecules that influence the ability of tumor-reactive T cells to kill or to otherwise become functionally suppressed, or tolerized (Dunn et al., 2004; Rabinovich et al., 2007; Swann and Smyth, 2007). These studies have largely relied on chemically-induced and spontaneous tumors in immunodeficient mice or on transplanted tumors. Such systems are limited because they fail to reproduce the complex interactions that exist among an emerging tumor, its microenvironment and the multiple elements of an intact immune system. More recently, genetically-engineered cancer models with tissue-specific expression of known antigens have been used to study T cells reactive to tumor antigens (Anderson et al., 2007; Drake et al., 2005; Huijbers et al., 2006; Muller-Hermelink et al., 2008; Nguyen et al., 2002; Willimsky and Blankenstein, 2005). Interestingly, these studies have led to divergent findings on T cell reactivity and tolerance to tumors, leading to the question of whether to attribute these differences to the immunobiology of specific tissues, the type of cancer under study, and/or the pattern in which antigens are expressed.

Tumor antigens are characterized as either tumor-specific (TSAs) or tumor-associated (TAAs). TSAs are derived from proteins to which the host immune system is naïve, such as mutant, germ cell-restricted, or viral proteins. TAAs, on the other hand, are derived from wild-type somatic proteins over-expressed or inappropriately expressed in tumors (e.g. CEA, ERBB2, hTERT, MUC1, P53). Experimental immune therapies for both TSAs and TAAs are being pursued as neither is clearly a more effective anti-cancer target (Dudley et al., 2005; June, 2007; Rosenberg et al., 2008). Targeting TSAs might appear preferable because this provides absolute selection for tumor cells; however, TSA expression is often limited to the individual tumors from

which they were originally identified (Novellino et al., 2005). Conversely, TAAs are commonly shared among tumor types and represent the majority of known tumor antigens (Stevanovic, 2002). Thus, therapies developed to TAAs may be applicable to patients across cancer types.

Lung cancer is the leading cause of cancer death worldwide. This is due to its high incidence and the failure of existing therapies to effectively treat advanced disease. Thus, there is great need to develop effective novel therapeutic strategies for lung cancer. Relative to other tumor types (e.g. melanoma, ovarian and prostate cancer) little is known about the immune response to lung cancer or the potential for immunotherapy for this cancer type (Fukuyama et al., 2006; Fukuyama et al., 2007; Mizukami et al., 2006; Raez et al., 2005; Woo et al., 2002). To begin to explore the immune response to lung cancer antigens and to build systems for testing immunotherapeutic strategies to treat lung cancer in humans, we have created a mouse model in which the well-characterized T cell antigen SIYRYYGL (Udaka et al., 1996) is over-expressed in autochthonous lung cancer. Based on analysis of T cells reactive to SIYRYYGL in this context, we have uncovered multiple levels of immune tolerance that limits an effective T cell response against a tumor-associated antigen (Figure S1).

RESULTS

Generation of $R26^{LSL\text{-}LSIY}$

To create a flexible system in which tumor-specific T cell responses could be carefully monitored during tumor progression, we used gene targeting to introduce a fusion of Firefly *luciferase* and SIYRYYGL (SIY) into the ubiquitously-expressed Rosa26 locus (Soriano, 1999) (Figure S2). Expression of luciferase-SIY fusion protein (termed LSIY) is controlled by a Lox-STOP-Lox, such that efficient expression can only be achieved following Cre-mediated excision

of the STOP element (Jackson et al., 2001; Tuveson et al., 2004). The *Rosa26-Lox-STOP-Lox-Luciferase-SIY* allele is hereafter referred to as $R26^{LSL\text{-}LSIY}$. We chose the synthetic peptide SIYRYYGL because many complementary reagents exist making it a tractable model antigen (Udaka et al., 1996) (Table S1). SIY was fused to luciferase to facilitate detection and quantification of antigen expression *in vitro* and *in vivo*. Cre-inducible expression from a ubiquitous promoter makes $R26^{LSL\text{-}LSIY}$ compatible with various mouse cancer models that bear Cre-regulated tumor-predisposition genes (Frese and Tuveson, 2007).

Adenovirus-Cre (Ad-Cre) infection of $R26^{LSL\text{-}LSIY/+}$ mouse embryonic fibroblasts (MEFs) yielded strong induction of luciferase activity (>10^3-fold over unrecombined MEFs), validating Cre-dependent regulation. Interestingly, in the absence of Ad-Cre, we consistently observed slightly elevated luciferase activity (~3-fold) in $R26^{LSL\text{-}LSIY/+}$ MEFs compared to controls (Figure 1A). This result suggests that $R26^{LSL\text{-}LSIY}$ expresses LSIY at low levels.

2C mice express a dominant TCR (recognized by the clonotypic antibody 1B2) that reacts with SIY bound by H-2K^b haplotype MHC class I (Kranz et al., 1984; Saito et al., 1984; Sha et al., 1988; Udaka et al., 1996). To test for LSIY expression *in vivo*, the fate of potentially self-reactive 2C T cells was assessed in $R26^{LSL\text{-}LSIY}$*;2C* mice. $CD8^+1B2^+$ cells were severely reduced in peripheral blood of these mice relative to $R26^{+/+}$*;2C* controls (Figure 1B). Furthermore, the proportion of thymic $1B2^+$ cells, in particular the $CD8^+CD4^-$ fraction, was diminished in $R26^{LSL\text{-}LSIY/+}$*;2C* mice. These data imply negative-selection of SIY-reactive cells during T cell development, or central tolerance, in $R26^{LSL\text{-}LSIY}$ mice.

$R26^{LSL\text{-}LSIY}$ exhibits Cre-dependent over-expression

Central tolerance could occur due to thymus-restricted or ubiquitous, somatic antigen expression. Various tissues were surveyed to distinguish between these possibilities. Luciferase

activity was 3-30-fold higher in both thymic and extra-thymic tissues of *R26*$^{LSL\text{-}LSIY/+}$ mice relative to background levels in controls (Figure 1C). By contrast, in *R26*$^{1Lox\text{-}LSIY/+}$ mice, in which the STOP element had been deleted by the *Meox2-Cre* transgene (Tallquist and Soriano, 2000), a 10^4-10^5-fold increase in reporter activity was detected in all tissues examined (Figure S2A; Figure 1C). These results show that *R26*$^{LSL\text{-}LSIY}$ exhibits low-level, ubiquitous expression of LSIY.

To further characterize the consequences of low-level expression from *R26*$^{LSL\text{-}LSIY}$, we assessed 2C cell reactivity to *R26*$^{LSL\text{-}LSIY/+}$ cells. Naïve 2C cells exhibited dose-dependent activation (measured by CD25 surface-expression) and proliferation upon co-culture with *R26*$^{LSL\text{-}LSIY/+}$, but not with *R26*$^{+/+}$, splenocytes (Figure S3A,B). Additionally, transfer of CFSE-labeled 2C cells into *R26*$^{LSL\text{-}LSIY/+}$ mice led to progressive dilution of CFSE and upregulation of CD44, a cell-adhesion molecule associated with T cell activation (Figure 1D; Figure S3C). These data establish that low-level LSIY expression from *R26*$^{LSL\text{-}LSIY}$ is sufficient to stimulate 2C cells *in vitro* and *in vivo*.

Incomplete central tolerance in *R26*$^{LSL\text{-}LSIY}$ mice

Because we detected CD8$^+$ T cells bearing 2C-TCR in blood and peripheral lymphoid organs of *R26*$^{LSL\text{-}LSIY/+}$*;2C* mice, central tolerance to SIY must be incomplete (Figure 1B; data not shown). To eliminate the possibility of an artifact of *2C* transgenic mice, we infected *R26*$^{LSL\text{-}LSIY/+}$ and control mice, that have normal TCR repertoires, with *Influenza* virus engineered to express SIY (WSN-SIY) (Li et al., 1993; Shen et al., 2008). Seven days after pulmonary WSN-SIY infection of *R26*$^{+/+}$ mice, robust induction of SIY-specific CD8$^+$ T cells in lungs and lymphoid tissues was evident by staining with SIY-loaded H-2K^b DimerX reagent (Figure 2A; data not shown). We also detected SIY-reactive T cells in lungs of WSN-SIY-infected *R26*$^{LSL\text{-}}$

$^{LSIY/+}$ mice, albeit at significantly lower levels compared to controls (Figure 2A). (SIY-reactive T cells were not detectable in uninfected mice (data not shown)).

Because SIY-reactive T cells in *R26*$^{LSL\text{-}LSIY}$ mice could be self-reactive, we probed the activity of these cells induced by WSN-SIY and found they were functional. When single-cell suspensions from lungs of WSN-SIY-infected mice were stimulated *in vitro* with SIY, a comparable fraction of cells secreted IFN-γ in both *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ cultures (Figure 2B). Furthermore, by assaying *in vivo* cytotoxicity (Figure S4) in WSN-SIY-infected mice, we found SIY-specific cytolysis in proportion to SIY-reactive T cells induced in *R26*$^{LSL\text{-}LSIY/+}$ mice (Figure 2C). As we observed long-term health without autoimmunity in WSN-SIY-infected *R26*$^{LSL\text{-}LSIY/+}$ mice, we investigated SIY-reactive T cell fate. Upon boosting with DC2.4.LSIY cells (a dendritic cell line expressing high levels of costimulatory molecules B7-1 and B7-2 (Shen et al., 1997), modified to additionally express LSIY), we did not detect surviving SIY-reactive T cells in WSN-SIY-infected *R26*$^{LSL\text{-}LSIY/+}$ mice. In contrast, memory cells were evident in WSN-SIY-infected wild-type mice (Figure 2D). Together, these results demonstrate that central tolerance to SIY is incomplete in *R26*$^{LSL\text{-}LSIY}$ mice and a transient, but functional, T cell response to SIY can be initiated.

Antigen over-expressing tumors progress normally

R26$^{LSL\text{-}LSIY}$ is a novel system in which over-expression of a self-antigen is induced, representing clinically-relevant tumor-associated antigens in human cancer (Novellino et al., 2005). Given the importance of TAAs as targets for cancer immunotherapy, *R26*$^{LSL\text{-}LSIY}$ in Cre-inducible cancer models provides a powerful tool to study T cell-tumor interactions. To explore the utility of this system, we used a model of human lung adenocarcinoma in which oncogenic K-ras is expressed from its endogenous locus after intranasal Ad-Cre administration (Jackson et

al., 2001; Tuveson et al., 2004). In this *K-ras*$^{LSL\text{-}G12D}$ model, focal activation of oncogenic K-ras leads to epithelial hyperplasia, which progresses to adenoma and adenocarcinoma over a defined time course resembling human non-small cell lung cancer (NSCLC).

We generated *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice and induced lung cancer in these animals and controls using Ad-Cre. *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice developed lung tumors histologically indistinguishable from their *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ littermates when examined at various times after Ad-Cre (Figure 3A; data not shown). Tumors dissected from *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice consistently had 10^3-10^4-fold higher luciferase activity than those from *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ mice, suggesting that LSIY expression was maintained in established tumors (Figure 3B). Luciferase activity was also detectable in large tumors several months after tumor initiation via *in vivo* bioluminescence imaging (Figure 3C).

Histological examination of tumors from *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ mice at 8, 12, and 16 weeks post-Ad-Cre revealed no evidence of an active anti-tumor T cell response in either genotype (Figure 3A; data not shown). Furthermore, flow cytometry of lungs and lymphoid tissues of *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice failed to detect SIY-reactive T cells either early after tumor initiation (within 12 days) or 3-4 months later during tumor progression (data not shown). Because this does not eliminate the possibility of an immune response below detection or at an unexamined time, we explored the effects that such a response might have had. Specifically, we compared tumor burdens and sizes between *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ and *Kras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ littermates, but no significant differences were observed (Figure 3D). These data suggest that although SIY-reactive T cells are elicited in WSN-SIY-infected *R26*$^{LSL\text{-}LSIY/+}$ mice (Figure 2), a response is not generated against SIY-over-expressing lung tumors.

Tumors maintain antigen presentation

Tumor-specific down-regulation of antigen presentation has been proposed as a mechanism to evade recognition by T cells (Algarra et al., 2004; Khong and Restifo, 2002; Pardoll, 2003). To measure MHC class I in tumors, lung tumor cell lines were derived from Ad-Cre-infected *K-ras*$^{LSL\text{-}G12D/+}$*;p53*$^{fl/fl}$*;R26*$^{LSL\text{-}LSIY/+}$ mice and *R26*$^{+/+}$ controls (Figure S5A,B). By flow cytometry, we detected comparably low levels of H-2K^{b}, which was induced 40-fold by IFN-γ treatment in all lines (Figure S5C). This indicates that antigen presentation is functional in tumors. Furthermore, we induced and examined lung tumors in mice on a *2C* transgenic background. Tumors in *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$*;2C* mice were highly infiltrated by lymphocytes in contrast to tumors induced in *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$*;2C* controls, demonstrating that antigen presentation by SIY-over-expressing tumors is sufficient to recruit 2C cells *in vivo* (Figure S5D). These observations imply that loss of SIY presentation is unlikely to account for the unproductive immune response to SIY-over-expressing tumors.

Naïve cells recognize but do not respond effectively to lung tumors

Our data indicate that SIY-reactive T cells are present in *K-ras*$^{LSL\text{-}G12D/+}$*; R26*$^{LSL\text{-}LSIY/+}$ animals, but fail to react to SIY-over-expressing lung tumors. There are several non-exclusive explanations for this observation. For example, neither tumor initiation nor progression may be a sufficient stimulus to induce T cell responses to tumor-associated antigens (Pardoll, 2003). Alternatively, peripheral tolerance to self-antigen may inhibit responses to over-expressed antigens in tumors (Redmond and Sherman, 2005). Finally, tumors over-expressing antigen could be actively suppressing reactive T cells (Rabinovich et al., 2007; Zitvogel et al., 2006). Because the low numbers of endogenous SIY-specific T cells in *R26*$^{LSL\text{-}LSIY}$ mice impedes

analyses, we investigated these possible mechanisms by transferring donor 2C cells into tumor-bearing and control animals.

Naïve 2C cells became activated and proliferated in tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ animals, similar to our observation in tumor-free *R26*$^{LSL\text{-}LSIY/+}$ mice (Figure 4A,B; Figure S3C; Figure S1F). Only in tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice, however, were 2C cells enriched in lung-draining mediastinal lymph nodes relative to non-draining mesenteric lymph nodes (Figure 4C). 2C cells are likely retained in mediastinal lymph nodes where SIY antigen presentation is increased due to SIY-over-expressing lung tumors.

Despite accumulation in high SIY areas, 2C cells did not infiltrate tumors, although 2C cells were detectable in lungs by flow cytometry (Figure 4C; data not shown). Consistent with the absence of tumor-infiltrating lymphocytes, *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice displayed no decrease in tumor burden, size, or number relative to *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ mice analyzed 4 weeks after 2C transfer (Figure 4D). These data demonstrate that 2C cells do not hinder tumor growth. Moreover, 2C cells were not detectable in any mice at this time-point whether they bore tumors or not (data not shown). These data indicate that 2C cells activated in *R26*$^{LSL\text{-}LSIY}$ mice do not form memory cells, as was observed in WSN-SIY-infected *R26*$^{LSL\text{-}LSIY}$ mice, potentially due to peripheral tolerance where persistent weak TCR stimulation leads to apoptosis (Redmond and Sherman, 2005). Therefore, in the presence of self-antigen, SIY-reactive T cells become activated and divide, but do not significantly kill tumor cells or persist.

SIY-reactive T cells are not cytotoxic in *R26*$^{LSL\text{-}LSIY}$ mice

Failure of T cells to kill SIY-over-expressing tumors could be due to a general defect in SIY-specific cytotoxicity or to a direct inhibition of T cells by tumors. To assess 2C cell functionality, we assayed *in vivo* cytotoxicity examining the ability of 2C cells to kill non-

malignant target cells (Figure S4; Figure 5A). As shown in Figure 5C panel 1, DC2.4.LSIY vaccination stimulated 2C cells to become highly cytotoxic in wild-type animals, a positive control for SIY-specific cytotoxicity. As a negative control, naïve 2C cells transferred into $R26^{+/+}$ without vaccination do not become activated and, thus, only displayed low SIY-specific cytolysis.

The assay was applied to tumor-free $R26^{LSL\text{-}LSIY/+}$ mice. Despite inducing activation markers and proliferating, 2C cells exhibited no more cytotoxicity in these animals than 2C cells in $R26^{+/+}$ mice, indicating that self-antigen-induced activation fails to yield full effector function (Figure 4A,B; Figure 5B). In tumor-bearing $K\text{-}ras^{LSL\text{-}G12D/+};R26^{LSL\text{-}LSIY/+}$ animals, SIY-specific cytotoxicity was similarly defective (Figure 5B). These data demonstrate generalized impairment in T cell cytotoxicity to antigenic targets, rather than specific inhibition of tumoricidal activity in $R26^{LSL\text{-}LSIY}$ mice.

TAA-over-expressing tumors suppress T cell cytotoxicity

Peptide presentation without costimulation often yields unproductive activation and T cell deletion, analogous to our own observations (Hernandez et al., 2001). To determine whether DC2.4.LSIY could provide the necessary costimulation to functionally activate 2C cells in animals in which SIY is a self-antigen, naïve 2C cells were transferred into $R26^{LSL\text{-}LSIY/+}$ animals concurrently with DC2.4.LSIY vaccination (Figure 5A). With this treatment, 2C cells exhibited high levels of SIY-specific cytotoxicity in tumor-free animals, despite low-level ubiquitous SIY (Figure 5B,C). This result demonstrates that self-antigen presented with ample costimulation can yield functional activation of potentially self-reactive T cells.

When 2C cells were transferred into tumor-bearing $K\text{-}ras^{LSL\text{-}G12D/+};R26^{LSL\text{-}LSIY/+}$ mice with DC2.4.LSIY vaccination, however, only marginal induction of SIY-specific cytotoxicity was

observed (Figure 5B,C). Thus, although costimulation enabled 2C cells to become highly cytotoxic to self-antigen in tumor-free animals, it failed to similarly induce SIY-specific cytotoxicity in the presence of tumors over-expressing SIY. Importantly, 2C transfer and DC2.4.LSIY vaccination of tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$ mice resulted in robust SIY-specific cytotoxicity (Figure 5B). This result strongly supports a mechanism by which tumors induce suppression of immune responses towards antigens over-expressed in those tumors. Furthermore, we observed comparably low cytotoxicity in mediastinal lymph nodes draining tumor-bearing lungs and mesenteric lymph nodes not draining tumors suggesting that tumor-induced inhibition of TAA-specific T cells occurs systemically (Figure 5C; data not shown).

Functional activation of TAA-reactive T cells requires stronger costimulation

The balance of immune stimulatory and inhibitory factors determines whether immune reactivity or tolerance is generated. Therefore, we reasoned that stronger immunological stimuli might induce SIY-specific cytotoxicity in tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice. DC2.4.LSIY vaccination failed to expand endogenous SIY-reactive T cells to detectable levels in *R26*$^{LSL\text{-}LSIY/+}$ mice (data not shown) whereas WSN-SIY was more potent (Figure 2). Thus, we used WSN-SIY instead of DC2.4.LSIY to vaccinate naïve 2C cell recipients (Figure 5A). In contrast to the case with DC2.4.LSIY vaccination, WSN-SIY vaccination caused 2C cells to exhibit high SIY-specific cytotoxicity in both tumor-free and tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice (Figure 5D). This result indicates that the threshold costimulation required to induce SIY-specific cytotoxicity is increased in the presence of SIY-over-expressing tumors. Despite being highly cytotoxic, however, 2C cells did not seem to exert a significant anti-tumor effect in WSN-SIY-infected *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ mice, although the number of animals tested was small (data not

shown). This phenomenon is likely a result of the relatively brief lifespan of 2C cells in *R26*$^{LSL\text{-}LSIY}$ mice.

SIY-reactive T cells blocked from death succumb to anergy

We have shown that SIY-reactive T cells can be functionally activated in tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice, but as these cells are short-lived, T cell death remains an obstacle to effective anti-tumor immunity. To overcome this barrier, we over-expressed anti-apoptotic Bcl2 by infecting SIY-stimulated 2C cells with a retrovirus carrying Bcl2 and EGFP (MIG-Bcl2). By observing enrichment of EGFP^{+} cells in culture, we confirmed that Bcl2-over-expressing 2C cells had enhanced survival over uninfected cells when deprived of IL-2 (Charo et al., 2005) (Figure 6A). When MIG-Bcl2-infected 2C cells were transferred into *R26*$^{LSL\text{-}LSIY/+}$ and *R26*$^{+/+}$ mice, Bcl2-over-expressing cells again became enriched over uninfected cells in both genotypes (Figure 6B). Despite this enrichment, 2C cells in *R26*$^{LSL\text{-}LSIY/+}$ mice were still significantly reduced and decreased over time compared to 2C cells in *R26*$^{+/+}$ recipients, suggesting that induction of both intrinsic and extrinsic apoptotic pathways account for loss of 2C cells.

Next, we examined the function of persisting Bcl2-over-expressing 2C cells. Upon *in vitro* SIY stimulation of splenocytes recovered from 2C recipients, we observed that 2C cells from *R26*$^{LSL\text{-}LSIY/+}$ mice had impaired IFN-γ secretion compared to controls (Figure 6C). Therefore, although Bcl2 over-expression can partially protect 2C cells from apoptosis in the presence of self-antigen, additional mechanisms act to induce anergy in these cells.

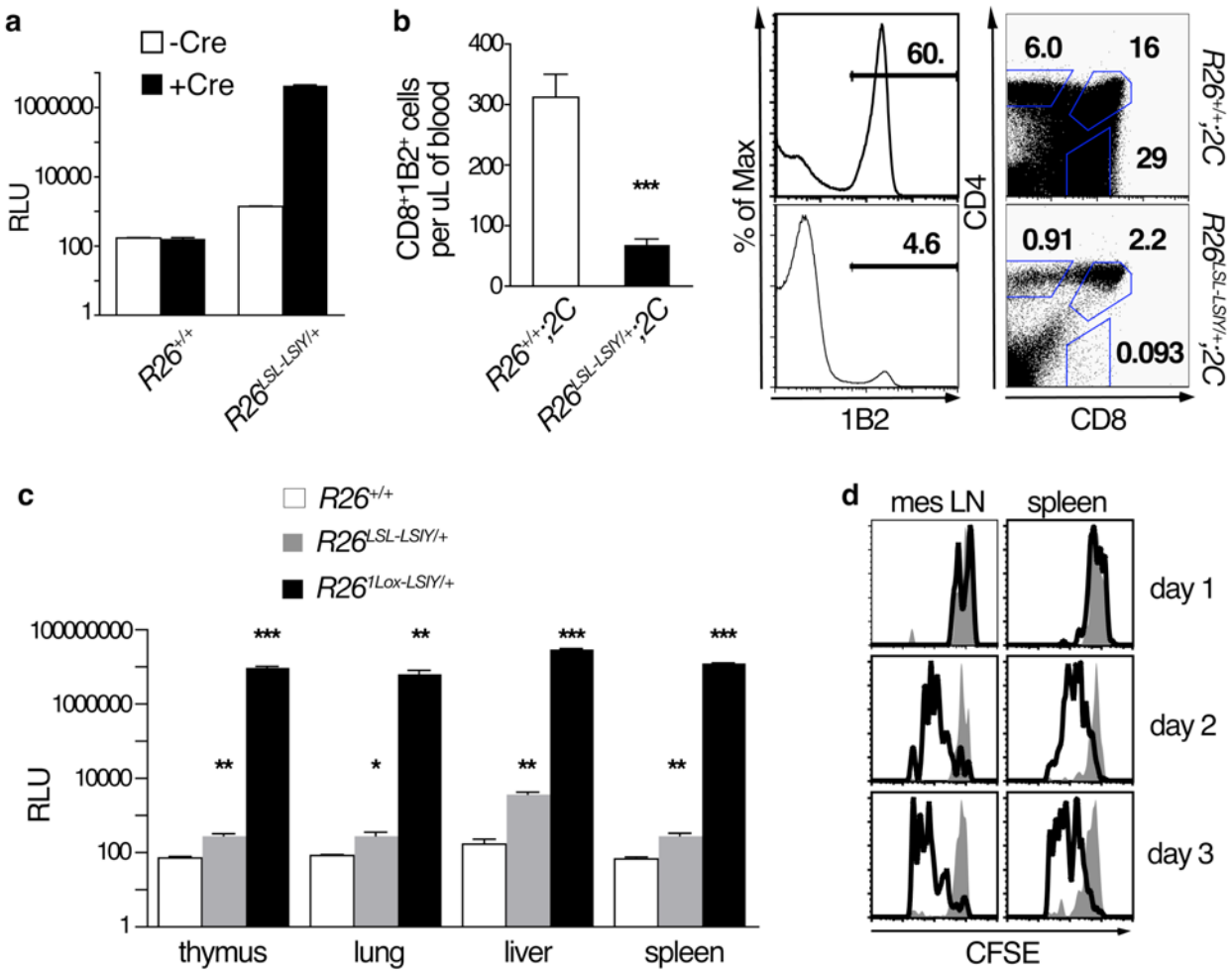


Figure 1. *R26*$^{LSL\text{-}LSIY}$ exhibits Cre-dependent over-expression and central tolerance to SIY. *A.* Luciferase activity in *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ MEFs uninfected (n = 2 and 12, *open bars*) or infected with Ad-Cre (n = 3 and 12, *filled bars*). *Bars*, mean + s.e.m., p <0.0001 by Student's *t* tests for both *R26*$^{LSL\text{-}LSIY/+}$ -Cre versus +Cre and -Cre *R26*$^{+/+}$ versus *R26*$^{LSL\text{-}LSIY/+}$. *B*. Number of CD8$^+$1B2$^+$ cells in tail tip bleeds of *R26*$^{+/+}$*;2C* and *R26*$^{LSL\text{-}LSIY/+}$*;2C* mice *(left)*. *Bars*, mean + s.e.m., p <0.0001 by Student's *t* test. 1B2, CD4, and CD8 staining of thymocytes from representative *R26*$^{+/+}$*;2C (top)* and *R26*$^{LSL\text{-}LSIY/+}$*;2C (bottom)* mice *(right)*. *C*. Luciferase activity in tissue lysates from whole thymus, lung, liver, and spleen from *R26*$^{+/+}$ (n = 4, *open bars*), *R26*$^{LSL\text{-}LSIY/+}$ (n = 5, *shaded gray bars*), and *R26*$^{1Lox\text{-}LSIY/+}$ (n = 2, *filled black bars*) mice. *Bars*, mean + s.e.m., *p <0.05, **p <0.005, ***p <<0.001 by Student's *t* test. *D*. Representative CFSE dilution in CD8$^+$1B2$^+$ cells recovered from mesenteric lymph nodes and spleens of *R26*$^{+/+}$ *(filled histograms)* and *R26*$^{LSL\text{-}LSIY/+}$ *(open histograms)* recipients of naïve 2C cells 1, 2, and 3 days after i.v. transfer.

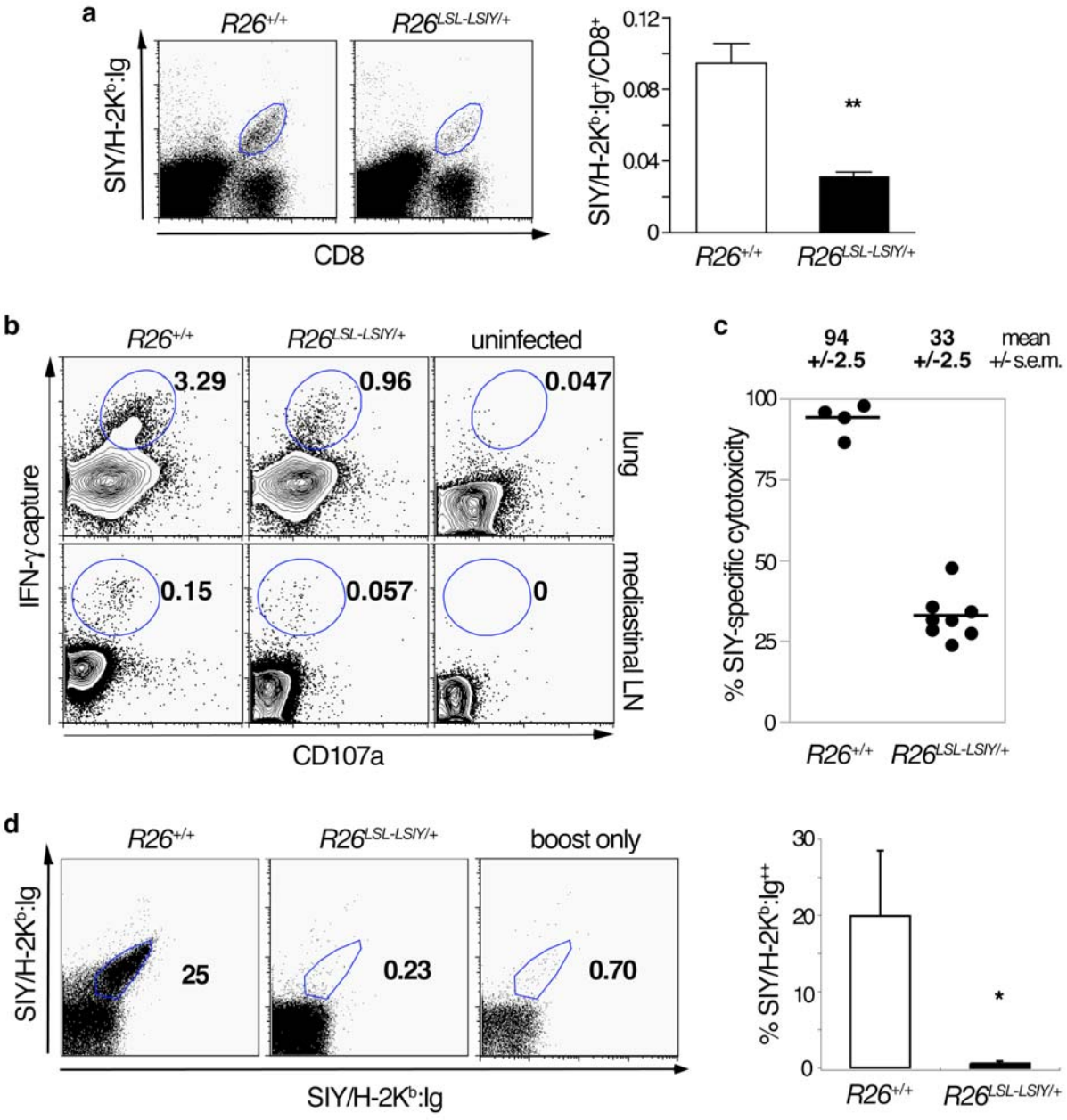

a
R26+/+
R26LSL-LSIY/+
SIY/H-2Kb:Ig
CD8
SIY/H-2Kb:Ig+/CD8+
0.12
0.08
0.04
0
**
R26+/+
R26LSL-LSIY/+
b
R26+/+
R26LSL-LSIY/+
uninfected
3.29
0.96
0.047
0.15
0.057
0
lung
mediastinal LN
IFN-γ capture
CD107a
c
94
+/-2.5
33
+/-2.5
mean
+/- s.e.m.
% SIY-specific cytotoxicity
100
75
50
25
0
R26+/+
R26LSL-LSIY/+
d
R26+/+
R26LSL-LSIY/+
boost only
25
0.23
0.70
SIY/H-2Kb:Ig
SIY/H-2Kb:Ig
% SIY/H-2Kb:Ig++
30
20
10
0
*
R26+/+
R26LSL-LSIY/+

Figure 2. Central tolerance to SIY is incomplete and functional SIY-reactive T cells can be transiently activated in the periphery. *A*. Representative plots of CD8 and BD DimerX SIY/H-2K^{b}:Ig stained whole lung cell suspensions from *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice 7 days after intranasal WSN-SIY Influenza A infection *(left)*. SIY/H-2K^{b}:Ig^{+} fraction of total CD8^{+} cells from WSN-SIY-infected lungs of *R26*$^{+/+}$ (n = 4, *open bar*) and *R26*$^{LSL\text{-}LSIY/+}$ (n = 4, *filled bar*) mice *(right)*. *Bars*, mean + s.e.m., p <0.005 by Student's *t* test. *B*. Representative plots of single cell suspensions from lungs *(top panels)* and mediastinal lymph nodes *(bottom panels)* of *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice 7 days post-WSN-SIY infection and uninfected controls. Cells were cultured and stimulated *in vitro* with 1μg/mL of SIY peptide and an IFN-γ capture assay was performed. CD8^{+}-gated cells are stained for CD107a and secreted IFN-γ. *C*. SIY-specific cytotoxicity in mediastinal lymph nodes of *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice 7 days after pulmonary WSN-SIY vaccination. Mean +/- s.e.m. specific cytolysis shown for each genotype. *D*. BD DimerX SIY/H-2K^{b}:Ig double-stained splenocytes from mice boosted with DC2.4.LSIY 5 days prior to analysis *(left)*. *R26*$^{+/+}$ *(left)* and *R26*$^{LSL\text{-}LSIY/+}$ *(middle)* mice were infected intranasally with WSN-SIY >30 days before boost or never infected *(right)*. Quantification of double-positive SIY/H-2K^{b}:Ig cells among splenocytes in *R26*$^{+/+}$ (n = 4, *open bar*) and *R26*$^{LSL\text{-}LSIY/+}$ (n = 3, *filled bar*) mice *(right)*. *Bars,* mean + s.e.m., p <0.05 by Student's *t* test.

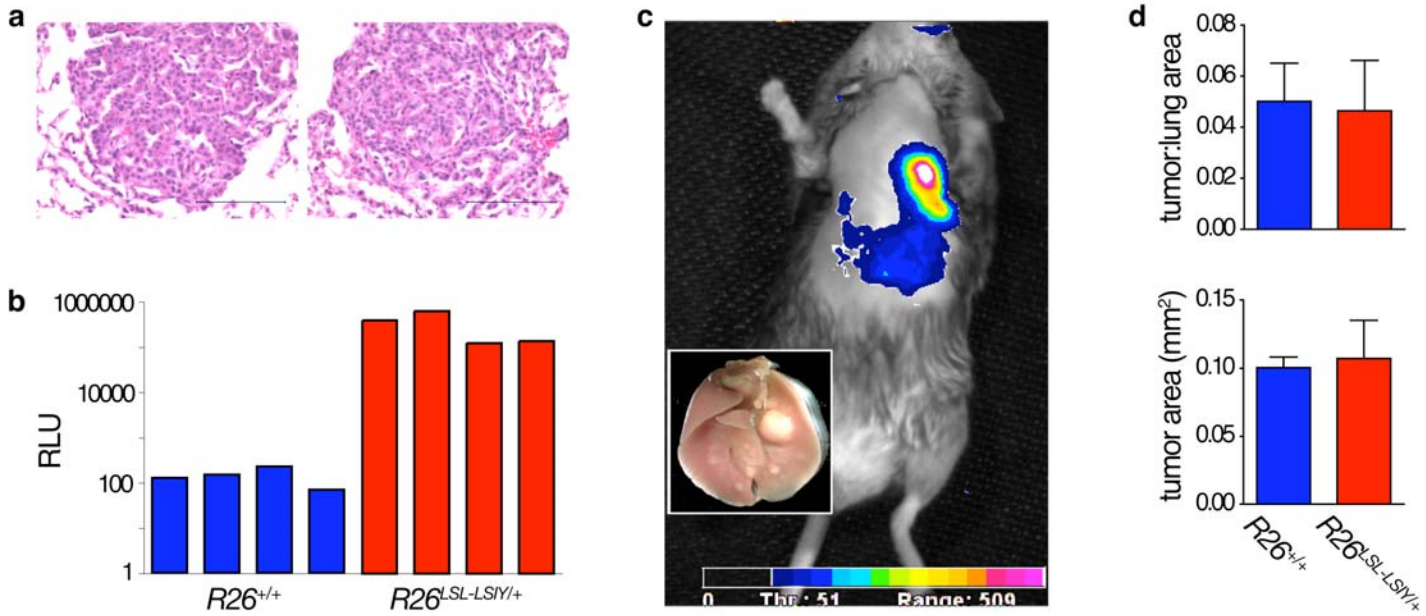


Figure 3. Antigen over-expressing tumors progress normally. *A*. Representative H&E-stained lung tumor histology from *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$ *(left)* and *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ *(right)* mice 12 weeks after intranasal Ad-Cre infection. *Scale bars*, 100μm. *B*. Luciferase activity of representative individual lung tumors dissected from *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$ *(blue bars)* and *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ *(red bars)* animals 16 weeks after intranasal Ad-Cre infection. *C*. *In vivo* bioluminescence imaging of a *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ mouse >6 months after intranasal Ad-Cre infection (*inset*: gross image of lungs after imaging). *D*. Quantification of lung tumor burden in *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$ (n = 3, *blue bars*) and *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ (n = 3, *red bars*) animals 12 weeks after intranasal Ad-Cre. *Bars,* mean + s.e.m., p = 0.89 and 0.83 for tumor-to-lung ratio and average tumor area by Student's *t* test.

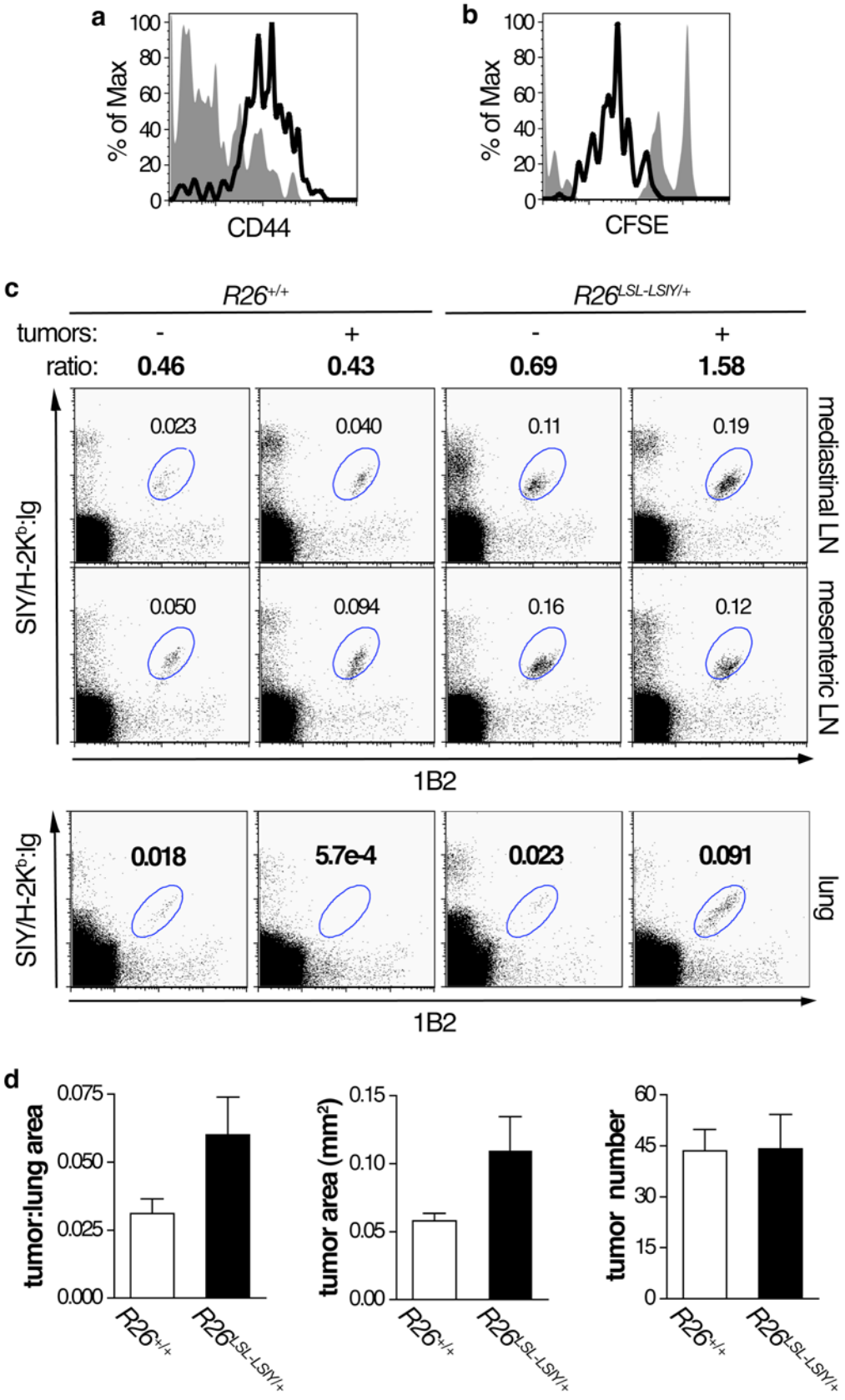

a
% of Max
CD44
b
% of Max
CFSE
c
R26+/+
R26LSL-LSIY/+
tumors:
ratio:
0.46
0.43
0.69
1.58
0.023
0.040
0.11
0.19
0.050
0.094
0.16
0.12
mediastinal LN
mesenteric LN
SIY/H-2Kb:Ig
1B2
0.018
5.7e-4
0.023
0.091
lung
d
tumor:lung area
tumor area (mm2)
tumor number
R26+/+
R26LSL-LSIY/+

Figure 4. Naïve 2C cells recognize, but do not response effectively to lung tumors. *A*. Representative plot of CD44 staining in $CD8^+1B2^+$ cells 5 days after naïve 2C cell transfer into *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ *(filled histogram)* and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ *(open histogram)* animals. *B*. Representative plot of CFSE dilution in $CD8^+1B2^+$ cells 3 days after naïve 2C cell transfer into *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ *(filled histogram)* and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ *(open histogram)* animals. *C*. Representative plots of 1B2 and BD DimerX SIY/H-$2K^b$:Ig stained cells recovered from lymph nodes of tumor-free and tumor-bearing *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice *(top)*. The ratio of $1B2^+$SIY/H-$2K^b$:Ig^+ cells in mediastinal to mesenteric lymph nodes is indicated. Double staining for 1B2 and DimerX enhances the signal-to-noise for 2C cells. Representative plots of whole lung cell suspensions from the same tumor-free and tumor -bearing *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice stained with 1B2 and BD DimerX SIY/H-$2K^b$:Ig *(bottom)*. *D*. Tumor-to-lung area in *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ (n = 9, *open bars*) and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ (n = 8, *filled bars*) animals 16 weeks after intranasal Ad-Cre infection *(left graph)*. Animals received naïve 2C cell i.v. transfer at either 4 weeks or 12 weeks after intranasal Ad-Cre. *Bars,* mean + s.e.m., p = 0.060 by Student's *t* test for tumor burden. Average area of tumors (p = 0.046 by Student's *t* test) in the same animals *(middle graph)* and average number of tumors per animal (p = 0.096 by Student's *t* test) *(right graph)*.

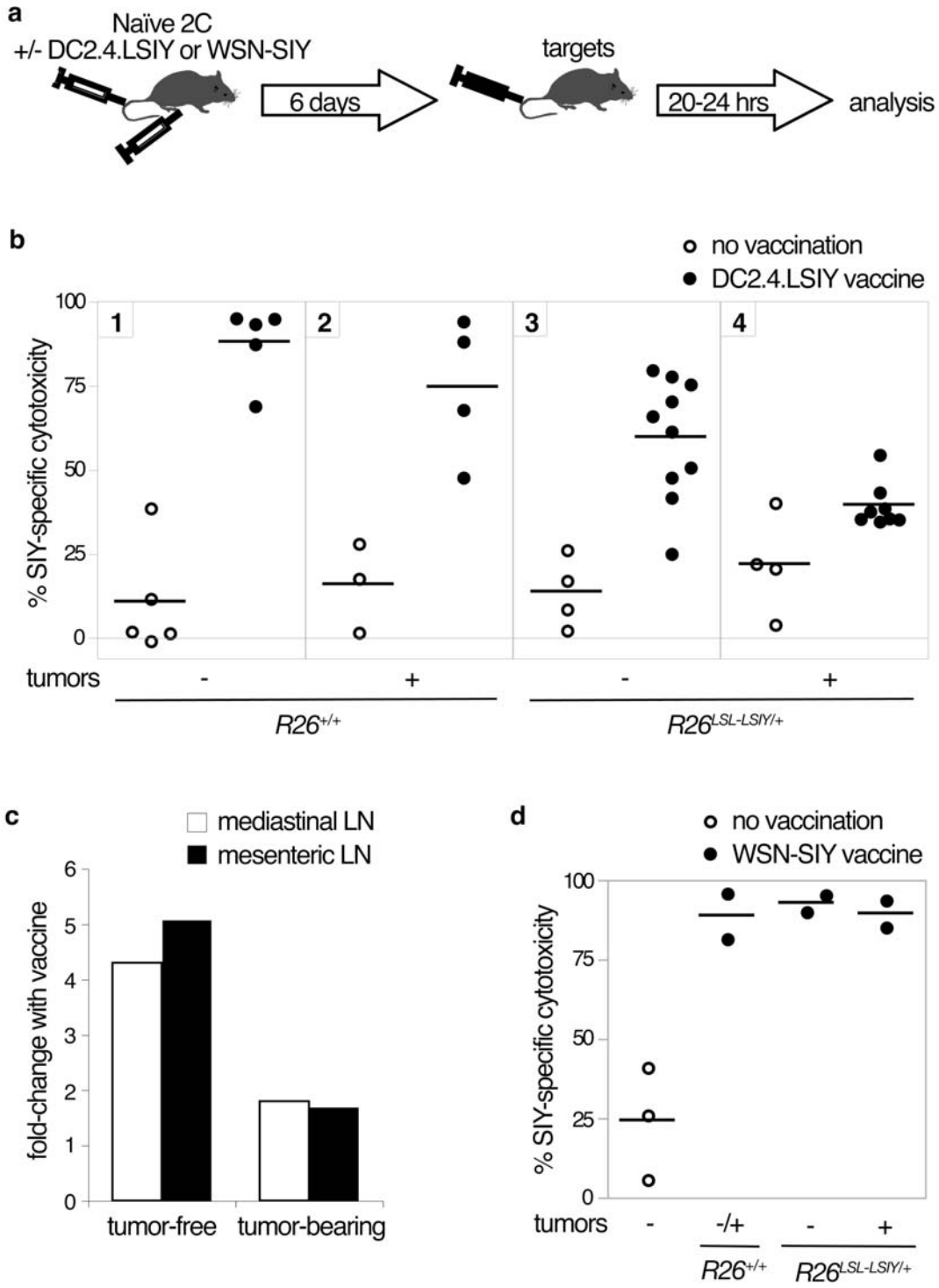

a
Naïve 2C
+/- DC2.4.LSIY or WSN-SIY
6 days
targets
20-24 hrs
analysis
b
no vaccination
DC2.4.LSIY vaccine
% SIY-specific cytotoxicity
100
75
50
25
0
1
2
3
4
tumors
-
+
-
+
R26+/+
R26LSL-LSIY/+
c
mediastinal LN
mesenteric LN
fold-change with vaccine
6
5
4
3
2
1
0
tumor-free
tumor-bearing
d
no vaccination
WSN-SIY vaccine
% SIY-specific cytotoxicity
100
75
50
25
0
tumors
-
-/+
-
+
R26+/+
R26LSL-LSIY/+

Figure 5. A higher threshold for co-stimulation is required in the presence of tumors over -expressing self-antigen. *A*. Schematic of experimental set-up for the *in vivo* cytotoxicity assay. Naïve 2C cells are intravenously injected into recipients with or without the addition of intraperitoneal DC2.4.LSIY or intranasal WSN-SIY vaccinations. Six days later, differentially -labeled $R26^{1LoxLSIY/+}$ target and $R26^{+/+}$ control cells are intravenously injected and animals are analyzed 20-24 hours later. *B*. SIY-specific cytotoxicity in mediastinal lymph nodes draining the lung 7 days after naïve 2C cell transfer into recipients with *(closed circles)* or without *(open circles)* DC2.4.LSIY vaccination. *C*. Fold change in SIY-specific cytotoxicity in mediastinal *(open bars)* and mesenteric lymph nodes *(filled bars)* of tumor-free and tumor -bearing $R26^{LSL-LSIY/+}$ mice when vaccinated with DC2.4.LSIY compared to unvaccinated mice. *D*. SIY-specific cytotoxicity in mediastinal lymph nodes 7 days after naïve 2C cell transfer into recipients with *(closed circles)* or without *(open circles)* WSN-SIY vaccination.

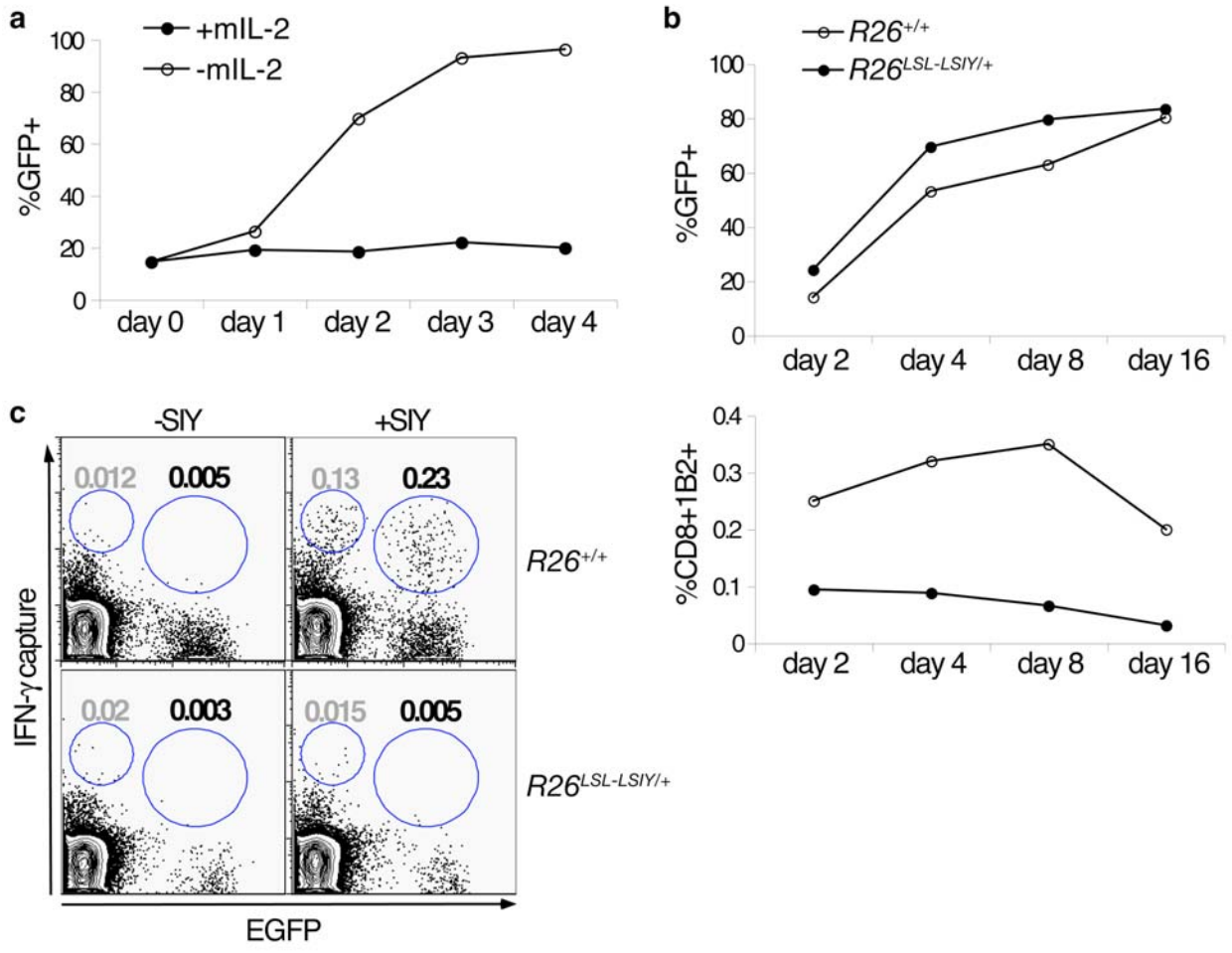


Figure 6. Inhibition of apoptosis results in T cell anergy in the presence of self-antigen. *A*. *In vitro* activated 2C cells infected with MIG-Bcl2 propagated in culture in the presence *(filled circles)* or absence *(open circles)* of survival cytokine IL-2. $CD8^+1B2^+$ cells were analyzed by flow cytometry for the percentage of $EGFP^+$ cells each day for 4 days after withdrawal of IL-2. *B*. *In vitro* activated 2C cells infected with MIG-Bcl2 intravenously transferred into either $R26^{+/+}$ *(open circles)* or $R26^{LSL\text{-}LSIY/+}$ *(filled circles)* recipient mice. Spleens were harvested and analyzed by flow cytometry at 2, 4, 8, and 16 days after transfer. $EGFP^+$ cells as a percentage of total $CD8^+1B2^+$ *(top panel)* and $CD8^+1B2^+$ cells as a percentage of total splenocytes *(bottom panel)*. *C*. Representative plots of $CD8^+$-gated splenocytes from $R26^{+/+}$ and $R26^{LSL\text{-}LSIY/+}$ mice 8 days after receiving MIG-Bcl2 infected activated 2C cells. Cells were stimulated *in vitro* with 1μg/mL of SIY peptide *(right panels)* or unstimulated *(left panels)* and an IFN-γ capture assay was performed. Population gates represent percentage of $EGFP^-$ IFN-γ-secreting *(gray gate values)* and $EGFP^+$ IFN-γ-secreting *(black gate values)* $CD8^+$ splenocytes.

DISCUSSION

We have described a novel system that allows inducible expression of a defined antigen in mice to mimic tumor-associated antigens (TAAs) in human cancer. We have characterized T cells reactive to the SIY TAA in lung adenocarcinoma and uncovered multiple levels of immune tolerance that limit an effective response against TAAs (model depicted in Figure S1). Due to low-level antigen expression, SIY-reactive T cells were subjected to central tolerance, eliminating the majority of potentially tumor- and self-reactive T cells during development. In the periphery, SIY-reactive cells that escaped thymic selection died soon after activation, likely a mechanism to limit auto-reactivity. Self-reactive T cells that were transiently blocked from apoptosis became anergic. Finally, in mice bearing autochthonous SIY-over-expressing lung tumors, strong immunological stimuli were required to activate transferred 2C cells to become highly cytotoxic toward targets. This is noteworthy because a weaker vaccine was able to impart cytotoxic activity on self-reactive T cells only in the absence of SIY-over-expressing tumors. Thus, in the context of a tumor-associated antigen, reactive T cells must overcome developmental negative selection, peripheral self-tolerance, and tumor-induced inhibition as barriers to effective anti-tumor immunity.

Our data suggest that neither prophylactic nor therapeutic vaccines to TAAs will be effective in preventing or treating lung cancer. Protection provided by prophylactic vaccination against an antigen relies on formation and maintenance of memory cells that can respond efficiently to tumors bearing that antigen (Lollini et al., 2006). In $R26^{LSL\text{-}LSIY}$ animals, where SIY is a self-antigen, we have demonstrated that SIY-reactive T cells do not persist and cannot result in immunological memory or protection to SIY (Figure 2D; data not shown). In contrast, when

targeting antigens to which the host is not tolerant, prophylactic vaccines protect against even large numbers of potentially tumorigenic cells (Willimsky and Blankenstein, 2005).

Therapeutic vaccines boost immune responses to tumor antigens in patients with established cancers (Lollini et al., 2006). To be effective, this requires both the presence of antigen-specific T cells and the ability to properly activate these cells. Based on our model, both elements are hampered in the context of TAAs. We have shown that central tolerance results in significant diminution of SIY TAA-specific T cells (Figure 1C,D). Additionally, we have demonstrated that DC2.4.LSIY vaccination yields only marginal induction of SIY-specific cytotoxicity in mice bearing tumors over-expressing SIY (Figure 5). Furthermore, although WSN-SIY can endow 2C cells with high cytotoxicity, activated 2C cells are anergized and die in the presence of self-antigen (Figure 2D; Figure 6). Our data are consistent with observations from human trials in which therapeutic vaccines have resulted in only rare cases of objective clinical response (Rosenberg et al., 2004). Importantly, therapeutic vaccines may still be effective in combination with other therapeutic strategies (Schlom et al., 2007).

Immune ignorance of tumors, immune avoidance by tumors, and active suppression of anti-tumor immunity are the prevailing explanations for defective T cell responses in cancer (Dunn et al., 2004; Pardoll, 2003; Rabinovich et al., 2007). Loss of antigen expression or presentation is often described as a means of immune avoidance (Algarra et al., 2004; Khong and Restifo, 2002). We have demonstrated sustained LSIY expression in lung cancer, even at advanced stages (Figure 3B,C). Moreover, we have shown that antigen presentation was not different between $R26^{LSL\text{-}LSIY/+}$ lung tumor cell lines and controls (Figure S5). Furthermore, we have demonstrated the inability of 2C cells to efficiently kill non-malignant cells expressing high

SIY in SIY-over-expressing tumor-bearing hosts (Figure 5). These data exclude immune avoidance by tumors as a major contributor to deficient anti-tumor T cell responses.

We cannot exclude the possibility of immune ignorance playing a role in early stages of tumorigenesis, but our data argue for active tumor-induced immune suppression. Inefficient 2C cell killing in mice bearing SIY-over-expressing tumors was observed even with coincident antigen presentation and costimulation provided by DC2.4.LSIY (Figure 5). This treatment is normally immune-activating and, moreover, is capable of overcoming self-tolerance in tumor-free $R26^{LSL\text{-}LSIY/+}$ mice. This implies that even if tumors are naturally ignored in our model, tumor-induced T cell suppression can dominate over immune-stimulatory signals.

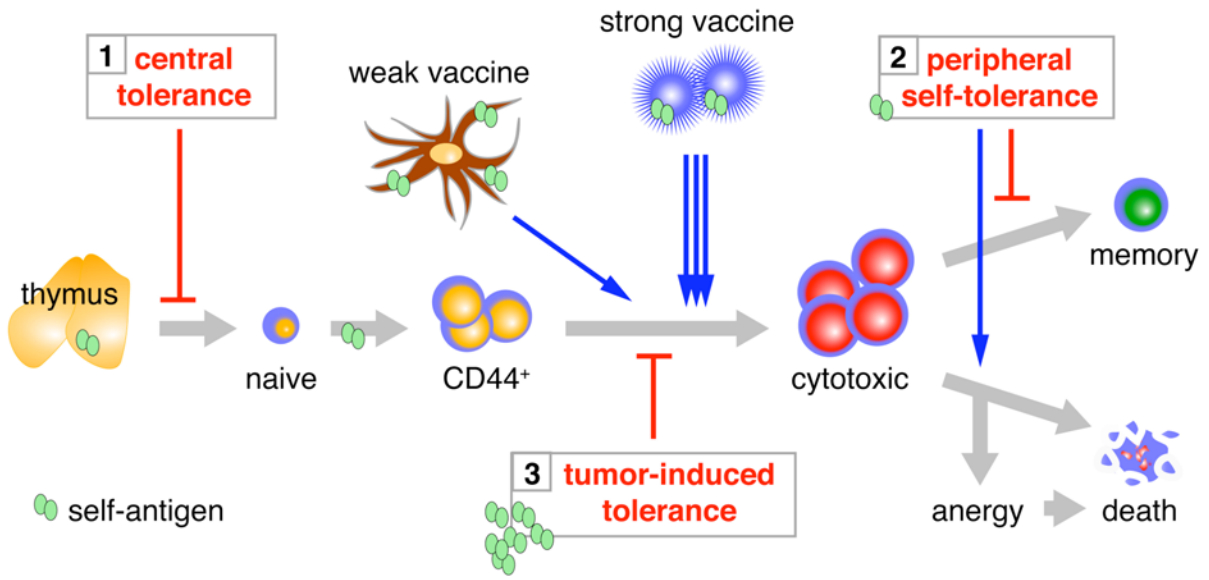
There are many mechanisms by which SIY-reactive T cells may be suppressed in $K\text{-}ras^{LSL\text{-}G12D/+};R26^{LSL\text{-}LSIY/+}$ mice (Rabinovich et al., 2007; Zitvogel et al., 2006). Suppression can occur by contact with tumors or by intermediaries, but since we observe systemic antigen-specific T cell suppression, direct contact between T cells and tumors is unlikely to be the dominant mechanism. Tumor suppression by intermediaries may involve cellular and/or soluble inhibitors of T cell cytotoxicity. These factors may be present normally to limit self-reactivity, but are increased in animals with self-antigen over-expressing tumors and thus induce greater suppression of antigen-reactive T cells. Consistent with this idea, we have shown that high levels of costimulation provided by WSN-SIY were required to effectively induce cytotoxicity in 2C cells (Figure 5).

We immunophenotyped animals to screen for potential cellular mediators of T cell suppression. Two putative immunosuppressive populations, $Gr1^{+}CD11b^{+}$ myeloid suppressor cells and $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells, were reproducibly expanded in lungs and lymphoid tissues of tumor-bearing mice relative to tumor-free mice (Figure S6). Interestingly,

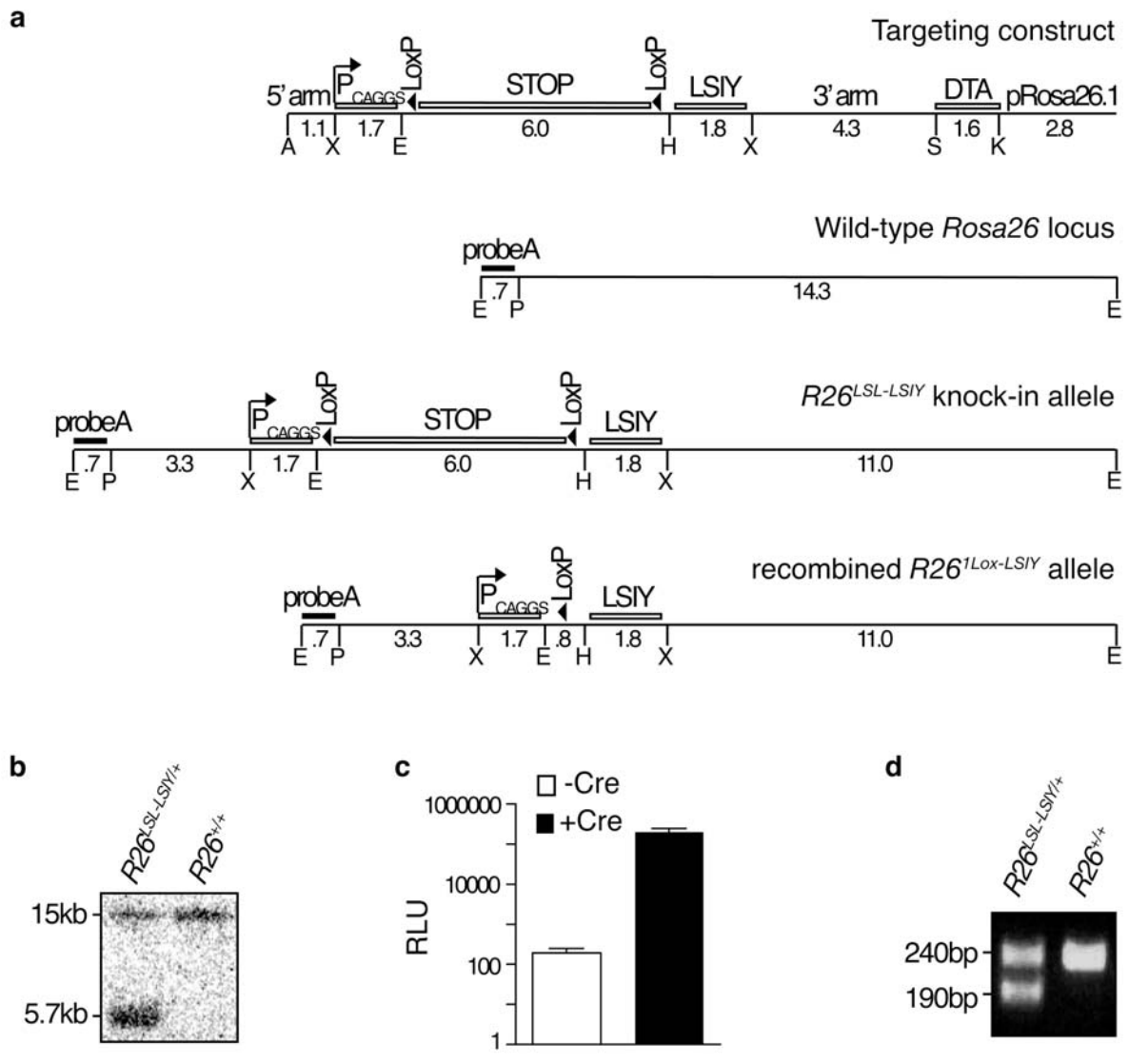
both immune cell types are overrepresented in NSCLC patients and appear to mediate systemic immune suppression (Almand et al., 2000; Perrot et al., 2007; Woo et al., 2001). Further studies are needed to determine if either of these cell types are playing a causal role in tumor-induced T cell suppression in our model.

Melanoma, ovarian and prostate cancers are more often treated with immunotherapy in the clinic than other cancers. This may reflect an inherent susceptibility of these cancers to immune attack or simply our limited experience in using the immune system to treat other cancers. Our observations of tolerance in the presence of tumors are similar to those made in TRAMP mice in which prostate-specific expression of SV40 TAg leads to spontaneous prostate cancer (Anderson et al., 2007; Drake et al., 2005). In contrast to our model, however, priming with a DC vaccine yielded more effective T cells that significantly reduced tumor burden in TRAMP mice (Anderson et al., 2007). Interestingly, T cell tolerance was not observed in a mouse model of insulinoma also driven by SV40 TAg (Nguyen et al., 2002). To learn more about tumor-immune interactions, it will be important to compare immune responses among different cancer types and even among tumors bearing different oncogenic alterations as particular pro-oncogenic events have been described to specifically modulate immune responses (Zitvogel et al., 2006).

Numerous Cre-regulated oncogenes and tumor suppressor genes have been generated in the context of human cancer models (Frese and Tuveson, 2007). Because $R26^{LSL\text{-}LSIY}$ is expressed ubiquitously and independent of oncogenic events, our model tumor antigen can be studied in several cancer models to gain greater understanding of context-dependent effects on anti-tumor immunity. Data derived from these studies will help define the best strategies to successfully stimulate the immune system to recognize and eliminate cancers of different origins.



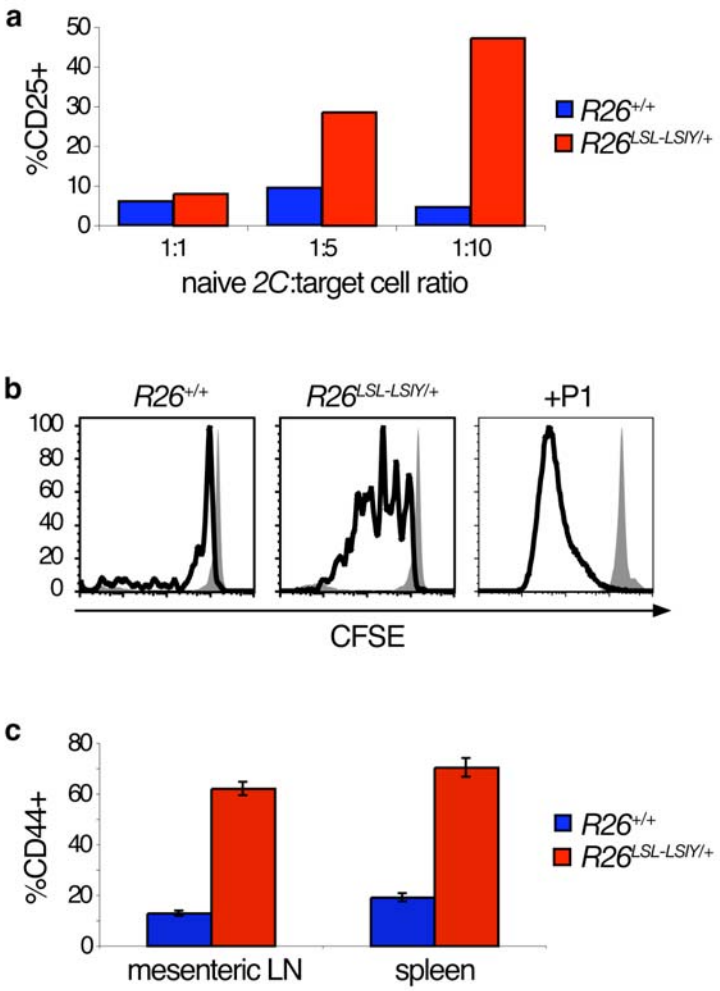
Supplementary Figure 1. Tumor reactive T cells face multiple levels of tolerance. We have uncovered multiple levels of immune tolerance that limit an effective immune response to tumor-associated antigens in lung cancer. *1*. Due to low level self-antigen expression, endogenous SIY-reactive T cells are subjected to central tolerance, eliminating the majority of potentially tumor- and self-reactive T cells during thymic development. In the periphery, SIY-reactive T cells that escape thymic selection do not become functionally activated without costimulation. *2.* Those T cells that are properly co-stimulated and activated are deleted by some mechanism of peripheral self-tolerance as these cells do not persist to maintain a memory population. If death is transiently blocked, persisting self-reactive T cells are anergized. *3*. In the presence of lung tumors over-expressing self-antigen, reactive T cells are not functionally activated and have a higher threshold for co-stimulation to become cytotoxic. Thus, in the context of a tumor-associated antigen, TAA-reactive T cells must overcome developmental negative selection, peripheral self -tolerance, and tumor-induced inhibition as barriers to effective anti-tumor immunity.



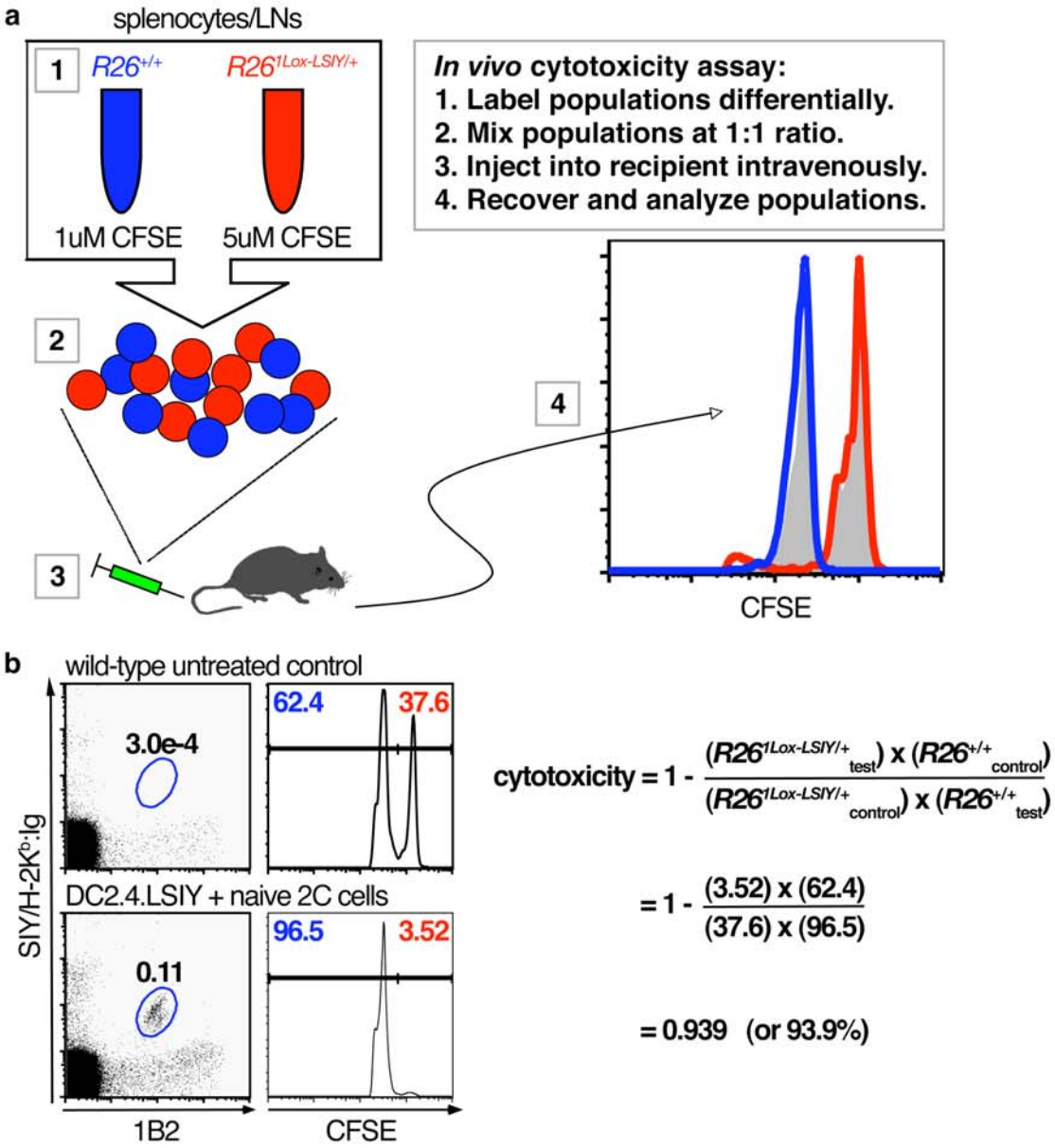
Supplementary Figure 2. Generation of the *R26*$^{LSL\text{-}LSIY}$ mouse. *A*. Restriction endonuclease maps of the pRosa26-Lox-STOP-Lox-Luciferase-SIY targeting construct, the wild-type *Rosa26* genomic locus, the unrecombined *R26*$^{LSL\text{-}LSIY}$ knock-in allele, and the recombined *R26*$^{1Lox\text{-}LSIY}$ allele. Note the hybridization position of Southern blot probe A. Size markers are in kilobases (kb). Restriction sites are as follows: A=*AscI*, E=*EcoRI*, H=*HindIII*, K=*KpnI*, P=*PacI*, S=*SalI*, and X=*XbaI*. *B*. Southern blot using radiolabeled probe A hybridized to correctly-targeted *R26*$^{LSL\text{-}LSIY}$ (5.7kb) and untargeted *R26*$^{+/+}$ (15kb) ES clone DNA after EcoRI digest. *C*. Luciferase activity in ES clones carrying *R26*$^{LSL\text{-}LSIY}$ allele transfected with pEGFP control plasmid (n = 3, *open bar*) or pOGEG Cre-expression plasmid (n = 3, *filled bar*). *Bars*, mean + s.e.m., p <0.01 by Student's *t* test. *D*. PCR genotyping of tail tip genomic DNA for *R26*$^{LSL\text{-}LSIY}$ (190bp) and *R26*$^{+}$ wild-type (240bp) alleles.

Reagent	Description	Reference
CTL clone 2C	Allo-MHC-specific cytotoxic T lymphocyte (CTL) clone from a H-2^{b} mouse	Saito et al 1984
1B2 mAb	Clonotypic antibody specific for the 2C TCR	Kranz et al Jan 1984; Kranz et al Dec 1984
2C TCR-tg mice	Mice expressing the $\alpha\beta$ antigen receptor from CTL clone 2C	Sha et al 1988
SIY peptide	Synthetic peptide SIYRYYGL, cross-reacts with 2C TCR in complex with H-2K^{b}	Udaka et al 1996
DimerX H-2K^{b}:Ig	Recombinant protein that can be loaded with SIY to detect SIY-specific T lymphocytes	BD Biosciences
WSN-SIY	*Influenza A* virus (WSN) engineered to express SIY peptide	Li et al 1993; Shen et al 2008
DC2.4.LSIY	Immortalized Dendritic cell line (DC2.4) transduced to express Luciferase-SIY	Shen et al 1997; DuPage and Jacks, unpublished
KP/LSIY cell lines	Cell lines derived from *K-ras$^{1Lox\text{-}G12D/+}$; p53$^{1Lox/1Lox}$;R26$^{1Lox\text{-}LSIY/+}$* lung tumors	Jackson et al 2005; Cheung and Jacks, reported here

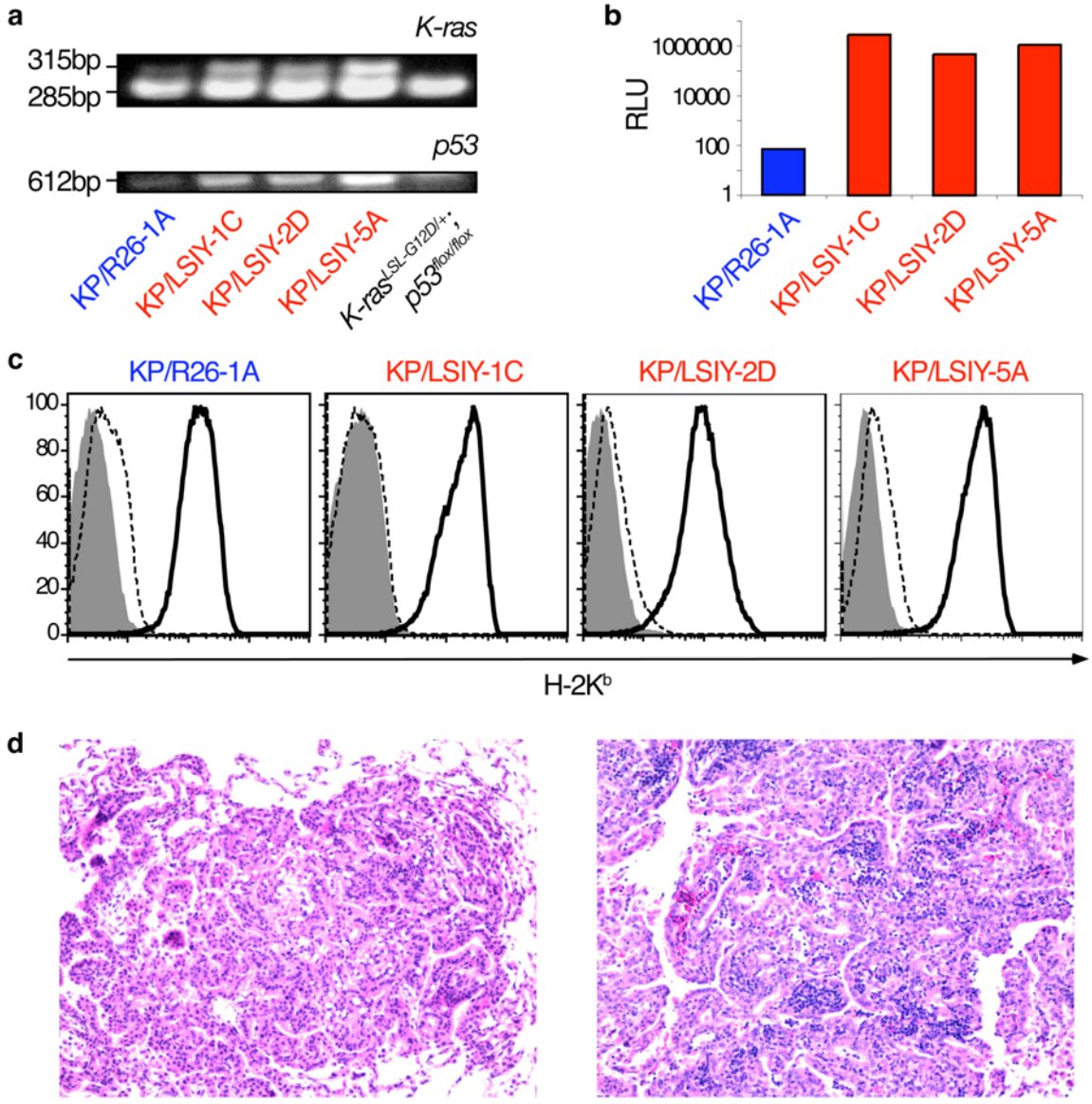
Supplementary Table 1. Reagents complementary to the *R26$^{LSL\text{-}LSIY}$* mouse. SIYRYYGL (or SIY) is a well-characterized CD8^{+} T cell antigen with many complementary reagents that make this system particular tractable to use. Cytotoxic T lymphocyte clone 2C and T cells from *2C* T cell receptor (TCR) transgenic mice recognize SIY antigen bound to H-2K^{b} complexes. The 1B2 monoclonal antibody specifically recognizes the 2C TCR, whereas the DimerX H-2K^{b}:Ig reagent loaded with SIY peptide can bind to all T cells that recognize SIY/H-2K^{b}. Double staining with 1B2 and SIY-loaded DimerX increases the signal-to-noise ratio for flow cytometric analysis. The WSN-SIY virus, DC2.4.LSIY cell line, and KP/LSIY cell lines are described in this paper.



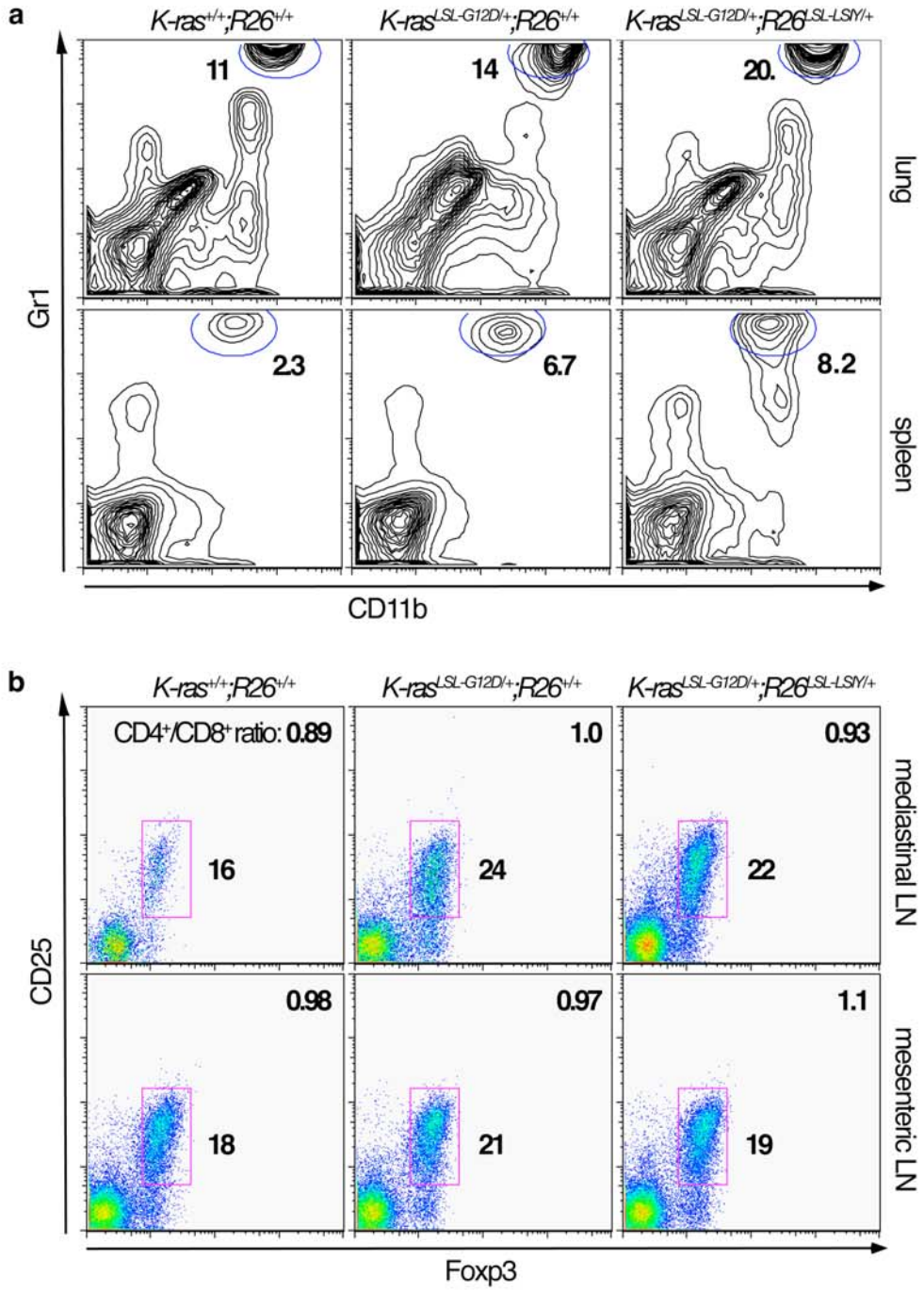
Supplementary Figure 3. 2C cells are activated by low level SIY expression. *A*. Percentage of $CD8^{+}1B2^{+}$ cells staining positive for the CD25 activation marker after 4 days of co-culture at 1:1, 1:5, and 1:10 naive 2C cell to target cell ratios with $R26^{+/+}$ *(blue bars)* and $R26^{LSL\text{-}LSIY/+}$ *(red bars)* splenocytes. *B*. Representative CFSE dilution in $CD8^{+}1B2^{+}$ cells after 1 day *(filled histograms)* and 4 days *(open histograms)* of co-culture with 10-fold excess $R26^{+/+}$ splenocytes *(left panel)*, co-culture with 10-fold excess $R26^{LSL\text{-}LSIY/+}$ splenocytes *(middle panel)*, and SIY peptide stimulation *(right panel)*. *C*. Percentage of $CD8^{+}1B2^{+}$ cells recovered from mesenteric lymph nodes and spleen that stain positive for the CD44 activation marker 4 days after transfer of naive 2C cells into $R26^{+/+}$ *(blue bars)* and $R26^{LSL\text{-}LSIY/+}$ *(red bars)* mice.



Supplementary Figure 4. *In vivo* cytotoxicity assay. *A*. Control *R26*$^{+/+}$ and target *R26*$^{1Lox\text{-}LSIY/+}$ splenocyte/lymph node primary cell suspensions were differentially-labeled with CFSE and mixed at a 1:1 viable cell ratio. These cells were then injected intravenously into recipient animals and harvested 20-24 hours later to be analyzed for the recovery efficiency of each CFSE-labeled population. *B*. As an example of the *in vivo* cytotoxicity assay, an untreated wild-type control mouse and a mouse that received naïve 2C cells and DC2.4.LSIY vaccine 7 days prior to analysis were assayed. The recovered cells were used to determine the degree of SIY-specific (*R26*$^{1Lox\text{-}LSIY/+}$) cytotoxicity compared to non-specific (*R26*$^{+/+}$) cytotoxicity in recipient mice. The equation normalizes the recovered populations in the test mouse to the populations recovered from the control mouse to account for differential survival of the two donor-derived populations.



Supplementary Figure 5. SIY antigen presentation is maintained in lung tumors. *A*. Cell lines were derived from lung tumors originating from Ad-Cre-infected *K-ras*$^{LSL\text{-}G12D/+}$;*p53*$^{fl/fl}$;*R26*$^{+/+}$ and *K-ras*$^{LSL\text{-}G12D/+}$;*p53*$^{fl/fl}$;*R26*$^{LSL\text{-}LSIY/+}$ mice. We used p53 deficient tumors as p53 proficient tumors do not seed cell lines efficiently. PCR genotyping for recombined *K-ras*$^{1Lox\text{-}G12D}$ (*upper panel*, 315bp), *K-ras*$^{+}$ (*upper panel*, 285bp) and recombined *p53*^{1Lox} (*lower panel*, 612bp) alleles. Control *K-ras*$^{LSL\text{-}G12D/+}$;*p53*$^{flox/flox}$ DNA has neither of the recombined alleles. *B*. Luciferase activity in individual cells lines: *R26*$^{+/+}$ *(blue bar)* and *R26*$^{1Lox\text{-}LSIY/+}$ *(red bars)*. *C*. Unstained *(filled histogram)* or H-2K^{b} stained cells lines untreated *(dashed line, open histogram)* or treated with 100U IFN-γ *(solid line, open histogram)*. *D*. Representative H&E-stained lung tumor sections from *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$;*2C (left)* and *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$;*2C (right)* mice 10 weeks post-Ad-Cre infection.



Supplementary Figure 6. Potential mediators of tumor-induced T cell suppression. *A*. CD11b (Mac1) and Gr1 (Ly-6G) staining of single cell suspensions from lung and spleen of representative tumor-free *(left panels)*, tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ *(middle panels)*, and tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ *(right panels)* mice. *B*. CD25 and intracellular Foxp3 staining of CD4$^+$ cells in the mediastinal and mesenteric lymph nodes of mice in *a*. The ratios of CD4$^+$ to CD8$^+$ lymphocytes are indicated.

METHODS

Mice. *2C* mice were provided by J. Chen, *Trp53*^{flox} mice by A. Berns, and *Meox-Cre* and *Rag2*$^{-}$ mice were purchased from Jackson Laboratories. *K-ras*$^{LSL-G12D}$ mice were generated in our laboratory (Jackson et al., 2001; Tuveson et al., 2004). *R26*$^{LSL-LSIY}$ mice and controls used were either pure 129S4/SvJae or enriched on C57BL/6 for 3-13 generations. Donor 2C cells for transfer experiments were from pure C57BL/6 *2C;Rag2*$^{-/-}$ mice and recipients were always C57BL/6 enriched. To induce tumors, mice were infected with Adenovirus-Cre intra-nasally as previously described (Jackson et al., 2001). WSN-SIY was provided by J. Chen and infections were done by intra-nasal instillation similar to Adenovirus-Cre. For tumor studies, lung histology was prepared and analyzed by Bioquant Image Analysis software as previously described (Jackson et al., 2005). Animal studies were approved by MIT's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

Cells. MEFs were derived from e13.5 embryos and grown as previously described (Tuveson et al., 2004). KP/KPLSIY cell lines were derived from *K-ras*$^{LSL-G12D/+}$*;p53*$^{flox/flox}$ mice (Jackson et al., 2005) 16 weeks post-Ad-Cre. Individual tumors were plucked from lungs, minced, trypsinized and cultured in MEF growing media until lines were established. DC2.4.LSIY cells were generated by infection of DC2.4 cells (Shen et al., 1997) with a lentivirus bearing *Luciferase-SIY* and selection of a single positive clone. *In vitro* activated 2C cells were made by stimulating *2C* splenocytes/lymphocytes suspensions with 1μg/mL SIY in DMEM-10 full

medium overnight, then culturing with 10ng/mL mIL-2 (R&D Systems cat#402-ML) for 6 days. Bcl2 over-expression was achieved by spin-infecting *in vitro* activated 2C cells twice (at days 3 and 4 of activation) with a pMSCV-IRES-GFP retrovirus carrying Bcl2, prepared as described (Schmitt et al., 2000).

Luciferase detection. Cells were lysed with Passive Lysis Buffer (Promega cat#E1941) and assayed with Luciferase Assay Reagent (Promega cat#E1501) according to manufacturer's instructions. Tissues and tumors were mechanically disrupted before lysis and tissue debris was pelleted before assay. *In vivo* bioluminescence imaging was performed on a NightOWLII LB983 (Berthold Technologies). Mice were shaved and intra-peritoneally injected with Beetle Luciferin (Promega cat#E1602) within 20 minutes prior to imaging.

Cell transfer and DC vaccination. Single-cell suspensions from lymph nodes and spleens of *2C;Rag2*$^{-/-}$, *R26*$^{1Lox\text{-}LSIY/+}$ and *R26*$^{+/+}$ mice were used for 2C transfer, *in vivo* cytotoxicity assay's target and control cells, respectively. Cells were counted by hemocytometer, resuspended in RPMI and transferred into recipients via tail vein. For CFSE-labeling, cells were resuspended at 4-6x10^6 cells/mL RPMI with 5% fetal calf serum containing 5μM (or 1μM for control cells in *in vivo* cytotoxicity assay) CFSE (Molecular Probes cat#C1157) for 10 minutes at 37^{o}C. Cells were then washed twice in RPMI with 5% fetal calf serum, followed by washes in serum-free RPMI before intravenous transfer into recipients. 3x10^6 cells were injected for naïve 2C transfer, 12x10^6 cells for *in vitro* activated 2C, and 6x10^6 cells each of CFSE-labeled target and control *in vivo* cytotoxicity cells. DC2.4.LSIY cells were washed and resuspended in RPMI for intra-peritoneal vaccination with 5x10^5 cells/mouse.

Cell isolation. Single-cell suspensions from lymph nodes, spleen and thymus were generated by mechanical disruption. For lung preparations, tissue samples were minced and digested at 37^{o}C for 30 minutes in 125U/mL of collagenase-typeI (Gibco) in phosphate-buffered saline before mechanical disruption and passage through a 70μm-pore filter (BD Falcon). Peripheral blood was collected by tail tip bleeds into 40μL of 50mM EDTA to prevent coagulation. Red blood cells were lysed with an aqueous solution containing 0.83% NH_4Cl, 0.1% $KHCO_3$, and 0.000037% Na_2EDTA at pH 7.2-7.4. Single-cell suspensions were passed through a 35μm-pore cell-strainer cap (BD Falcon) before culture, intravenous transfer, or staining for flow cytometry.
Reagents and flow cytometry. SIYRYYGL peptide was synthesized by MIT's Biopolymers Laboratory. Recombinant murine Interferon-γ (cat#315-05) was purchased from PeproTech Inc. The following antibodies were purchased from BD Pharmingen: αCD4 (H129.19), αCD8 (53-6.7), αCD11b (M1/70), αCD16/CD32 (2.4G2), αCD25 (7D4 and PC61), αCD44 (IM7), αCD69 (H1.2F3), αCD107a (1D4B), αGr-1 (RB6-8C5), αH-2K^{b} (AF6-88.5), and αIgG$_1$ (X56). Phycoerythrin-conjugated and unconjugated DimerX I reagents were purchased from BD Pharmingen and prepared according to manufacturer's instructions. Intracellular Foxp3 staining was performed with FITC-α-mouse/rat Foxp3 staining set from eBioscience and secreted IFN-γ capture/detection was performed with Mouse IFN-γ Secretion Assay Detection kit (order#130-090-516) from Miltenyi Biotec, according to manufacturers' instructions. Biotinylated 1B2 monoclonal antibody was provided by J. Chen. Streptavidin-allophycocyanin and streptavidin-phycoerythrin conjugates were purchased from BD Pharmingen. Cells were read on a FACSCalibur (BD Biosciences) and analyzed using Flowjo 8.1 software (Tree Star Inc). Dead cells were excluded by 1μg/mL propidium iodide staining (Sigma).

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CHAPTER 3:

Adoptive cell transfer therapy against tumors over-expressing a self-antigen breaks tolerance without inducing autoimmunity

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The author performed all of the experiments described. All experiments were performed in the laboratory of Tyler Jacks.

ABSTRACT

Adoptive cell transfer (ACT) therapy for cancer has demonstrated efficacy in clinical trials, particularly for the treatment of metastatic melanoma. There is great potential in broadening the application of ACT to treat other cancer types, but the threat of severe autoimmunity may limit its use. Immunosuppressive agents can be used to reduce autoimmune side effects, but this would undoubtedly also diminish immune reactivity against tumors. Studies in model systems have demonstrated successful induction of anti-tumor immunity against self-antigens without detrimental effects on normal somatic tissue. These results are likely due to preferential recognition of tumor over normal tissue by activated T cells. We previously described the generation of a mouse model system in which a defined self-antigen is highly over-expressed in cancer. Here, we applied adoptive T cell transfer therapy combined with lymphodepletion pre-conditioning to treat autochthonous lung tumors in this system. With this treatment, we can overcome peripheral tolerance, successfully inducing large number of functional anti-tumor T cells. Furthermore, these T cells are able to effect lung tumor over-expressing self-antigen. Importantly, despite large numbers of potentially self-reactive T cells, we do not observe overt autoimmunity. In addition, we have potentially identified LAG-3 as an additional factor that serves to limit anti-tumor T cell activity.

INTRODUCTION

Cancer immune therapy highlights new potential in the treatment of cancer. In particular adoptive cell transfer (ACT) therapy has received great attention due to its success in treating some patients with metastatic melanoma (Dudley et al., 2005). There is, however, a strong correlation between autoimmunity and the effectiveness of immune therapy against cancer (Yee et al., 2000). When lineage-specific differentiation antigens are targeted by cytotoxic T lymphocytes (CTLs), as in ACT therapy for melanoma and prostate cancer, self-reactivity is tolerable. On the other hand, autoimmunity against essential tissues, such as to lung or pancreas, would not be acceptable. Thus, to expand the applicability of immune therapy to cancer of vital organs, we must learn more about the rules governing the balance between anti-tumor immunity and autoimmunity.

Effective immune therapy against self-antigens in cancer has been demonstrated without resulting in autoimmunity in some animal models (Eck and Turka, 2001; Morgan et al., 1998; Vierboom et al., 1997). In other cases, detrimental autoimmunity has resulted (Ludewig et al., 2000). Whether or not autoimmunity is induced likely depends on the features of the therapy as well as the differential specificity of target antigens for tumors versus somatic tissue. For example, non-specific immune modulation, such as with the use of anti-CTLA-4 blocking antibodies, results in autoimmune symptoms in patients at high frequencies (Attia et al., 2005; Peggs et al., 2008). Furthermore, self-antigens that are highly over-expressed in cancer, but have relatively low expression in somatic tissue, such as peptides from p53 and cyclin B1, are attractive targets for tumor antigen-directed immune therapy (Egloff et al., 2006; Theobald et al., 1995).

Immune therapy has been used successfully to slow the growth and even eradicate established tumors in transplant models (Hanson et al., 2000; Overwijk et al., 2003). Conversely, autochthonous tumors have proven more difficult to treat and reports of successful immune therapy against established lesions are rare (Hurwitz et al., 2000; Nguyen et al., 2002). Transplanted cells are commonly injected under the skin, an organ that is surveyed by Langerhans cells, a dendritic cell subset described to be relatively potent at activating immune responses (Merad et al., 2008). Furthermore, tumor cell injection results in tissue damage, death to transplanted cells, and an acute burden of tumor antigens that is likely to be highly immunogenic. Moreover, transplanted tumors are unlikely to possess a realistic tissue microenvironment due to their ectopic location and rapid growth rate, which can influence immune therapy. Thus, to better recapitulate the state of immune tolerance that exists in human cancer, immune therapy necessitates evaluation in autochthonous cancer.

Previously, we described a novel allele $R26^{LSL\text{-}LSIY}$ that allows inducible expression of a defined antigen SIYRYYGL (SIY) in mice to mimic an over-expressed self-antigen in human cancer (Cheung et al., 2008). In the context of autochthonous lung cancer, SIY-reactive T cells encounter multiple barriers to effective anti-tumor immunity. Here, we investigated adoptive T cell transfer with lymphodepletion pre-conditioning, an essential component for successful therapy, in $R26^{LSL\text{-}LSIY}$ mice (Dudley et al., 2002; North, 1982). We examined the ability of lymphodepletion to support transferred T cells reactive to a self-antigen and we determined the capacity of these T cells to influence self-antigen over-expressing tumors. Furthermore, we explored the conditions governing heterogeneous response to immune therapy.

RESULTS

Established B16-LSIY tumors can be controlled without overt autoimmunity

B16-F10 melanoma, a poorly immunogenic cell line syngeneic to C57BL/6 mice, expresses a number of melanocyte differentiation antigen genes and has been used widely as an experimental tumor model. Adoptive transfer of pmel-1 transgenic cytotoxic T lymphocytes (CTLs) specific for melanocyte antigen $gp100_{25\text{-}33}$ causes regression of established B16-F10 tumors and treatment is enhanced in mice with prior non-myeloablative immunodepletion (Gattinoni, 2005; Overwijk et al., 2003). CTL-mediated tumor killing, however, is associated with autoimmune melanocyte destruction in the skin and eye (Overwijk et al., 2003). Although autoimmunity to tumor differentiation antigens in non-essential tissues can be tolerated, autoimmune reactivity to self-antigens expressed ubiquitously in vital organs can be fatal. Thus, we wanted to determine if we could achieve effective anti-tumor immunity against a self-antigen over-expressed in tumors without fatal autoimmunity in mice.

First, we assessed if transplanted B16-F10 cells could be controlled in mice receiving lymphodepleting radiation followed by adoptive transfer of activated T cells specific for a tumor over-expression antigen as was demonstrated with transfer of T cells reactive to melanocyte differentiation antigens (Gattinoni, 2005). SIY is an H-$2K^b$-restricted self-antigen expressed ubiquitously at low levels in $R26^{LSL\text{-}LSIY}$ mice (Cheung et al., 2008). We generated a SIY over-expressing B16-F10 line B16-LSIY and a control line B16-0 by retroviral infection of B16-F10 cells with a MSCV-based vector expressing Luciferase-SIY (LSIY) or an empty vector, respectively, followed by drug selection. B16-LSIY cells exhibit high-level luciferase activity that is decreased by approximately 10-fold after subcutaneous *in vivo* passage (Figure S1). B16-LSIY cells have equally reduced luciferase after 19 days in either $R26^{+/+}$, $R26^{LSL\text{-}LSIY/+}$, or

R26$^{1Lox\text{-}LSIY/+}$ hosts suggesting that LSIY, the puromycin resistance gene product, or both are immunogenic in the context of a transplanted cell line despite peripheral self-tolerance in *R26*$^{LSL\text{-}LSIY/+}$ and *R26*$^{1Lox\text{-}LSIY/+}$ mice. Regardless of the reduction of LSIY expression in these lines, *in vivo* passaged B16-LSIY lines still retain considerable reporter expression and so the parental lines were, therefore, utilized further.

Animals were challenged subcutaneously with $2x10^5$ B16-LSIY or B16-0 cells and tumors were allowed 10 days to grow before treatment. Treated animals received 5 Grays total body lymphodepleting irradiation (IR) several hours prior to intra-peritoneal vaccination with $5x10^5$ DC2.5.LSIY cells (DC) and intravenous adoptive transfer of $1\text{-}1.5x10^7$ *in vitro* activated 2C cells (2C). B16-LSIY tumors in untreated *R26*$^{LSL\text{-}LSIY/+}$ mice and B16-0 tumors in IR/DC/2C-treated *R26*$^{LSL\text{-}LSIY/+}$ mice grew progressively until animals were sacrificed. B16-LSIY tumors in IR/DC/2C-treated *R26*$^{LSL\text{-}LSIY/+}$ mice, however, exhibited significantly stunted growth, increasing only 3.5-fold in the 10 days following treatment (Figure 1A). Flow cytometry analysis of inguinal lymph nodes revealed preferential accumulation of $1B2^+$ cells, which identifies the 2C T cell receptor (TCR), in ipsilateral lymph nodes draining B16-LSIY tumors, but not a B16-0 tumor, compared to contralateral lymph nodes. Furthermore, 2C cells were found in varying proportions in B16 tumors 5 days after treatment by flow cytometry (Figure S2). This result indicates that adoptive T cell transfer boosted by vaccination and preceded by lymphodepletion can reduce growth of transplanted melanoma tumors by targeting an over-expressed self-antigen. Inhibition of tumor growth was SIY-specific and did not result in overt autoimmunity.

We also examined B16-LSIY tumors in *R26*$^{+/+}$ mice where SIY represents a tumor-specific neo-antigen. B16-LSIY tumors in untreated *R26*$^{+/+}$ mice had reduced growth compared to those in *R26*$^{LSL\text{-}LSIY/+}$ hosts demonstrating there is endogenous immune reactivity to B16-LSIY

tumors in the absence of peripheral self-tolerance. Furthermore, when $R26^{+/+}$ mice bearing B16-LSIY tumors were treated with IR/DC/2C, most tumors regressed (Figure 1A). One B16-LSIY tumor that did progress, albeit slowly over the 10 days following treatment, failed to exhibit luciferase activity indicating that loss of antigen expression allowed this tumor to escape immune attack (Figure S1). None of the other explanted tumors examined, regardless of host or treatment conditions, had lost antigen expression. Thus, these data suggest that immune therapy targeting a tumor-specific antigen is more effective than targeting a tumor over-expression antigen, but such a potent response can lead to tumor escape variants (Khong and Restifo, 2002).

Lymphodepletion, vaccination, adoptive transfer function together to limit tumor growth

To determine the individual contributions of lymphodepletion, vaccination, and transferred T cells in limiting B16-LSIY tumor growth in $R26^{LSL\text{-}LSIY}$ mice, we treated animals with IR, DC, and 2C in combination, with IR and 2C without DC, with DC and 2C without IR, or with IR alone. We observed that tumors grew similarly whether mice received DC/2C dual-therapy or were left untreated, demonstrating that adoptive transfer of tumor-reactive T cells even with a weak vaccine is ineffective in this context without lymphodepletion (Figure 1B). Furthermore, we found similarly reduced tumor growth whenever animals received total body irradiation treatment either as a monotherapy or in combination. This may suggest induction of endogenous immunity or an effect on tumors unrelated to immune response and will require further investigation. 2C following IR resulted in slightly less tumor growth then IR alone and the combination of DC and 2C following IR yielded the least tumor growth (Figure 1B). The ability of self-antigen-presenting dendritic cells to boost the activity of effector CTLs has been described (Lou et al., 2004). Thus, each component was needed to maximize the effectiveness of immune therapy in this context.

Adoptively transferred 2C cells persist better and are functional after lymphodepletion

Previously, we demonstrated that activated 2C cells were short-lived in *R26*$^{LSL\text{-}LSIY}$ mice, dying rapidly so that they were barely detectable after a week *in vivo*. Ectopic expression of the anti-apoptotic protein Bcl2 extends viability transiently, but 2C cells become anergic to antigen stimulation in this context (Cheung et al., 2008). Lymphopenia in immunodepleted mice increases the persistence of anti-tumor T cells (Gattinoni, 2005; Wang, 2005). Furthermore, ablation of lymphocytic suppressor cells and release of natural adjuvants by total body irradiation supports anti-tumor T cell function (Antony et al., 2005; North, 1982; Paulos et al., 2007). Thus, we sought to determine if lymphodepletion could enhance 2C cell viability and function in *R26*$^{LSL\text{-}LSIY}$ mice.

Tumor-free *R26*$^{LSL\text{-}LSIY/+}$ mice and *R26*$^{+/+}$ littermates received *in vitro* activated 2C cells with DC.2.4.LSIY vaccine either with or without prior immunodepletion and were analyzed following treatment. Analysis of inguinal lymph nodes of *R26*$^{LSL\text{-}LSIY/+}$ mice revealed on average 5-6 times more 2C cells 7 days after treatment in treated versus untreated mice (Figure 2A). On day 15 after treatment, 2C cell number was maintained in treated *R26*$^{LSL\text{-}LSIY/+}$ mice such that on average they numbered more than 15 times that in untreated. Thus, lymphodepletion does indeed enhance 2C viability in SIY-expressing mice. Lymphodepletion, however, does not eliminate all sources of peripheral self-tolerance, as 2C cells are maintained at even higher numbers in *R26*$^{+/+}$ mice, with and without treatment (Figure 2A). Interestingly, we did not observe a significant reduction in Foxp3^{+}CD25^{+}CD4^{+} T regulatory cells, a potent mediator of peripheral tolerance, after sub-lethal total body irradiation (Figure S3). It is unclear if Tregs are resistant to irradiation or if they divide rapidly to recover, although the relatively small decline in

number at days 4 and 7 after lymphodepletion might suggest the former. This matter will require further investigation.

We then inquired about the function of persisting 2C cells in tumor-free *R26*$^{LSL\text{-}LSIY/+}$ mice. Upon *in vitro* stimulation with SIY peptide, a significant fraction of 2C cells recovered from splenocytes of IR/DC/2C-treated *R26*$^{LSL\text{-}LSIY/+}$ mice 6 days after treatment were able to secrete IFN-γ (Figure 2B). A variable proportion of 2C cells recovered from treated *R26*$^{LSL\text{-}LSIY/+}$ animals maintained the ability to secrete IFN-γ upon antigen stimulation for at least 2 weeks (data not shown). In addition, 2C cells from treated *R26*$^{+/+}$ mice also secreted IFN-γ upon SIY stimulation, but untreated *R26*$^{LSL\text{-}LSIY/+}$ mice never demonstrated cytokine secretion beyond background levels (Figure 2B). We went on to examine 2C function in mice by an *in vivo* cytotoxicity assay (Cheung et al. 2008). Despite reduced numbers of functional 2C cells in IR/DC/2C-treated *R26*$^{LSL\text{-}LSIY/+}$ mice compared to *R26*$^{+/+}$ mice, comparable levels of target cell cytolysis were observed in treated mice 2 weeks post-treatment (Figure 2C). Not surprisingly, untreated *R26*$^{LSL\text{-}LSIY/+}$ mice had low levels of *in vivo* SIY-specific cytotoxicity. Therefore, lymphodepletion treatment enhances the survival and maintains the function of 2C cells transferred into animals expressing SIY self-antigen.

K-ras^{G12D}-driven lung tumors further support 2C persistence and function

Previously, we demonstrated a higher threshold of stimulation to functionally activate SIY-reactive T cells in the context of autochthonous lung tumors over-expressing SIY (Cheung et al., 2008). Tumor-induced tolerance was only investigated in the context of naïve 2C cells, so we wondered if SIY over-expressing lung tumors would negatively affect activated 2C cells transferred into lymphodepleted mice. We infected *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice intra-tracheally with an Adenovirus vector carrying Cre (Ad-Cre) to

generate autochthonous lung tumors that do not express and those that over-express SIY self-antigen, respectively (Jackson, 2001). We then treated tumor-bearing $R26^{+/+}$ and $R26^{LSL\text{-}LSIY/+}$ mice with 5 Gy total body irradiation followed by DC2.4.LSIY vaccination and transfer of activated 2C cells. When we examined these mice 2 weeks after treatment, we noticed that a high proportion of $CD8^+$ T cells staining positive for 1B2 in lungs, lung draining lymph nodes (LNs) and non-draining LNs of $R26^{+/+}$ mice (Figure 3A). These $1B2^+CD8^+$ cells likely represent a greater population than is found in tumor-free $R26^{+/+}$ mice, but we have yet to perform a direct comparison.

Interestingly, in tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice 2 weeks after treatment, the proportion of $1B2^+CD8^+$ cells found in the lungs is comparable to that in tumor-bearing $R26^{+/+}$ mice (Figure 3A). This population exhibits great variability, but continues to be significant 3 weeks post-treatment in lungs of $R26^{LSL\text{-}LSIY/+}$ mice, whereas $1B2^+CD8^+$ cells are considerably diminished in untreated mice (Figure 3B). In addition, a higher proportion of 2C cells is found in the tumor-draining LN of tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice than in non-draining LNs, although this accumulation is variable (Figure 3A). The distribution of 2C cells in lymph nodes of tumor-bearing $R26^{+/+}$ mice, however, does not exhibit this bias. Furthermore, we noted a correlation between SIY over-expressing tumor burden and 2C T cell accumulation. Specifically, reduced tumor burdens were found in $R26^{LSL\text{-}LSIY/+}$ mice with lower proportions of $1B2^+CD8^+$ T cells in their lung-draining LNs (data not shown). We observed a similar preference of 2C cells for lymph nodes draining SIY over-expressing tumors when studying B16-LSIY tumors (Figure S2).

To test for 2C function after lymphodepletion, we examined the ability of recovered cells to produce IFN-γ after *in vitro* SIY stimulation. Similar proportions of 2C cells recovered 2 weeks following treatment from lymph nodes of tumor-bearing and tumor-free $R26^{LSL\text{-}LSIY/+}$ mice

secreted IFN-γ upon antigen stimulation (Figure 3C; Figure 2B). At 3 weeks post-treatment, 2C cells from tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice continued to be functional (Figure 3C). Although the proportion of IFN-γ-secreting cells was higher in $R26^{+/+}$ mice, this disparity did not differ between tumor-free and tumor-bearing mice (Figure 3C; Figure 2B). Thus, rather than having a negative effect of activated 2C cells, lung tumors over-expressing SIY self-antigen in lymphodepleted animals appear to further support persistence and function of transferred 2C cells.

Autochthonous SIY over-expressing lung tumors are effected by 2C ACT after lymphodepletion

Since functional 2C cells are maintained at high numbers and accumulate in lungs of lung tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice after lymphodepletion treatment, we sought to determine whether this combination immune therapy could be effective against autochthonous lung tumors. We infected $K\text{-}ras^{LSL\text{-}G12D/+};R26^{LSL\text{-}LSIY/+}$ mice with Ad-Cre intra-tracheally to generate SIY over-expressing lung tumors. At 18 weeks post-infection, we divided tumor-bearing animals into 3 groups to be treated with 10^7 *in vitro* activated 2C cells plus DC2.4.LSIY vaccine (DC/2C), 5 Gy sub-lethal total body irradiation only (IR), or a combination of activated 2C cells and vaccine preceded by lymphodepleting radiation (IR/DC/2C). Animals received a single dose of treatment and were analyzed 3 weeks later. To quantitatively assess the effects of therapy, we performed luciferase assays on whole lung lysates after bronchoalveolar lavage (BAL) to reduce biases caused by variable tumor distributions, which would influence surface counts and assessment of tumor burden by histological sections. Lungs of tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice treated with IR/DC/2C exhibited a statistically significant reduction of 66% in luciferase activity (** $p < 0.005$ by Student's *t* test) when compared to mice treated with DC/2C (Figure 3D). IR-

treated animals had an intermediate level of luciferase activity, but because an untreated control group was not included in this experiment we cannot conclude if IR alone reduced luciferase or if DC/2C somehow increased luciferase. In addition, we cannot determine at this time if a reduction in luciferase activity reflects a decrease in tumor burden, a decrease in luciferase expression by tumor cells, or a combination of the two, as we were unable to effectively assess tumor burden. Furthermore, if reduced luciferase does indeed mirror decreased tumor burden, whether or not this is an antigen-specific phenomenon remains to be determined, as we could not examine $R26^{+/+}$ tumor burden by luciferase assay. In the future, longitudinal studies should be performed to assess tumor burden prior to and after treatment in order to address these uncertainties.

LAG-3 upregulation may contribute to heterogeneous T cell function

Activated 2C cells transferred into tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice after lymphodepletion pre-conditioning persist, retain function, and potentially influence tumors over-expressing SIY. We have, however, observed considerable variability in each of these measurements, particularly when compared to tumor-bearing $R26^{+/+}$ mice when applicable (Figure 3A,C). These results suggest the involvement of immune suppressive mechanisms that function in an antigen-specific manner. Many inhibitory molecules have been described to limit T cell function. Thus, we have started to examine expression of candidate inhibitory receptors. CTLA-4, upregulated by activated T cells, blocks co-stimulatory molecule CD28 and inhibits TCR signaling (Peggs et al., 2008). We stained 2C cells from tumor-bearing mice after IR/DC/2C treatment for CTLA-4, but failed to observe an increase in surface expression of this molecule in cells from $R26^{LSL\text{-}LSIY/+}$ mice compared to $R26^{+/+}$ mice (data not shown).

Next, we examined the expression of LAG-3, another negative regulator of T cell homeostasis (Grosso et al., 2007; Workman and Vignali, 2005). We found increased staining for LAG-3 on 2C cells derived from lungs of tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice, but not on 2C cells from $R26^{+/+}$ mice (Figure 4). Individual lymphodepleted animals yielded heterogeneous levels of LAG-3 expression consistent with the heterogeneity in 2C cell persistence and function. We have not, however, been able to correlate these measurements. Interestingly, 2C cells in lung draining lymph nodes from either genotype had consistently low levels of LAG-3 (Figure 4). LAG-3 upregulation may represent a contributor to 2C cell heterogeneity in tumor-bearing $R26^{LSL\text{-}LSIY/+}$ mice.

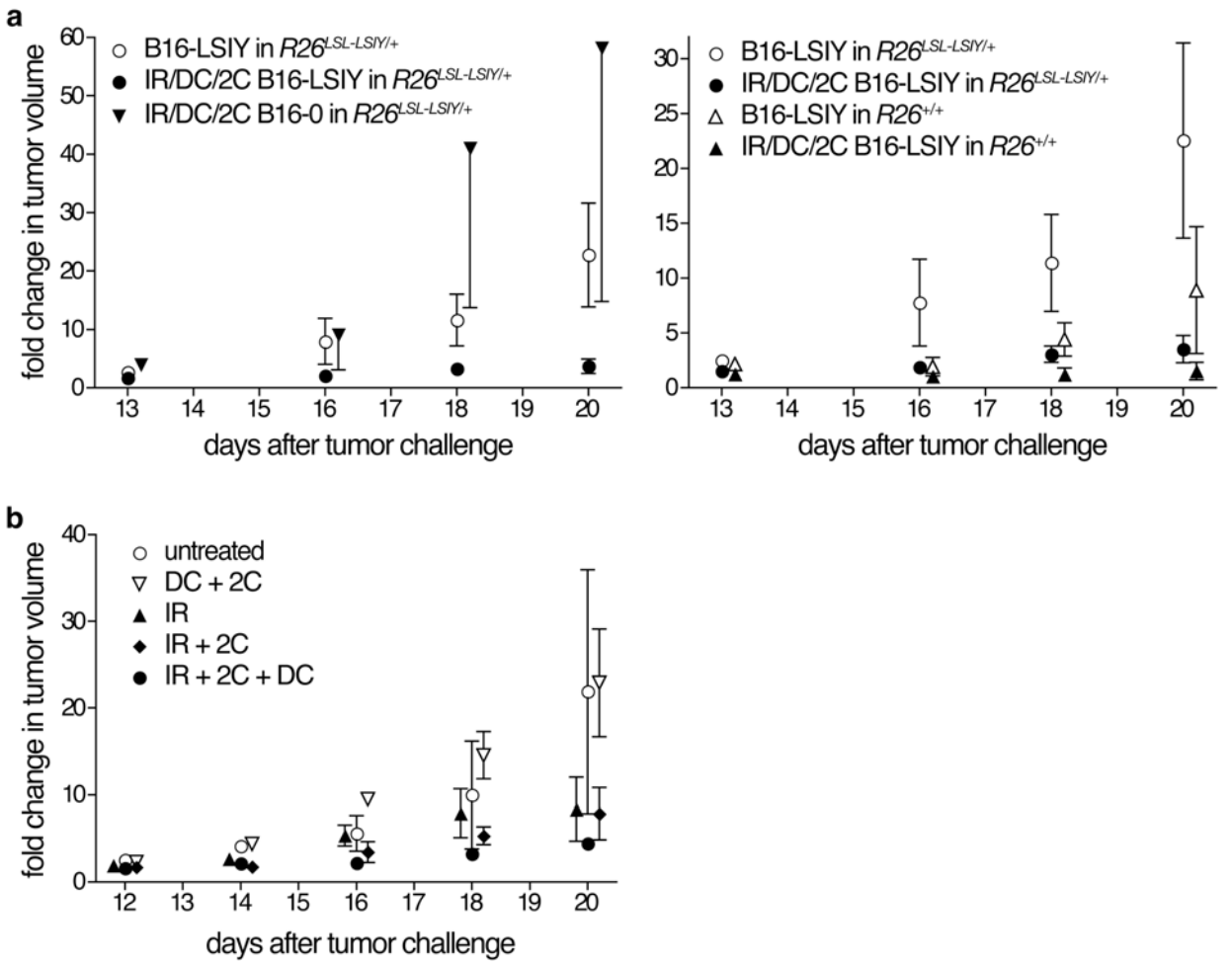
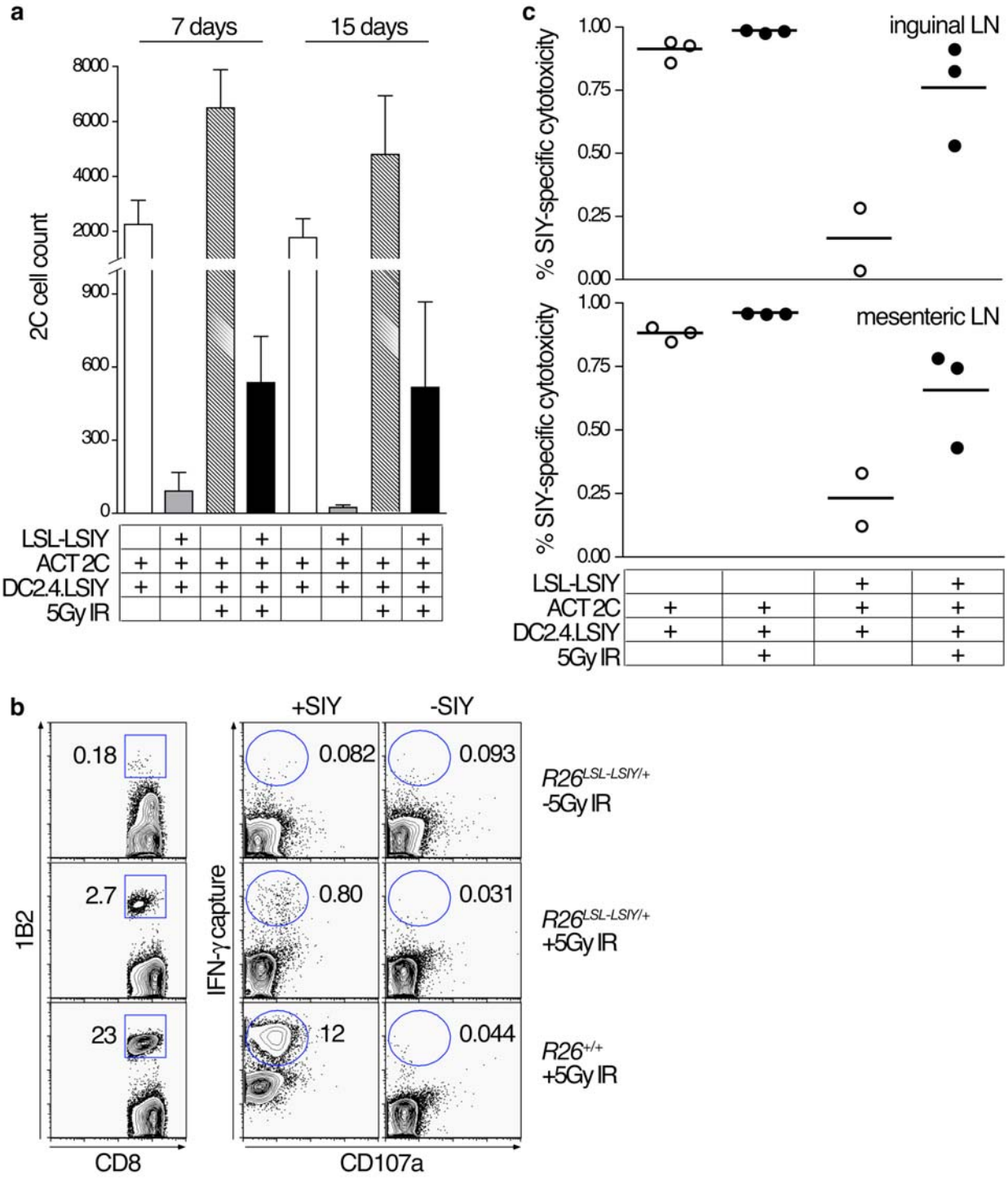


Figure 1. Adoptive T cell transfer is effective against B16 melanoma over-expressing SIY self-antigen only after immunodepletion pre-conditioning. *A.* Fold change in tumor volumes from day 10 after tumor challenge when mice were treated with 5Gy total body irradiation followed by DC2.4.LSIY vaccination and activated 2C cell transfer. *R26*$^{LSL\text{-}LSIY/+}$ mice bearing B16-LSIY tumors left untreated *(open circles)* or treated *(closed circles)* are shown in both plots. *R26*$^{LSL\text{-}LSIY/+}$ mice bearing empty vector transduced B16-0 cells *(closed triangles)* were also treated *(left). R26*$^{+/+}$ mice bearing B16-LSIY tumors were left untreated *(open triangles)* or treated *(closed triangles) (right)*. *Bars*, mean + s.e.m. *B*. Fold change in B16-LSIY tumor volumes growing in *R26*$^{LSL\text{-}LSIY/+}$ hosts from day 10 after tumor challenge when mice were left untreated *(open circles)*, vaccinated with DC2.4.LSIY and given 2C cells *(open triangles)*, treated with 5Gy total body irradiation only *(closed triangles)*, irradiated then given 2C cells alone *(closed diamonds)*, or irradiated then given both DC2.4.LSIY vaccine and activated 2C cells *(closed circles)*. *Bars*, mean + s.e.m.


a
7 days
15 days
2C cell count
8000
6000
4000
2000
900
600
300
0
LSL-LSIY
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DC2.4.LSIY
5Gy IR
c
% SIY-specific cytotoxicity
1.00
0.75
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inguinal LN
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b
+SIY
-SIY
0.18
0.082
0.093
2.7
0.80
0.031
23
12
0.044
1B2
IFN-γ capture
CD8
CD107a
R26LSL-LSIY/+
-5Gy IR
R26LSL-LSIY/+
+5Gy IR
R26+/+
+5Gy IR

Figure 2. Transferred 2C cells persist better after immunodepletion pre-conditioning in *R26*$^{LSL\text{-}LSIY}$ mice and retain function. *A.* Number of 2C cells recovered from the inguinal lymph nodes of *R26*$^{+/+}$ (*open bars*) and *R26*$^{LSL\text{-}LSIY/+}$ (*gray bars*) mice without or with 5Gy total body irradiation (*hatched bars and filled bars, respectively*) prior to adoptive transfer of *in vitro* activated 2C cells and DC2.4.LSIY vaccination. *Bars*, mean + s.e.m. *B*. Representative plots of splenocytes 6 days after activated 2C cell transfer and DC2.4.LSIY vaccination of unirradiated *R26*$^{LSL\text{-}LSIY/+}$ *(top panels)*, irradiated *R26*$^{LSL\text{-}LSIY/+}$ *(middle panels)*, and irradiated *R26*$^{+/+}$ *(bottom panels)* mice. Percentage of 1B2^{+} stained cells within CD8^{+} splenocytes *(left)*. Cells were cultured *in vitro* in the presence or absence of 1μg/mL SIY peptide and an IFN-γ capture assay was performed. CD8^{+}-gated cells are stained for CD107a and secreted IFN-γ *(right). C. In vivo* cytotoxicity assay for SIY-specific target cytolysis 2 weeks after *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ animals received activated 2C transfer and DC2.4.LSIY vaccine either without *(open circles)* or with *(closed circles)* prior 5Gy sub-lethal total body irradiation. 1.5x10^{7} of each CFSE-labeled *R26*$^{+/+}$ control and *R26*$^{1Lox\text{-}LSIY/+}$ target cells were injected 20 hours prior to analysis of inguinaland mesenteric lymph nodes.

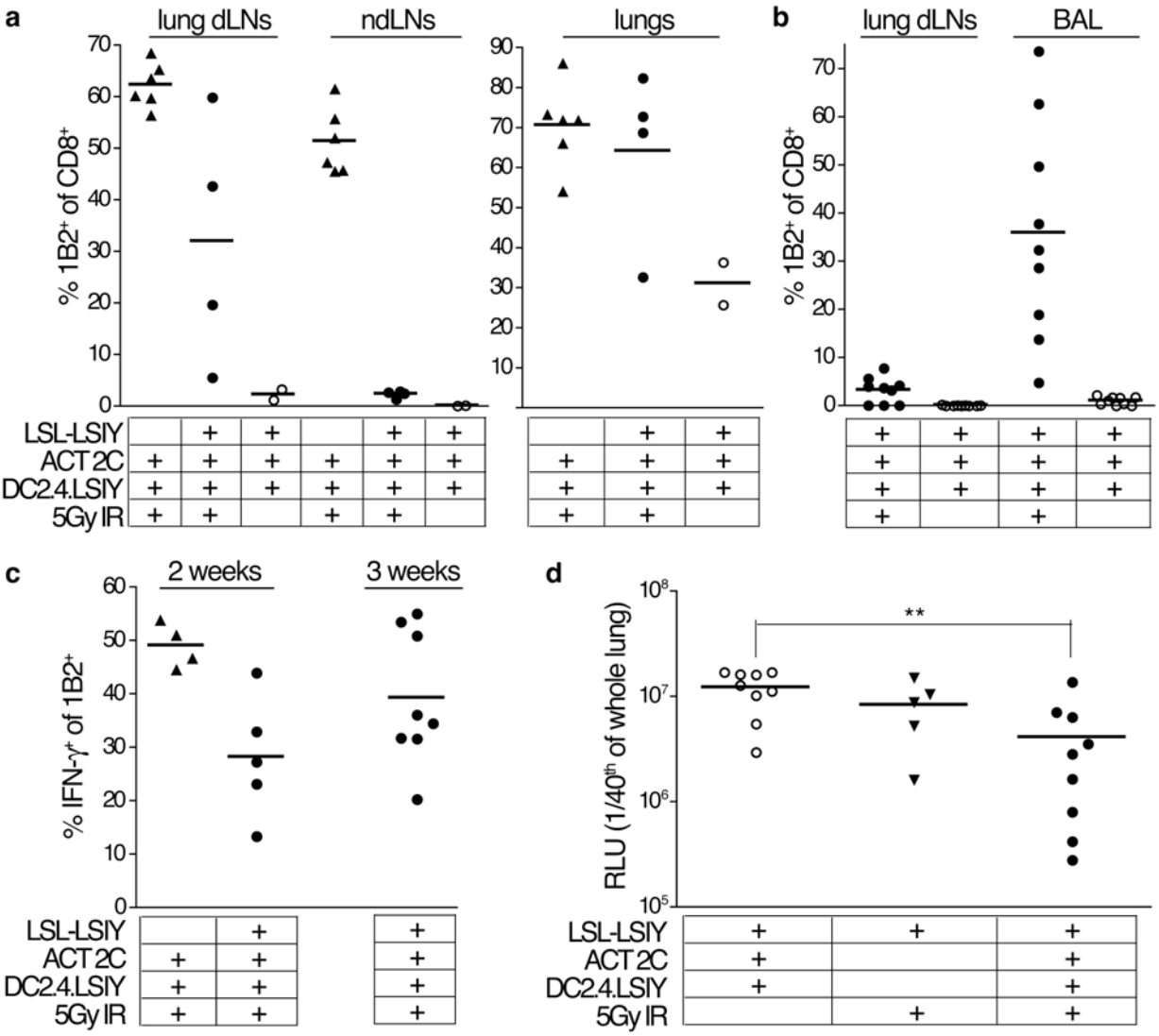


Figure 3. Transferred 2C cells in pre-conditioned *R26*$^{LSL\text{-}LSIY}$ mice bearing tumors that over-express SIY are maintained at high levels, retain function over weeks, and result in reduced luciferase reporter activity in lungs. *A.* Percentage of CD8$^+$ cells that stain positive for 1B2 in lung draining lymph nodes *(left)*, non-draining mesenteric lymph nodes *(middle)*, and lungs *(right)* of *R26*$^{+/+}$ (*triangles*) and *R26*$^{LSL\text{-}LSIY/+}$ (*circles*) mice 2 weeks after adoptive transfer of *in vitro* activated 2C cells and DC2.4.LSIY vaccination with *(closed symbols)* or without *(open symbols)* prior pre-conditioning with 5Gy total body irradiation. *B*. Same as in *a*, but analysis of lung draining lymph nodes *(left)* and bronchoalveolar lavage *(right)* of *R26*$^{LSL\text{-}LSIY/+}$ mice 3 weeks after treatment. *C*. Lung draining lymph nodes of *R26*$^{+/+}$ (*triangles*) and *R26*$^{LSL\text{-}LSIY/+}$ (*circles*) mice were stimulated *in vitro* with SIY peptide. Percentage of 1B2$^+$ cells secreting IFN-γ at 2 weeks *(left)* or staining positive for intracellular IFN-γ at 3 weeks *(right)* after combination treatment. *D*. Luciferase activity in 1/40th of whole lung from lung tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice 3 weeks after treatment with 2C transfer+DC2.4.LSIY vaccine *(open circles)*, 5Gy total body irradiation alone *(closed triangles)*, or combination of 2C transfer, DC.2.4.LSIY vaccine, and total body irradiation *(closed cirlces)*. *Bars*, mean,**p <0.005 by Student's *t* test.

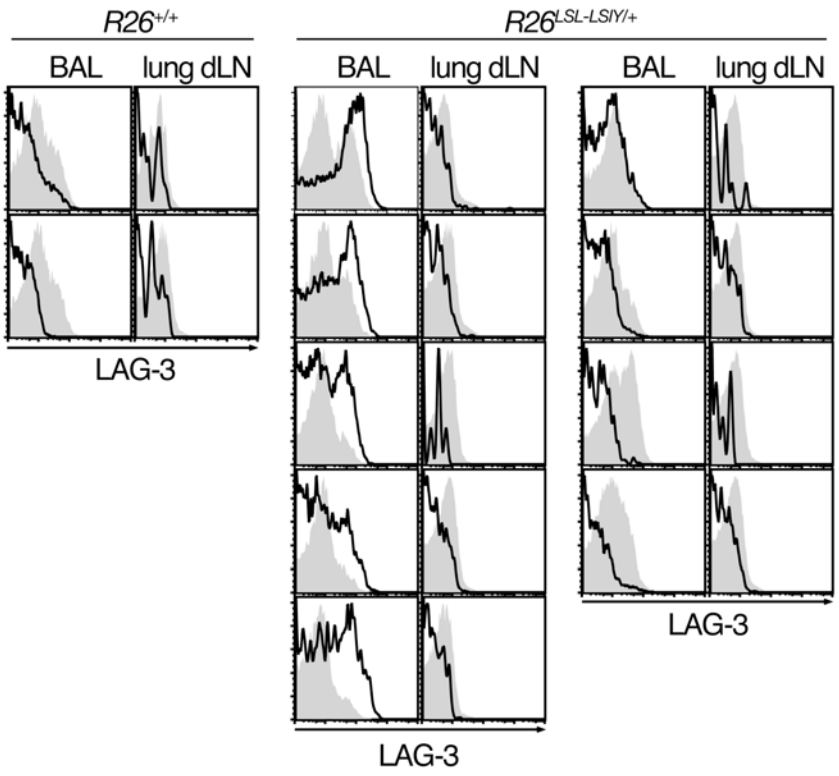


Figure 4. 2C cells in the lungs of tumor-bearing *R26*$^{LSL\text{-}LSIY}$ mice upregulate LAG-3 variably. LAG-3 staining in 2C *(open histograms)* and non-2C CD8^{+} T cells *(shaded histograms)* from the bronchoalveolar lavage and lung draining lymph nodes of lung tumor-bearing *R26*$^{+/+}$ *(left)* and *R26*$^{LSL\text{-}LSIY/+}$ mice *(middle and right)* 3 weeks after immunodepletion pre-conditioning, adoptive transfer of activated 2C cells, and DC2.4.LSIY vaccination.

DISCUSSION

We have demonstrated for the first time that adoptive T cell transfer influences established autochthonous tumors that over-express a ubiquitous self-antigen. Lymphodepletion pre-conditioning had an essential role in therapeutic efficacy, allowing the persistence of functional self-antigen reactive T cells. In addition, tumors in lymphodepleted animals further supported the persistence of these T cells. Moreover, treated mice do not exhibit overt signs of autoimmunity. It is likely that the differential expression of self-antigen in tumors and somatic tissues contributes to the observation of anti-tumor immunity in the absence of autoimmunity. Furthermore, self-reactive T cells were found to exhibit heterogeneity in population size and function despite lymphodepletion, suggesting the existence of additional immune tolerizing factors. We discovered variable upregulation of the LAG-3 inhibitory molecule on self-reactive T cells that might contribute to the observed immune suppression.

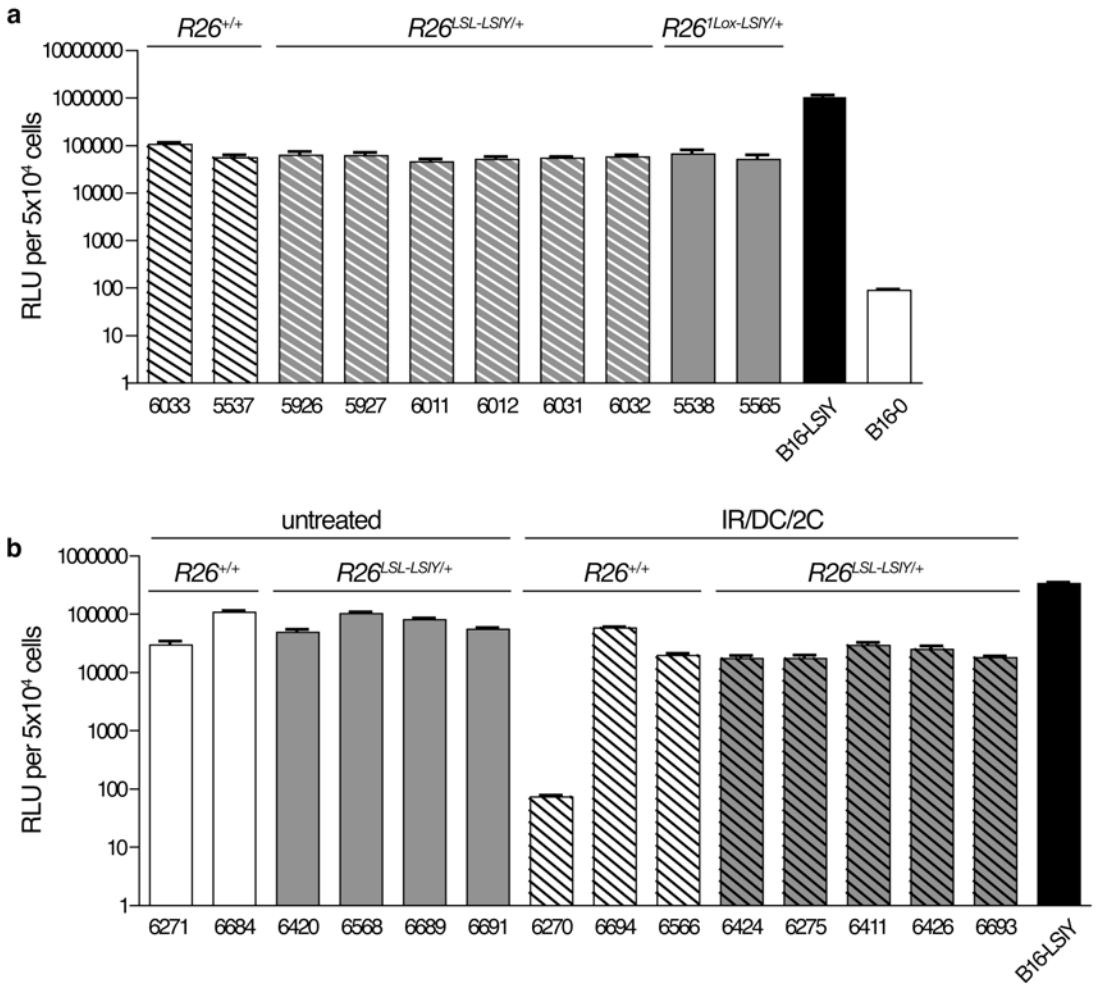
Recently, we described a novel allele $R26^{LSL\text{-}LSIY}$ in mice that allows inducible expression of a defined antigen SIYRYYGL (SIY) to mimic tumor-associated antigens (TAAs) in human cancer (Cheung et al., 2008). In $R26^{LSL\text{-}LSIY}$ mice, SIY is a self-antigen expressed at low-levels in somatic tissues throughout the animal. Upon Cre-mediated recombination of the allele, SIY expression is increased tremendously, copying the expression of known TAAs, such as p53 and cyclin B1 (Egloff et al., 2006; Theobald et al., 1995). This system is compatible with the many Cre-loxP-regulated mouse cancer models available, which closely recapitulate important features of human cancer (Frese and Tuveson, 2007). We characterized SIY-reactive T cells in the context of $K\text{-}ras^{G12D}$-driven lung adenocarcinoma, revealing multiple levels of immune tolerance that together serve to limit an effective response against SIY (Jackson, 2001).

Previously, we demonstrated that activated 2C cells were short-lived in $R26^{LSL\text{-}LSIY}$ mice, dying rapidly so that they were barely detectable after a week *in vivo*. Ectopic expression of the anti-apoptotic protein Bcl2 extended viability transiently, but 2C cells became anergic to antigen stimulation in this context. In contrast, we have shown here that lymphodepleting radiation prior to T cell transfer enhances that persistence 2C cells that retains functionality. Moreover, we demonstrated tumor-induced tolerance earlier, such that in the presence of autochthonous lung tumors over-expressing self antigen, naïve T cells reactive to self-antigen could not be induced to become highly cytotoxic when boosted with a weak vaccine. A stronger vaccine, in the form of a replicating *Influenza* virus, was required to overcome this tumor-induced tolerance. Here, rather than having a negative effect of SIY-reactive T cells, we observe that the presence of tumors actually enhances the persistence of activated 2C cells and their homing to the tumor site when hosts are lymphodepleted.

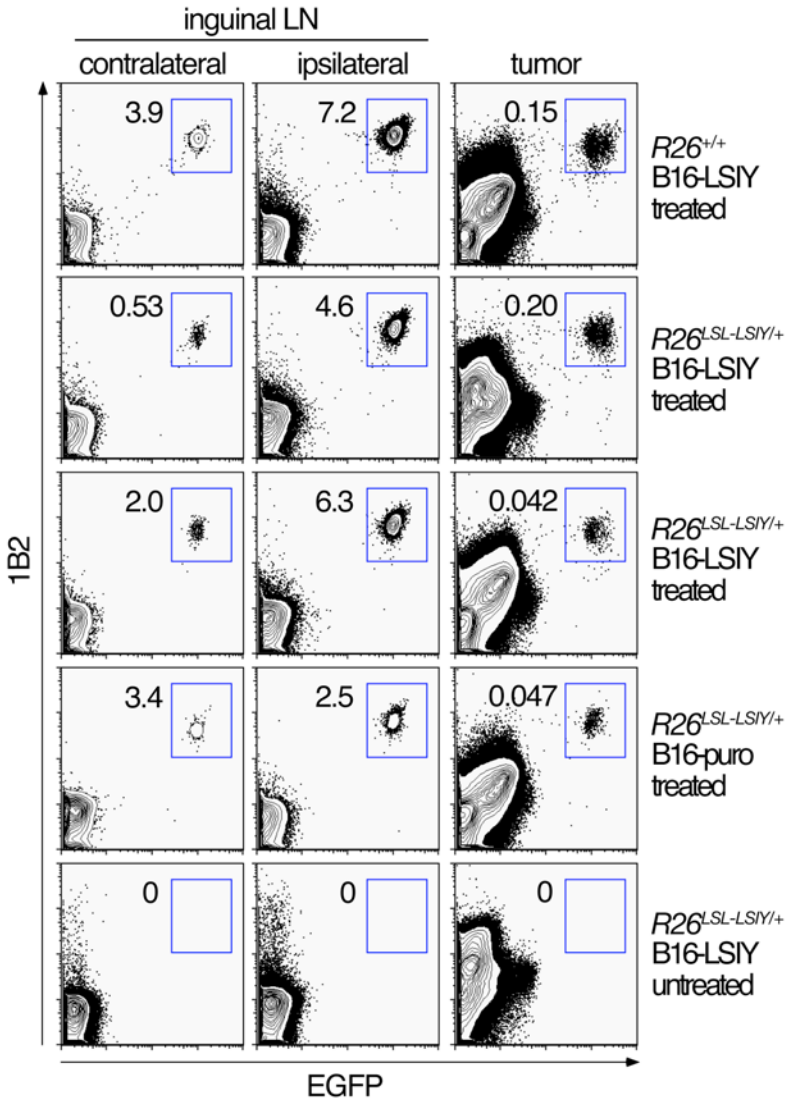
Total body sub-lethal irradiation has profound consequences on 2C cell survival, function, and anti-tumor effects in $R26^{LSL\text{-}LSIY}$ mice. It is not clear at this time exactly how pre-conditioning produces all of these results, but rationales for this phenomenon have been elucidated. The generation of immunological space for homeostatic T cell proliferation and the elimination of lymphocytes that consume supportive cytokines have been found to have potent roles in the effects of lymphodepletion (Dummer, 2002; Gattinoni, 2005). Furthermore, the elimination of lymphocytic suppressor cells, such as T regulatory cells, by lymphodepletion has also been demonstrated (Antony et al., 2005; North, 1982). Interestingly, T regulatory cells are not significantly reduced in number after irradiation of tumor-free mice in our hands (Figure S3). Release of lipopolysaccharides, potent immune adjuvants, from gut microbes has also been shown to mediate the effects of lymphodepletion, as well (Paulos et al., 2007). Furthermore,

non-cytotoxic doses of radiation have been demonstrated to upregulate Fas death receptor and MHC class I antigen presentation on tumor cells (Chakraborty et al., 2003; Garnett et al., 2004). Moreover, low-dose ionizing radiation has been shown to enhance innate immune cell activity (Ren et al., 2006). Finally, it is possible that low dose radiation can alter the tumor microenvironment to be immune supportive. Many of these possibilities may account for our observation of efficacy with 5 Gy total body irradiation treatment alone on B16-LSIY tumors (Figure 1B).

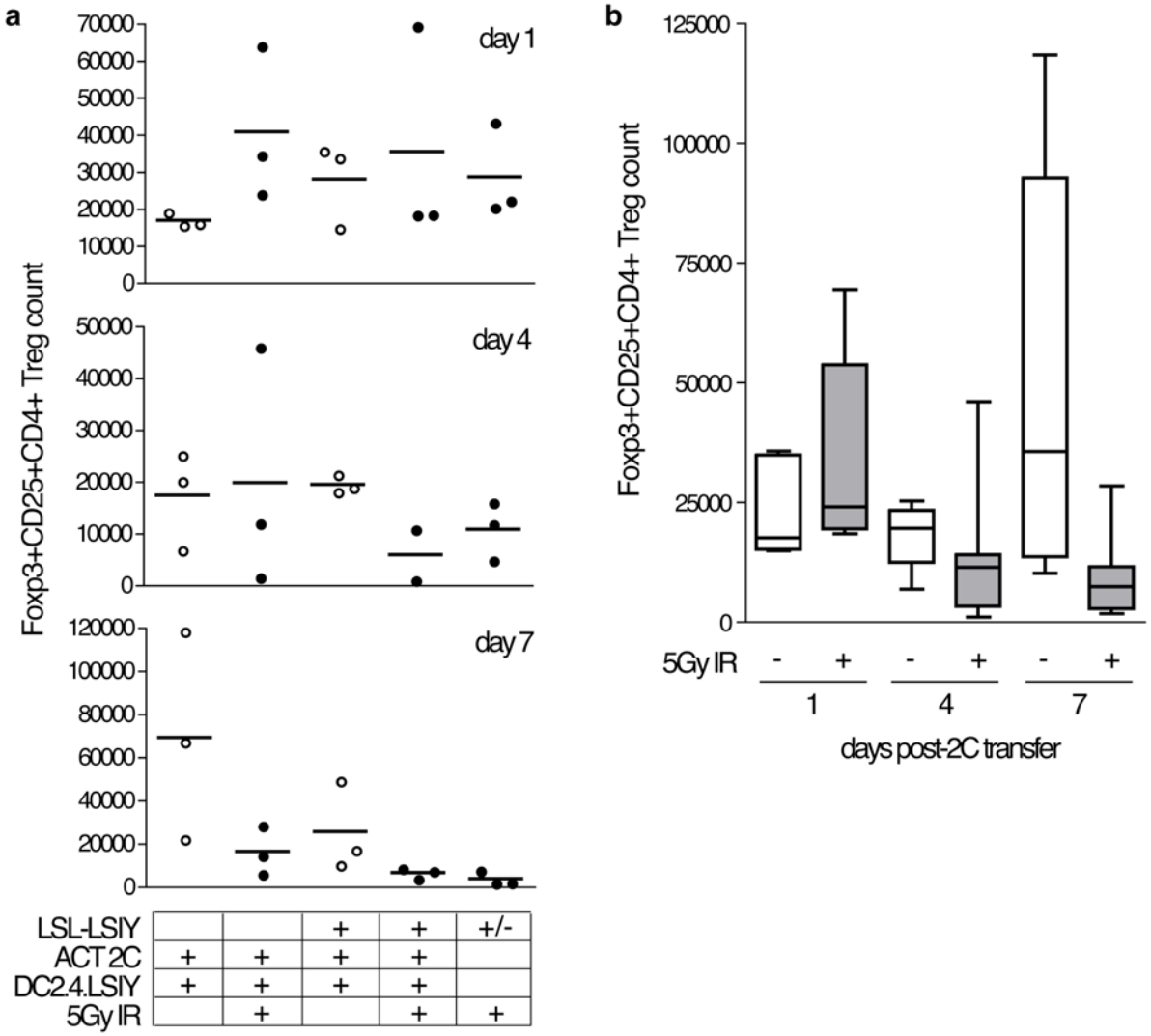
Further experiments are needed to investigate the actual effect of 2C ACT with prior lymphodepletion on autochthonous lung tumors. Longitudinal studies will allow us to determine whether tumors are losing antigen expression or actually being killed and whether the effects we observe are SIY specific. Moreover, additional studies are required to determine if LAG-3 upregulation correlates with decreased SIY-reactive T cell number or function and whether additional inhibitory molecules are involved. Longitudinal studies will be helpful with respect to this detail, as well. If expression of LAG-3 or other inhibitory molecules correlate with reduced therapeutic efficacy in tumor-bearing $R26^{LSL\text{-}LSIY}$ mice, we may be able to apply blocking antibodies to enhance immune therapy (Blackburn et al., 2009; Grosso et al., 2007; Keir et al., 2007).



Supplementary Figure 1. Luciferase assay detects antigen loss variants. *A.* B16-LSIY cells were transplanted into $R26^{+/+}$ *(white hatched bars)*, $R26^{LSL\text{-}LSIY/+}$ *(gray hatched bars)*, and $R26^{1Lox\text{-}LSIY/+}$ *(solid gray bars)* hosts and explanted 3 weeks later. Explanted tumors were cultured for 5days before luciferase assay along with B16-LSIY parent line and B16-0 control. *B.* B16-LSIY cells were transplanted into $R26^{+/+}$ *(white bars)* and $R26^{LSL\text{-}LSIY/+}$ hosts *(gray bars)* left untreated or $R26^{+/+}$ *(white hatched bars)* and $R26^{LSL\text{-}LSIY/+}$ hosts *(gray hatched bars)* treated with 5Gy total body irradiation, DC2.4.LSIY vaccine, and activated 2C cell transfer 10-12days after tumor challenge. Tumors were explanted 9-10days later and cultured for 3-5days before luciferase assay along with B16-LSIY parent line. Data are compiled from two independent experiments.



Supplementary Figure 2. Transferred 2C cells are enriched in tumors and tumor-draining lymph nodes of mice bearing subcutaneous B16-LSIY tumors. Inguinal lymph nodes and tumors were analyzed from mice 5 days after combination treatment with 5Gy total body irradiation, activated 2C cell (EGFP+) transfer, and DC2.4.LSIY vaccination or untreated 10 days after subcutaneous tumor innoculation. Percentages of live cells that were $1B2^{+}EGFP^{+}$ are indicated from contralateral and ipsilateral lymph nodes and tumors. Host genotypes, the B16 tumor injected, and treatment are indicated.



Supplementary Figure 3. Immunodepletion pre-conditioning does not significantly reduce T regulatory cell number. *A.* Number of Foxp3^{+}CD25^{+}CD4^{+} cells in inguinal lymph nodes from individual tumor-free *R26*$^{+/+}$ and *R26*$^{LSL\text{-}LSIY/+}$ mice either un-irradiated (*open circles*) or treated with 5Gy total body irradiation (*filled circles*) prior to adoptive transfer of *in vitro* activated 2C cells and vaccination with DC2.4.LSIY. Analysis performed 1, 4, and 7 days after 2C transfer. *Lines*, mean value. *B*. Box and whisker plots of the data from *a*, but grouped by treatment without *(open bars)* or with total body irradiation *(shaded bars)*, irrespective of genotype, 1, 4, and 7 days after 2C cell transfer and vaccination.

METHODS

Mice. *2C* mice were provided by J. Chen and *Rag2*$^{-}$ mice were purchased from Jackson Laboratories. *R26*$^{LSL\text{-}LSIY}$ and *R26*$^{1Lox\text{-}LSIY}$ mice were generated in our laboratory (Cheung et al., 2008). *K-ras*$^{LSL\text{-}G12D}$ mice were also generated in our laboratory (Jackson, 2001; Tuveson et al., 2004). Donor 2C cells for transfer experiments were from pure C57BL/6 *2C;Rag2*$^{-/-}$ mice and all recipients and controls used were also on pure C57BL/6 background. To induce tumors, mice were infected with Adenovirus-Cre intra-tracheally. Non-myeloablative immunodepleting irradiation was performed with 5 Grays (500 rads) total body irradiation upon exposure of animals to ^{137}Cesium in a Gammacell 40 irradiator. Irradiation was always performed within 6-14 hours prior to T cell transfer or vaccination. Animal studies were approved by MIT's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

Cell lines. B16-F10 cells were purchased from ATCC. B16-LSIY and B16-0 derivatives were generated by infection of B16-F10 parental cells with amphotropic pseudotyped retroviruses made with sub-cloned pMSCV-LSIY-puro and empty pMSCV-puro transfer vectors, followed by puromycin selection. Melanoma tumors were generated by subcutaneous injection of $2x10^5$ B16-LSIY or B16-0 cells into C57BL/6 mice. Tumor masses were measured by calipers and volumes were determined by assuming an ellipsoid shape; volume = (4/3)*pi*x*y*z, with x, y, and z being the widths of each perpendicular axis. Tumor-bearing mice were treated with therapy on day 10 after tumor innoculation and sacrificed within 3 weeks after challenge.

DC2.4.LSIY cells were previously described (Cheung et al., 2008). DC2.4.LSIY cells were washed and resuspended in RPMI for intra-peritoneal vaccination with $5x10^5$ cells/mouse. Single-cell suspensions from lymph nodes and spleens of *2C;Rag2*$^{-/-}$, *R26*$^{1Lox\text{-}LSIY/+}$ and *R26*$^{+/+}$ mice were used for 2C transfer, *in vivo* cytotoxicity assay's target and control cells, respectively. Cells were counted by hemocytometer, resuspended in RPMI and transferred into recipients via tail vein. $1.5x10^7$ of CFSE-labeled target and control cells were injected per mouse for the *in vivo* cytotoxicity, which has been previously described (Cheung et al., 2008). *In vitro* activated 2C cells were made by stimulating *2C* splenocytes/lymphocytes suspensions with 1μg/mL SIY in DMEM-10 full medium overnight, then culturing with 10ng/mL mIL-2 (R&D Systems cat#402-ML) for 6 days. $1\text{-}1.5x10^7$ *in vitro* activated 2C cells were injected for adoptive T cell transfer therapy.

Luciferase detection. *In vivo* passaged B16-LSIY and B16-0 cells were explanted and cultured in DMEM-10 full medium for a maximum of 5 days before assay. Cells were trypsinized, washed, and lysed with Passive Lysis Buffer (Promega cat#E1941). Assayed were performed with Luciferase Assay Reagent (Promega cat#E1501) according to manufacturer's instructions. Tumor-bearing lungs were minced before lysis and tissue debris was pelleted before assay.

Cell isolation. Single-cell suspensions from lymph nodes, spleens, and B16 tumors were generated by mechanical disruption. For lung preparations, tissue samples were minced and digested at 37^{o}C for 30 minutes in 125U/mL of collagenase type I (Gibco) in phosphate-buffered saline before mechanical disruption and passage through a 70μm-pore filter (BD Falcon). Red blood cells were lysed with an aqueous solution containing 0.83% NH_4Cl, 0.1% $KHCO_3$, and

0.000037% Na_2EDTA at pH 7.2-7.4. Single-cell suspensions were passed through a 35μm-pore cell-strainer cap (BD Falcon) before culture, intravenous transfer, or staining for flow cytometry.

Reagents and flow cytometry. SIYRYYGL peptide was synthesized by MIT's Biopolymers Laboratory. Recombinant murine interleukin-2 (cat#402-ML) was purchased from R&D Systems, Inc. The following antibodies were purchased from BD Pharmingen: αCD4 (H129.19), αCD8 (53-6.7), αCD16/CD32 (2.4G2), αCD25 (7D4 and PC61), αCD107a (1D4B), αCTLA-4 (UC10-4F10-11), αIgG_1 (X56), and αLAG-3 (C9B7W). Intracellular Foxp3 staining was performed with FITC-α-mouse/rat Foxp3 staining set from eBioscience and secreted IFN-γ capture/detection was performed with Mouse IFN-γ Secretion Assay Detection kit (order#130-090-516) from Miltenyi Biotec, according to manufacturers' instructions. Biotinylated 1B2 monoclonal antibody was provided by J. Chen. Streptavidin-allophycocyanin and streptavidin-phycoerythrin conjugates were purchased from BD Pharmingen. Cells were read on a FACSCalibur (BD Biosciences) and analyzed using Flowjo 8.1 software (Tree Star Inc). Dead cells were excluded by 1μg/mL propidium iodide staining (Sigma).

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CHAPTER 4:

Overview and Future Directions

APPLYING *R26*$^{LSL\text{-}LSIY}$

In Chapter 2 of this thesis, I described the generation of a new model system *R26*$^{LSL\text{-}LSIY}$ that recapitulates a self-antigen SIY that becomes over-expressed in cancer. We have identified many sources of tolerance faced by SIY-reactive T cells, which collectively limit anti-tumor immunity, in the context of a mouse model of lung adenocarcinoma. With this new model, one can now explore the specific mechanisms that contribute to tolerance in the context of autochthonous cancer. In addition, *R26*$^{LSL\text{-}LSIY}$ can be used as a platform to examine therapeutic strategies currently in clinical and pre-clinical trials as well as to explore novel immune therapies. Finally, as *R26*$^{LSL\text{-}LSIY}$ is independent of specific oncogenes/tumor suppressor genes and specific tissue types, tumor-associated antigens can be studied in other Cre-loxP-inducible mouse cancer models.

Elucidating mechanisms of tolerance

In the Introduction, I described several means by which tumors can evade immunity and tolerance to cancer can be mediated. We have already detected an expansion of myeloid suppressor cells and T regulatory cells in tumor-bearing mice compared to tumor-free mice, described in Chapter 2. In addition, we observed upregulation of the inhibitory molecule LAG-3 on SIY-reactive T cells in tumor-bearing *R26*$^{LSL\text{-}LSIY}$, described in Chapter 3. Furthermore, we have preliminary evidence of other immune regulatory factors, including additional immune cell types and inhibitory molecules, which could potentially be mediating tolerance to lung tumors. Despite our observations, we have not yet demonstrated that any of these mechanisms are actually contributing to immune tolerance to cancer. Thus, *R26*$^{LSL\text{-}LSIY}$ can be applied specifically for this purpose and to determine the relative contributions of individual immune suppressive agents.

Many groups have developed reagents to facilitate the interrogation of immune suppressive factors. To interrogate specific cell populations or proteins, genetic tools have been developed which one can utilize to acutely delete the factor in question. For example, we have initiated experiments with Foxp3-DTR transgenic and knock-in mice, which will deplete T regulatory cells upon administration of diphtheria toxin (DT) to mice, described in Appendix 1 (Kim et al., 2007; Lahl et al., 2007; Quezada et al., 2008). In addition, conditional alleles can be used to delete genes coding for specific factors or to specifically express DTR in defined cell types to be subsequently depleted with DT administration (Buch et al., 2005). In addition to genetic tools, blocking antibodies have been widely used. In Appendix 1, we described our efforts to deplete T regulatory cells, which express high levels of CD25, with a CD25-specific antibody (Zou, 2006). Moreover, contingent upon additional experiments discussed in Chapter 3, we can potentially use blocking antibodies to LAG-3 to improve T cell expansion and function in the context of adoptive cell transfer and immunodepletion (Grosso et al., 2007). Furthermore, one can manipulate T cells to over-express, reduce expression, or block function of specific gene products to interrogate T cell-intrinsic molecules (Kershaw et al., 2005). For example, expression of short hairpin (sh)-RNAs to LAG-3 or T cell-specific gene knockout of LAG-3 will also allow interrogation of LAG-3 function in limiting anti-tumor T cell function.

Evaluating immune therapies for cancer

Many immune therapies for cancer are currently being tested in clinical trials and, likely, more in pre-clinical trials. Although, immune therapies have been used successfully against established tumors in transplant models, the likelihood of such wide success in clinical trials in low. Transplanted tumors results in tissue damage, death to transplanted cells, and an acute burden of tumor antigens that is likely to be highly immunogenic. In addition, the

microenvironment of the tumors are unlikely to be realistic due to the ectopic location and rapid growth rate of the transplanted tumor cells, which can influence immune therapy. Thus, to better recapitulate the state of immune tolerance that exists in human cancer, immune therapy necessitates evaluation in autochthonous cancer. Genetically-modified mouse cancer models, to which $R26^{LSL\text{-}SIY}$ is compatible, closely recapitulate many aspects of human cancer (Frese and Tuveson, 2007). As described in Chapter 3, we have evaluated adoptive cell transfer therapy with lymphodepletion pre-conditioning in lung tumor-bearing $R26^{LSL\text{-}SIY}$ mice. Whereas subcutaneous, SIY-expressing melanoma tumors were relatively easy to control, autochthonous lung tumors demonstrated a wide-ranging response. Furthermore, we have identified LAG-3 as a potential contributor to heterogeneous T cell function. Studies such as this will not only help in predicting the efficacy of cancer immune therapies in the clinic, but also facilitate the identification of additional points of intervention that may be useful for combination therapies.

Discovering context-dependency in immune reactivity and tolerance

An open question in cancer immunology concerns the immunogenicity of antigens among tissue sites. For example, the skin has been described as a particularly immunogenic site, while the lung has been labeled immune suppressive (O'Mara and Allen, 2004). Direct comparison of individual studies on tumor tissue microenvironments can be misleading due to many variables. For example, two transgenic tumor models both based on oncogenic SV40 large T antigen have demonstrated divergent findings relating to immune tolerance. In RIP-Tag2 mice, which develop spontaneous insulinomas, tumor reactive T cells are not tolerized despite large tumor burdens and mice remain responsive to therapeutic vaccination (Nguyen et al., 2002). In contrast, tumor-reactive T cells in TRAMP mice, which develop prostate cancer, become dysfunction and vaccination is only effective in boosting T cell activity briefly

(Anderson et al., 2007). The difference in tolerance induction between the models could be related to the tissue in which the cancer arises. Alternatively, the difference may be attributable to the strain background or model antigens being investigated as T cells reactive to an antigen derived from a glycoprotein of lymphocytic choriomeningitis virus (LCMV-GP) is used in RIP-Tag2 mice while T cells reactive to an epitope of T antigen is TRAMP mice.

R26$^{LSL\text{-}LSIY}$ offers researchers the ability to compare the same tumor antigen within two or more different tumor microenvironments. As *R26*$^{LSL\text{-}LSIY}$ is independent of specific tissue types, SIY can be studied as a model tumor-associated antigen in all Cre-loxP-regulated cancer models. For example, conditional expression of oncogenic K-ras^{G12D} and mutation/loss of the tumor suppressor p53 contributes to both lung adenocarcinoma and soft tissue sarcoma (Jackson, 2005; Kirsch et al., 2007). Thus, comparing tolerance to SIY in these contexts will be informative in learning about how tissue microenvironments contribute to tolerance induction and maintenance.

Another open question is the extent to which driving mutations in tumors influence tolerance. For example, expression of oncogenic K-ras has been found to induce expression of various cytokines and chemokines (Ancrile et al., 2008; Ji et al., 2006). In addition, as mentioned in the Introduction, Stat-3, induced downstream of various growth factor receptors, establishes an immune suppressive program (Yu et al., 2007). Since *R26*$^{LSL\text{-}LSIY}$ is expressed independently from tumor-predisposing alleles, one could use this system to investigate the impact of cancer genetics on immune tolerance.

IMPROVING UPON *R26*$^{LSL\text{-}LSIY}$ FOR MORE ADVANCED CANCER MODELING

I have described a model system in which a defined antigen SIYRYYGL (SIY) has been genetically engineered to represent a self-antigen that is expressed ubiquitously at low levels and

presented by H-2K^{b} MHC class I molecules in mice. Upon Cre expression in somatic cells of Cre-loxP-regulated mouse cancer models, oncogene activation and/or tumor suppressor gene inactivation is coordinated with over-expression of SIY in tumors (Chapter 2). This system recapitulates what take places with naturally occurring self-antigens that are over-expressed in cancer. One can make efforts to improve aspects of the current system as well as to use this as a foundation for constructing new systems.

Making use of additional antigens

As described in the Introduction, CD4^{+} T cell help is needed to license dendritic cells (DCs) in order for DCs to properly activate CD8^{+} T cell. CD4^{+} T cells also support an active cytotoxic T cell response through cytokines and co-stimulatory molecules and promote the formation of functional memory T cells (Bevan, 2004; Kennedy and Celis, 2008). In *R26*$^{LSL\text{-}LSIY}$ mice, *Firefly* luciferase is a protein fusion partner with SIY and, thus, luciferase is over-expressed in tumors alongside SIY. It is expected that luciferase contains peptide epitopes that can be presented on MHC class II molecules, which are recognized by CD4^{+} T cells. In fact, pulmonary infection of *K-ras*$^{LSL\text{-}G12D/+}$ mice with integrating lentiviruses expressing luciferase and Cre results in lung tumors with substantial lymphocytic infiltration, whereas relatively few lymphocytes are observed with lentiviruses bearing Cre alone (DuPage and Jacks, unpublished). Thus, to examine CD4^{+} T helper cells in the context of tumors over-expressing luciferase-SIY, one could clone such T helper cells and identify their cognate MHC class II-restricted antigens. With these additional reagents, one can better examine and manipulate the induction of anti-tumor immunity in *R26*$^{LSL\text{-}LSIY}$ mice. Furthermore, suppressor lymphocytes could potentially be examined with these reagents as T helper cells can be converted into Foxp3^{+}CD4^{+} T regulatory cells (Andersson et al., 2008). In addition, one can assess whether natural T regulatory cells to

LSIY are generated in the thymus due to LSIY expression there. Finally, it would also be useful to identify addition $CD8^+$ T cell clones and their cognate MHC class I-restricted antigens from luciferase-SIY.

Including tumor-specific antigens

Tumor cells contain numerous antigens that can potentially be recognized by the immune system. These antigens likely fall into both tumor-associated antigen and tumor-specific antigen classes, which were described in the Introduction. Most tumor-specific antigens are not found coincident between individual tumors, even if they are from the same type of cancer (Segal et al., 2008). Despite this, use of some cancer immune therapies, such as autologous whole tumor cell vaccines, can boost immune responses to undefined tumors-specific antigens (Gruijl et al., 2008). Furthermore, activation of immune responses to one tumor antigen can result in induced reactivity to associated antigens via epitope spreading (Pilon et al., 2003). Thus, a system in which multiple types of antigens can be studied within the same tumor would be useful. I have described a model system utilizing the defined antigen SIY as a tumor-associated antigen in lung cancer. An alternative system has been developed in the lab that makes use of SIY as a tumor-specific antigen (DuPage and Jacks, unpublished). In this case, SIY is introduced into mice, again as a fusion with luciferase, but via an integrating lentivirus that also carries Cre with it. This system results in restriction of SIY expression to cells that will become tumors by way of the conditional *K-ras*$^{LSL\text{-}G12D}$ allele. Neo-antigen expression of SIY results in induction and infiltration of endogenous SIY-reactive T cells into tumors, a phenotype of immunogenic tumors (DuPage and Jacks, unpublished). In contrast, when SIY is introduced via lentivirus into *R26*$^{LSL\text{-}LSIY}$ mice, lymphocytic infiltration into tumors is not observed, indicative of SIY representing a self-antigen in this situation (Cheung, DuPage, and Jacks, unpublished). Thus, with the same

lentivirus used to infect *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{+/+}$ and *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice, we can generate tumors bearing SIY as a tumor-specific antigen or as a tumor-associated antigen. Furthermore, when we use a lentivirus carrying ovalbumin antigens in addition to SIY and Cre to infect *K-ras*$^{LSL\text{-}G12D/+}$*;R26*$^{LSL\text{-}LSIY/+}$ mice, we generate a lung tumors with coincident expression of tumor-associated antigens (specifically SIY and epitope in luciferase) and tumor-specific antigens (specifically known antigens derived from ovalbumin) (Cheung, DuPage, and Jacks, unpublished). With this system one can learn how immune reactivity to one type of tumor antigen influences another.

Studying naturally occurring tumor antigens

Tumor immunologists have learned a great deal from the use of model antigens. These systems have been attractive because of the many established reagents on hand that can be used to interrogate these defined antigens in various contexts. Defined antigens in mouse cancer models, however, may not fully recapitulate naturally occurring tumor antigens, particularly when the model antigens are derived from a different species or are synthetic. *R26*$^{LSL\text{-}LSIY}$ suffers from both these short-comings, as luciferase is derived from *Firefly* and SIY is synthetic, not yet found to occur naturally in any species (Whittaker and Eisen, unpublished). To more faithfully recapitulate the immunology of cancers, one can potentially clone $CD8^+$ and $CD4^+$ T cells from tumor-vaccinated mice. In addition, cognate antigens can be identified by screening T cell clones. This can be achieved with peptides derived from dendritic cells that have engulfed and processed tumor antigens. Furthermore, with nuclear transfer and 4-factor reprogramming technology, transgenic T cell receptor (TCR) mice that produce T cells recognizing these naturally occurring tumor antigens can be generated.

Aside from this unbiased approach to identifying useful tumor antigens and anti-tumor T cell clones, one can use our established knowledge of protein products that are mis-expressed or commonly mutated in cancer. I will use our lab's mouse models of human lung adenocarcinoma based on K-ras^{G12D} alone or in combination with mutant or deleted p53 as examples (Jackson, 2001; Jackson, 2005; Olive et al., 2004; Tuveson et al., 2004). Using these cancer models, T cells reactive to known lung tumor-specific antigens and tumor-associated antigens can be generated. To potentially produce tumor-specific T cells, one can vaccinate naïve animals with mutant peptides/genes for our genetically-defined mutations: *K-ras*^{G12D}, p53^{R172H}, and p53^{R270H}. Vaccination should be performed on various genetic backgrounds bearing different MHC class I haplotypes to increase the likelihood of mutant epitope presentation. Furthermore, one can utilize our tumor-versus-normal lung tissue gene expression data to choose somatic genes that are specifically over-expressed in our models' lung tumors (Shaw and Jacks, unpublished; Sweet-Cordero et al., 2004). In our p53 point mutant tumors, the tumor suppressor p53 is over-expressed due to disregulation of a negative feedback loop involving Mdm2 (Jackson, 2005; Olive et al., 2004). In addition to being highly relevant to human cancer, p53 is generally undetectable in normal somatic tissues, and, thus, represents an ideal candidate to which to generate T cell reactivity. In fact, both cytotoxic T cell and helper T cell clones specific for un-mutated epitopes of the tumor suppressor p53 have already been identified (Vierboom et al., 1997; Zwaveling et al., 2002). One could apply these T cell clones to examine immune therapy and tolerance in various cancer models (Frese and Tuveson, 2007).

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APPENDIX 1:

Depletion of T regulatory cells enhances anti-tumor cytotoxic T cell function

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The author and Michel DuPage performed all of the experiments described together, except for Figure 1A, which the author performed alone. Yuelei Shen and Dan Littman generated the *Foxp3-DTR* mice used and generously provided them for our experiments. All experiments were performed in the laboratory of Tyler Jacks.

ABSTRACT

Immune tolerance thwarts efforts to utilize immune therapy against cancer. Numerous mechanisms have been attributed to the phenomenon of tolerized anti-tumor cytotoxic T cells in both mouse and human disease. T regulatory cells (Tregs) have stood out among these due to their critical role in maintaining peripheral tolerance and preventing autoimmune disease. In many studies, depletion of Tregs has yielded increased anti-tumor T cell activity and tumor regression when Tregs are lost prior to tumor establishment. The importance of T regulatory cells in maintaining suppression to anti-tumor T cells once cancer is established, however, is still an open issue. We have documented an expansion of Tregs in mice bearing autochthonous lungs tumors that correlates with tumor burden. Furthermore, we have devised a combination depletion strategy that yields substantial T regulatory cell depletion for an extended length of time. Finally, we have demonstrated that depletion of T regulatory cells enhances anti-tumor cytotoxic T cell function in the presence of established lung cancer.

INTRODUCTION

Expanded populations of T regulatory cells (Tregs) have been found within tumors, in tumor-draining lymph nodes, and in peripheral blood of patients with various types of cancer (Ichihara et al., 2003; Liyanage et al., 2002; Ormandy et al., 2005; Viguier et al., 2004; Woo et al., 2001). Patient-derived Tregs were found to constitutively express the inhibitory co-signaling molecule CTLA-4 and produce immunosuppressive cytokines TGF-β and IL-10. When isolated from patient tumors, Tregs inhibited proliferation and cytokine secretion of autologous $CD4^+$ and $CD8^+$ T lymphocytes *in vitro*, demonstrated their ability to suppress adaptive immune effectors (Ormandy et al., 2005; Viguier et al., 2004; Woo et al., 2002). High numbers of tumor-infiltrating $Foxp3^+$ T regulatory cells predicted reduced survival compared to patients with low numbers of Tregs in ovarian cancer (Curiel et al., 2004). Conversely, in breast cancer, complete responders after neoadjuvant chemotherapy exhibited a reduced number of tumor-infiltrating Tregs and increased cytotoxic T cell responses to tumors (Ladoire et al., 2008).

$CD4^+CD25^+$ T regulatory cells are specifically identified by expression of the transcription factor Foxp3, which represses genes directly involved in T cell activity (Marson et al., 2007; Schubert et al., 2001; Zheng et al., 2007). Spontaneous mutation of *Foxp3* was found to be causative in neonatal autoimmune syndromes *scurfy* and *IPEX*, in mouse and humans, respectively (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). In addition, targeted deletion of *Foxp3* in mice also resulted in autoimmunity and early lethality, confirming the critical role of Foxp3 in immune regulation (Fontenot et al., 2003). Adoptive transfer of either natural $CD4^+CD25^+$ Tregs or $CD4^+CD25^-$ T cells transduced to express Foxp3 rescued animals from disease. Moreover, specific deletion of $Foxp3^+$ Tregs in adult mice also led to autoimmune disease, supporting an essential role for T regulatory cells in immune suppression

throughout life (Kim et al., 2007; Lahl et al., 2007). Based on the requirement for Tregs in maintaining peripheral tolerance, it is not unexpected that there is now a great deal of evidence for T regulatory cell participation in tolerance to cancer.

T regulatory cells are described to exert immune suppressive function in three general ways. First, since Tregs express high levels of CD25, direct competition for IL-2 can limit anti-tumor effector T cell survival and proliferation, particularly in the tumor microenvironment (Antony et al., 2006; Zhang et al., 2005). Second, Tregs may directly kill cross-presenting antigen-presenting cells or effector T cells via granzymes and/or perforin (Cao et al., 2007; Gondek et al., 2005; Grossman et al., 2004; Zhao, 2006). Finally, described through a number of means and involving both direct contact and immunosuppressive cytokines, Tregs can suppress T cell activity or induce myeloid cells to become suppressive of T cell activity (Chen et al., 2005; Sakaguchi et al., 2008; Zou, 2006). The relative importance of these activities and the contexts under which each functions are still under investigation.

In animal models, the functional relationship between T regulatory cells and suppressed anti-tumor immunity has been investigated. The first demonstration of this association was through the use of monoclonal antibodies against the IL-2 receptor α-chain, CD25, which depletes Tregs based on their high expression of CD25. Depletion in mice before or a day after tumor cell inoculation led to tumor rejection that was dependent on either $CD8^+$ T cells or natural killer cells (Onizuka et al., 1999; Shimizu et al., 1999). Since then, Treg depletion via CD25-specific antibodies has been found to similarly enhanced anti-tumor immunity in other transplant tumor models (Zou, 2006). Interestingly, depletion of Tregs in the context of established tumors has not been reported to facilitate immune rejection of tumors.

RESULTS and DISCUSSION

T regulatory cells are expanded in tumor-bearing mice

We examined $Foxp3^+CD25^+CD4^+$ T regulatory cells (Tregs) in *K-ras*^{G12D} lung tumor-bearing mice by flow cytometry (Cheung et al., 2008; Jackson, 2001). We observed an expanded population of Tregs in lungs and lung-draining mediastinal lymph nodes (LNs) in tumor-bearing mice compared to age-matched, tumor-free controls (Figure 1A). The proportion of Tregs increased gradually after tumor initiation and, therefore, correlated with tumor progression. Twenty-two weeks after lung tumor initiation, Tregs had doubled on average in their representation among $CD4^+$ T cells in lungs that bore tumors. In addition, a slightly increased proportion of Tregs in non-draining LNs was observed at this time (Figure 1A).

We then went on to examine Tregs in an immunogenic lung cancer model in which stably-integrating lentiviruses expressing Cre recombinase and tumor antigens are used to infect lungs of *K-ras*$^{LSL-G12D}$ mice (DuPage and Jacks, unpublished). Cre induces expression of oncogenic K-ras and, thus, tumor initiation. In these same tumor-initiating cells, defined antigens fused to *Firefly* luciferase are also expressed and are, hence, tumor-specific antigens. In *K-ras*$^{LSL-G12D}$ mice infected with a lentivirus bearing Cre and a Luciferase-SIY fusion (Lv-LSIY-Cre), we again observed expanded populations of Tregs by flow cytometry. When counted, this amounted to a 4-fold increase in Treg number in mediastinal LNs and a 3-fold increase in lungs of Lv-LSIY-Cre tumor-bearing mice over age-matched, tumor-free controls (Figure 1B).

Low-grade lentivirus-induced, immunogenic lung tumors are infiltrated by high numbers of lymphocytes, in contrast to non-immunogenic lung tumors induced by non-integrating adenoviruses expressing Cre (DuPage and Jacks, unpublished). These lymphocytes are heterogeneous in type, but tumor antigen-specific $CD8^+$ T cells are among the tumor-infiltrates.

We speculated that perhaps a higher proportion of T regulatory cells was needed to maintain suppression of tumor-specific T cells, as they did not appear to substantially impede progression of established tumors. *R26*$^{LSL\text{-}LSIY/+}$ mice express LSIY ubiquitously at low levels and exhibit both central and peripheral tolerance to SIY (Cheung et al., 2008). We infected *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{+/+}$ and *K-ras*$^{LSL\text{-}G12D/+}$;*R26*$^{LSL\text{-}LSIY/+}$ mice with Lv-LSIY-Cre to generate matched animals with immunogenic and non-immunogenic lung tumors, respectively. We refer to these animals as bearing tumors with tumor-specific antigens (TSAs) or tumor-associated self-antigens (TAAs). When we evaluated these mice 20 weeks after infection, we observed no difference in the proportions of T regulatory cells in either bronchoalveolar lavage (BAL) or lung-draining LN (Figure 1C). We then counted Tregs and found more Tregs on average from lungs of animals bearing immunogenic tumors than those with non-immunogenic tumors (data not shown). Because of the small number of animals analyzed and the variability in numbers of tumors induced, we investigated whether this effect could be due to differences in tumor burden. Indeed, when we counted the number of tumors on the surface of each lung, we found that tumor multiplicity directly correlated with the proportion of Tregs found in their BALs (data not shown). Thus, T regulatory cells expand in lung tumor-bearing mice and the degree of expansion correlates with tumor burden rather than with the apparent immunogenicity of tumors.

DT depletes Tregs in Foxp3-DTR mice, but chronic DT treatment results in resistance

Depletion of T regulatory cells has resulted in increased anti-tumor T cell responses in many mouse cancer models (Zou, 2006). These anti-tumor responses have led to prevention of tumor outgrowth or tumor regression when Tregs were depleted before tumor establishment (Onizuka et al., 1999; Shimizu et al., 1999). Treg depletion as a successful mono-therapy leading to tumor regression has not been reported, however, in established tumors

(Degl'Innocenti et al., 2008). One possibility for this result is inefficient depletion of T regulatory cells. Recently, two groups have demonstrated highly efficient Treg depletion using genetically-modified mice that express the diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter (Kim et al., 2007; Lahl et al., 2007). Diphtheria toxin (DT) administration in these mice resulted in severe autoimmunity similar to mice genetically deficient for Treg production. We acquired a different transgenic Foxp3-DTR that was unpublished at the time, generated in Dan Littman's laboratory, for our experiments (Quezada et al., 2008).

We examined the efficiency of Treg depletion by DT administration in *Foxp3-DTR* transgenic mice. Testing various doses of DT, we found saturating level of Treg depletion 24 hours after a dose of 40ng per mouse (data not shown). This single dose resulted in ~85% depletion of Tregs in lymph nodes and ~75% in the spleen. In spite of this, when we examined the ability to sustain depletion, we discovered resistance after multiple administrations of DT (Figure 2A). After 4 doses administered every other day, we found only ~50% of Tregs were being depleted in mesenteric LNs. In fact, in the spleen, animals had nearly no reduction in Treg proportions after just 2 doses and after 4 doses, Tregs were actually almost twice as numerous as in undepleted mice (Figure 2A). This suggests not only resistance to Treg depletion by DT administration, but, perhaps, an active mechanism that compensates when Tregs are lost. We did not observe any appreciable changes to Treg population size in the thymus regardless of the number of DT treatments.

α-CD25 antibody administration improves long-term Treg depletion efficiency

Clearly, diphtheria toxin administration alone to deplete T regulatory cells in *Foxp3-DTR* mice would be insufficient for studies on anti-tumor efficacy. Thus, we sought to combine DT administration with α-CD25 antibody treatment. α-CD25 antibodies have been widely used to

deplete Tregs in mice as Tregs are known to express high levels of the IL-2 receptor α-chain, CD25 (Zou, 2006). We found that a single dose of 250μg α-CD25 antibody per mouse was optimal, resulting in sustained depletion of ~65% of Tregs in mesenteric lymph nodes and ~50% in spleens (Figure 2B). Interestingly, higher doses resulted in a reduction in depletion efficiency (data not shown). Furthermore, when we examined Tregs that remained in α-CD25-treated mice, we noticed a significant reduction in the level of CD25 staining on cell surfaces (data not shown). As Tregs function is dependent on IL-2, it is possible that remaining Tregs have reduced function (Antony et al., 2006).

We compared animals a day after a single 40ng/g dose of DT to animals 7 days after 250μg α-CD25 antibody, and we detected similar level of Treg depletion in the mesenteric LNs and somewhat better depletion in the spleen with DT. When the two treatments were combined, we observed greater depletion than either treatment alone in both the mesenteric LNs and spleens (Figure 2C). In addition, in some animals, we administered CD25-specific antibody 7 days before analysis followed by 3 doses of 40ng/g DT given every other day starting 5 days before analysis. This resulted in a similar degree of Treg depletion as α-CD25 antibody followed by a single dose of DT when analyzed a day after the last DT treatment, but presumably multiple doses yielded reduced Treg numbers for 5 days prior to analysis (Figure 2C). Thus, we chose this combined treatment regimen as we could sustain Treg depletion to ~80% in mesenteric LNs of *Foxp3-DTR* transgenic mice.

Tumor-specific T cells are more functional after Treg depletion in tumor-bearing mice

Using this combined method of Treg depletion, we proceeded to determine how this would affect T cell activity in tumor-bearing mice. We crossed *Foxp3-DTR* transgenic mice to *K-ras*$^{LSL\text{-}G12D/+}$ mice to generated *K-ras*$^{LSL\text{-}G12D/+}$;*Foxp3-DTR* animals, which we infected with

lentiviruses carrying ovalbumin antigens and Cre (Lv-OVA-Cre). This resulted in lung tumors that express ovalbumin antigens as tumor-specific antigens and that can be recognized by $CD8^+$ T cells expressing the OT-I TCR from OT-I transgenic mice (DuPage and Jacks, unpublished). We either treated tumor-bearing animals with combined α-CD25/DT regimen or with α-CD25 alone or left mice untreated. All animals also received transfer of $1x10^6$ naive $CD45.1^+$ OT-I T cells 7 days after the start of treatment and were analyzed 7 days after T cell transfer.

We found <50% Treg depletion in lung-draining mediastinal LNs of α-CD25/DT-treated mice compared to untreated animals upon analysis, but only ~20% depletion with α-CD25 alone (Figure 3A). When we stimulated mediastinal LNs *in vitro* with SIINFEKL (SIN) peptide, to which OT-I $CD8^+$ T cells respond, we discovered that a greater proportion of OT-1 cells (detected by the CD45.1 marker) from α-CD25/DT-treated mice stained positively for intracellular cytokines than from untreated mice. Specifically, there was a ~60% increase in the proportion of IFN-γ^+ cells and nearly a 200% increase in the proportion of TNF-α^+IFN-γ^+ double-positive cells (Figure 3B). Interestingly, α-CD25 alone failed to result in a change in cytokine staining, despite having an intermediate level of Treg depletion. This suggests a threshold level of T regulatory cell depletion that is necessary to have an effect on anti-tumor T cell activity.

Anti-tumor T cells are reduced in α-CD25 administered mice

We went on to examine if Treg depletion would allow greater proliferation of anti-tumor OT-I T cells. Rather than detecting a greater proportion of $CD8^+$ T cells staining positive for CD45.1, we actually observed a reduction of ~40% under both treatment conditions compared to untreated mice (Figure 3C). α-CD25/DT combination treatment and α-CD25 alone had comparable proportions of OT-I cells, indicating that antibody administration alone could cause

loss of OT-I cells. As activated T cells express CD25 on their surfaces, albeit to a lesser extent than T regulatory cells, this is not an unexpected finding.

Finally, we examined how the reduction in OT-I$^+$ cells influenced the proportion of CD8$^+$ cells that were capable of effector function towards SIN-presenting targets. On average, we noticed almost equivalent percentages of CD8$^+$ cells staining for IFN-γ after SIN stimulation in untreated and α-CD25/DT-treated mice (Figure 3D). TNF-α$^+$IFN-γ$^+$ double-positive cells still represented an increased fraction of CD8$^+$ cells, though this was less significant now. In mice treated with just α-CD25, both IFN-γ$^+$ and TNF-α$^+$IFN-γ$^+$ double-positive cells were actually reduced in proportion compared to untreated mice, suggesting that CD25-specific antibody treatment alone could actually be detrimental to anti-tumor activity (Figure 3D). Taken together, although we can achieve substantial T regulatory cell depletion with combined α-CD25/DT treatment, some of these effects are negated due to the concomitant reduction in anti-tumor effector T cell numbers.

A reagent for improved T regulatory cell depletion

We hypothesize that the mice we used here were inefficient in Treg depletion because of epigenetic silencing of the *Foxp3-DTR* transgene. This would allow T regulatory cells that expressed low levels or failed to express DTR to have reduced susceptibility to death via diphtheria toxin. As mentioned, two groups have demonstrated highly efficient Treg depletion using genetically-modified mice containing Foxp3-driven diphtheria toxin receptor expression (Kim et al., 2007; Lahl et al., 2007). One of these groups targeted the *DTR* gene to the 3' end of the endogenous *Foxp3* gene (Kim et al., 2007). Thus, in order to decrease or silence expression of DTR in these knock-in mice, Tregs would have an associated loss of Foxp3 expression. As Foxp3 is essential for T regulatory cell function as immune suppressors, this method assures

efficient depletion of Tregs. We have since acquired these animals and are currently breeding these knock-in animals to *K-ras*$^{LSL\text{-}G12D/+}$ mice for our experiments.

Considerations in interpreting results of Treg depletion in Foxp3-DTR mice

The expression of Foxp3 has been demonstrated in epithelial cells. Foxp3 is described to be a cell-autonomous tumor suppressor, directly repressing expression of the growth factor receptor ErbB2/Her2 and the E3 ubiquitin ligase complex component Skp2 oncogenes in mouse mammary cancer (Zuo et al., 2007a; Zuo et al., 2007b). In contrast, expression of Foxp3 in pancreatic cancer promotes tumor progression in a non-cell-autonomous fashion by suppressing the proliferation of anti-tumor T cells, similar to the function of T regulatory cells themselves (Hinz et al., 2007). Regardless of the role of Foxp3 expression in epithelial cells, expression itself could render epithelial cells susceptible to diphtheria toxin-induced death in *Foxp3-DTR* mice. Furthermore, DT-induced death to large number of Tregs could potentially results in an immune activating adjuvant effect independent of the effect produced by loss of immune suppressive cells. Both indirect consequences of DT administration in Foxp3-DTR mice can be controlled for by transfer of Foxp3^{+} Tregs that do not express DTR. Possible synergistic effects between Treg depletion and DT-induced death of cancer cells or an adjuvant effect of massive cell death will be more difficult to rule out.

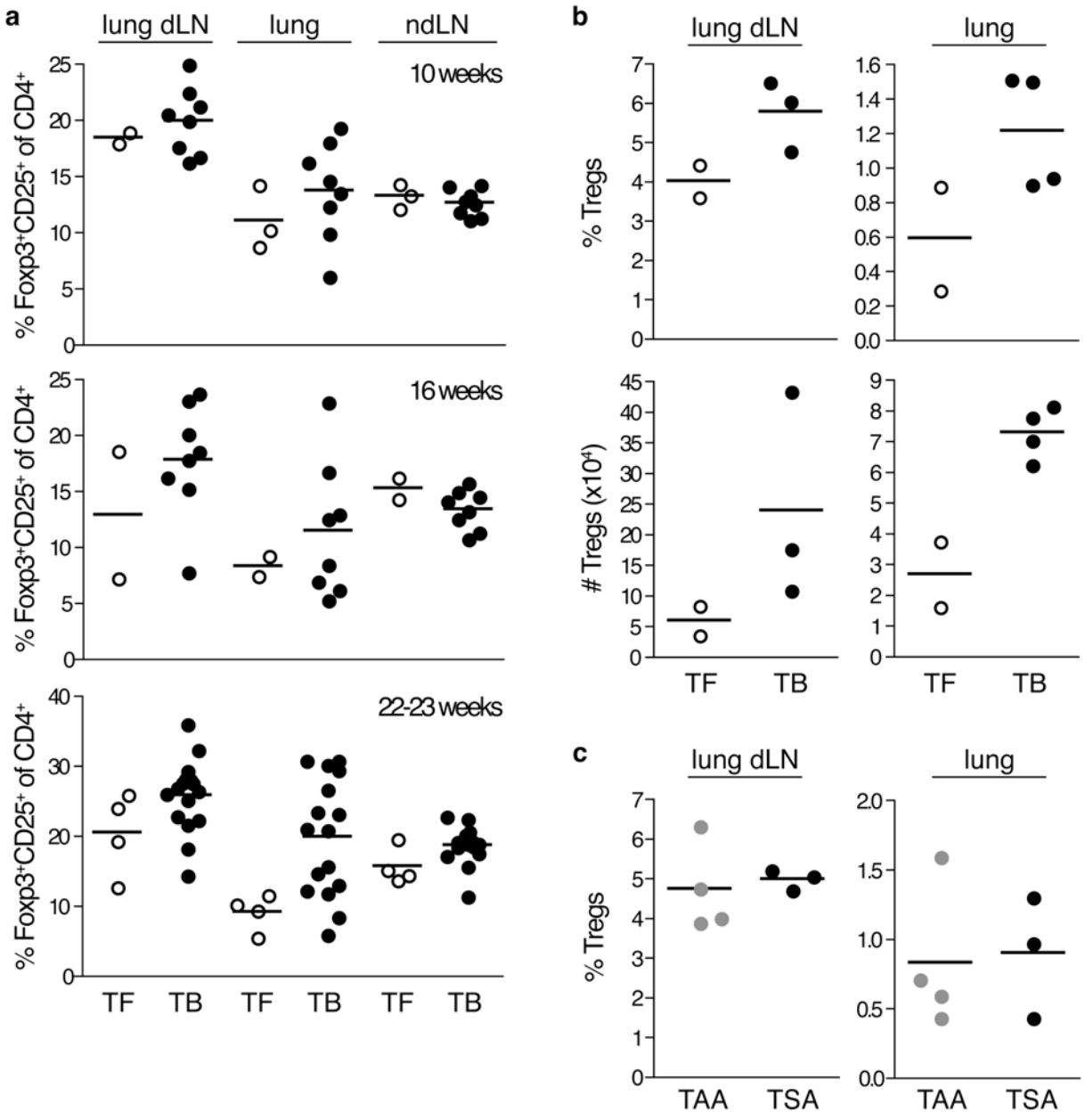

Figure 1. T regulatory cells are expanded in lungs and lung draining lymph nodes of tumor-bearing mice. *A.* Percentage of $CD4^+$ lymphocytes positive for both Foxp3 and CD25 in lung draining lymph nodes (dLN), lungs, and non-draining mesenteric LN (ndLN) of tumor-free *(*TF*, open circles)* and *K-ras*^{G12D} lung tumor-bearing mice *(*TB, *closed circles)* 10, 16, and 22-23 weeks after Ad-Cre infection. *B*. Percentage (top graphs) and number (bottom graphs) of $Foxp3^+CD25^+CD4^+$ Tregs in lung dLNs and lungs of mice without tumors *(*TF*, open circles)* and mice with immunogenic lung tumors 28 weeks after Lv-LSIY-Cre infection*(*TB, *closed circles)*. *C*. Percentage of $Foxp3^+CD25^+CD4^+$ Tregs in lung dLNs and lungs of mice 20 weeks after Lv-LSIY-Cre infection. Mice bear lung tumors expressing SIY as a tumor-associated antigen *(*TAA*, gray circles)* or tumor-specific antigen *(*TSA, *black circles)*. *Lines*, mean values.

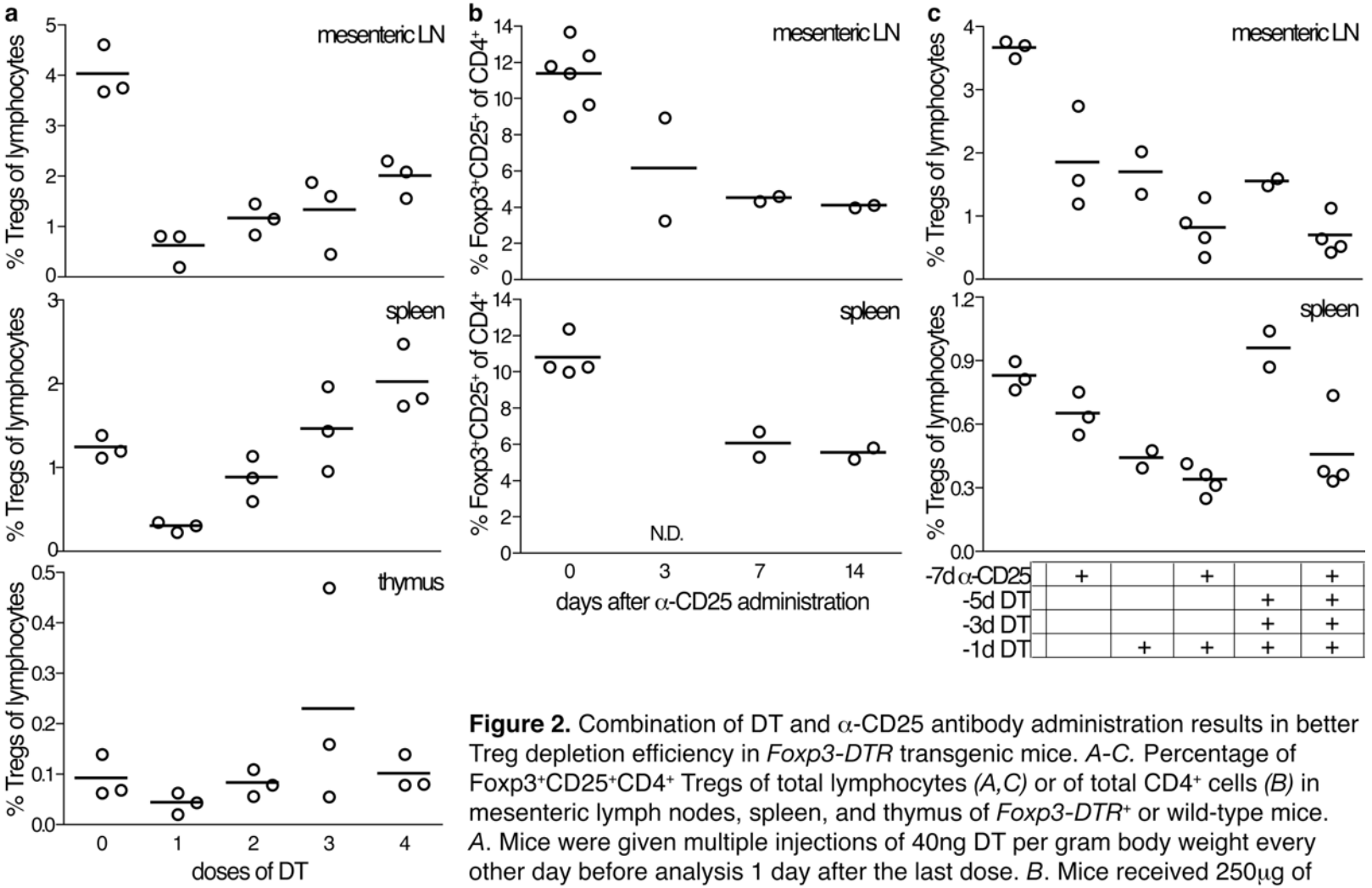

Figure 2. Combination of DT and α-CD25 antibody administration results in better Treg depletion efficiency in *Foxp3-DTR* transgenic mice. *A-C.* Percentage of Foxp3⁺CD25⁺CD4⁺ Tregs of total lymphocytes *(A,C)* or of total CD4⁺ cells *(B)* in mesenteric lymph nodes, spleen, and thymus of *Foxp3-DTR*⁺ or wild-type mice. *A*. Mice were given multiple injections of 40ng DT per gram body weight every other day before analysis 1 day after the last dose. *B*. Mice received 250μg of α-CD25 (PC-61) antibody and were analyzed 3, 7, and 14 days afterwards. *C*. Mice received a combination of DT and α-CD25 antibody as shown and were analyzed on day 0. *Lines*, mean values.

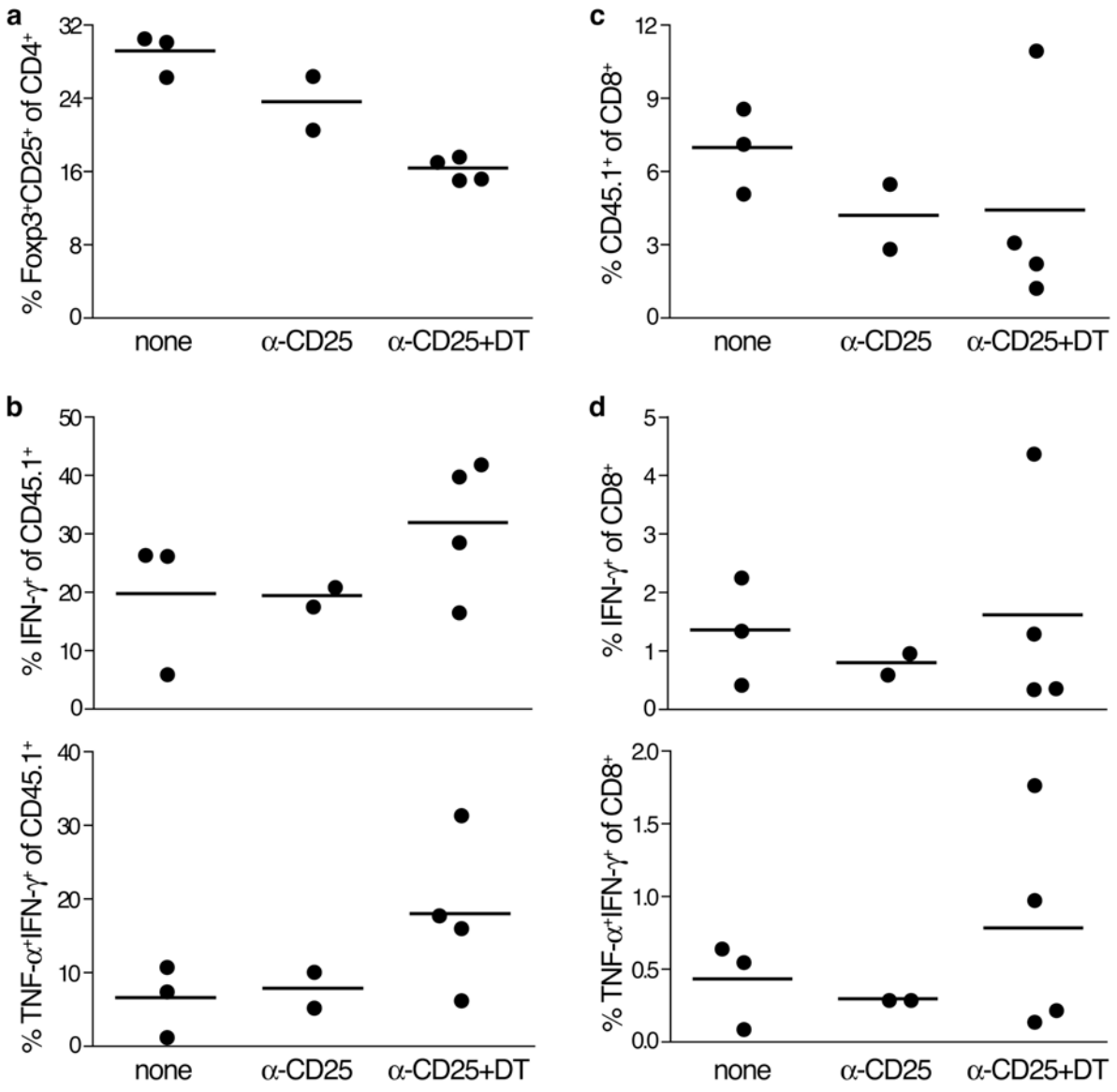

Figure 3. Naive tumor antigen-specific T cells gain more function when transferred into Treg depleted mice, but α-CD25 antibody reduces the proportion of tumor-antigen specific T cells. *A-D.* Lv-OVA-Cre infected mice bearing lung tumors are not treated, treated with 250μg of α-CD25 antibody alone, or α-CD25 in combination with 4 doses of 40ng DT per gram body weight given every other day starting 6 days after α-CD25. $1x10^6$ naive OT-1$^+$CD45.1$^+$ T cells were transferred into animals 7 days after α-CD25 and animal's lung draining lymph nodes were analyzed on day 14. *A*. Percentage of CD4$^+$ lymphocytes positive for both Foxp3 and CD25. *B*. Percentage of CD45.1$^+$ cells positive for intracellular IFN-γ *(top)* or double positive for TNF-α and IFN-γ *(bottom)* after *in vitro* stimulation with SIN. *C*. Percentage of CD8$^+$ that are CD45.1$^+$. *D.* Percentage of CD8$^+$ cells with intracellular IFN-γ *(top)* or TNF-α and IFN-γ double positive *(bottom)* after *in vitro* stimulation with SIN. *Lines*, mean values.

METHODS

Mice. *OT-I* mice were provided by J. Chen, *Foxp3-DTR* mice were a gift from D. Littman, and *CD45.1*$^{+}$ and *Rag2*$^{-}$ mice were purchased from Jackson Laboratories. *K-ras*$^{LSL-G12D}$ mice were generated in our laboratory (Jackson, 2001; Tuveson et al., 2004). *R26*$^{LSL-LSIY}$ mice were also generated in our laboratory (Cheung et al., 2008). Donor OT-I cells for transfer experiments were from C57BL/6 *OT-I;CD45.1*$^{+/+}$*;Rag2*$^{-/-}$ mice and recipients were C57BL/6. To induce tumors, mice were infected with Adenovirus-Cre, Lv-LSIY-Cre, or Lv-OVA-Cre intra-tracheally. Animal studies were approved by MIT's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

T regulatory cell depletion. Diphtheria toxin was purchased from Sigma (cat# D0564) and dissolved in PBS for injection. α-CD25 antibody (PC61) was produced from a hybridoma cell lines purchased from ATCC. Hybridomas were grown up in spinner flasks in DMEM-10 full medium. PC61 antibodies were prepared collecting and filtering the hybridoma growth media, then purifying PC61 via a protein G column. The antibody concentration was determined by spectrophotometer and purified antibody was diluted in PBS to be injected.

Cell isolation. Single-cell suspensions from lymph nodes, spleen and thymus were generated by mechanical disruption. For lung preparations, tissue samples were minced and digested at 37^{o}C for 30 minutes in 125U/mL of collagenase type I (Gibco) in phosphate-buffered saline before

mechanical disruption and passage through a 70μm-pore filter (BD Falcon). Red blood cells were lysed with an aqueous solution containing 0.83% NH_4Cl, 0.1% $KHCO_3$, and 0.000037% Na_2EDTA at pH 7.2-7.4. Single-cell suspensions were passed through a 35μm-pore cell-strainer cap (BD Falcon) before culture, intravenous transfer, or staining for flow cytometry.

Reagents and flow cytometry. SIINFEKL peptide was synthesized by MIT's Biopolymers Laboratory. The following antibodies were purchased from BD Pharmingen: αCD4 (H129.19), αCD8 (53-6.7), αCD16/CD32 (2.4G2), αCD25 (7D4 and PC61), and αCD45.1 (A20). Intracellular Foxp3 staining was performed with FITC-α-mouse/rat Foxp3 staining set from eBioscience and intracellular IFN-γ and TNF staining was performed with BD Cytofix/Cytoperm Fixation/Permeabilization kit, according to manufacturers' instructions. Streptavidin-allophycocyanin and streptavidin-phycoerythrin conjugates were purchased from BD Pharmingen. Cells were read on a FACSCalibur (BD Biosciences) and analyzed using Flowjo 8.1 software (Tree Star Inc). Dead cells were excluded by 1μg/mL propidium iodide staining (Sigma).

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APPENDIX 2:

The adaptive immune system promotes lung tumor progression

Ann F. Cheung[1], Michel J.P. DuPage[1], H. Katie Dong[1], Denise Crowley[1,2] and Tyler Jacks[1,2]

[1]Koch Institute and Department of Biology and [2]Howard Hughes Medical Institute, MIT, Cambridge, MA.

The author, with some help from Michel DuPage, bred *K-ras*^{LA2} and *Rag2*$^{-}$ mice and performed the experiments described. Katie Dong, an undergraduate student supervised by the author, assisted in the quantification of tumor burden. Denise Crowley processed and cut the numerous sections used for tumor quantification. All experiments were performed in the laboratory of Tyler Jacks.

ABSTRACT

The adaptive immune system has been described to have paradoxical roles in cancer. Just as it serves to eliminate pathogens, T and, perhaps, B cells can function to suppress tumor growth. On the other hand, both cell types have been described to facilitate chronic inflammation, which promotes tumor progression. We have used a spontaneous model of human adenocarcinoma in the mouse to assess the role of adaptive immunity in cancer development. We observe that adaptive immuntiy promotes tumorigenesis, such that fewer tumors are detected in the absence of B and T lymphocytes.

INTRODUCTION

The adaptive immune system consists of B and T lymphocytes, both of which have antigen-specific recognition receptors. In an antigen-specific manner, both cell types are known to possess essential roles in the response to and elimination of pathogens. Additionally, the adaptive immune system has been described to have paradoxical roles in cancer development. In the same manner by which pathogen and pathogen-infected cells are eliminated, T cells and, perhaps, B cells can target malignant cancer cells by way of mutant, mis-expressed, or over-expressed antigens. Consistent with this notion, immune-deficient animals have been reported to develop spontaneous and carcinogen-induced tumors at higher frequency than fully immune competent mice (Dunn et al., 2004; Swann and Smyth, 2007). In fact, there is great hope in utilizing the immune system therapeutically against cancer (Rosenberg et al., 2008; Stagg et al., 2007).

On the other hand, chronic inflammation has been observed to contribute to cancer (Mantovani et al., 2008). While this phenomenon has been described to be mediated primarily by innate immune cells, cells of the adaptive immune system can certainly promote innate immunity. In fact, B cell secretion of immunoglobulins was observed to promote chronic inflammation in the skin that supports cancer development (de Visser et al., 2005). Furthermore, there is evidence that a low level of T cell reactivity to tumors actually encourages tumor progression (Prehn and Prehn, 2008). We sought to determine the effect of the adaptive immune system in a spontaneous model of lung cancer that does not rely on chronic inflammation for oncogene activation.

RESULTS and DISCUSSION

The adaptive immune system promotes lung tumor progression

The *K-ras*^{LA2} allele is a duplication of the first coding exon of *K-ras*, both copies of which bear the G12D oncogenic mutation, intervened by a neomycin drug resistance gene (Johnson et al., 2001). In its germline state, *K-ras*^{LA2} is silent. Oncogenic K-ras is only expressed after spontaneous recombination of the allele, which causes loss of the drug resistance gene and restoration of mutant exon 1 to a single copy. This event occurs in a sporadic manner and results in tumor-prone animals that develop lung tumors with full penetrance.

To determine the role of the adaptive immune system in lung tumor development, we crossed tumor-prone *K-ras*^{LA2} mice onto *Rag2* heterozygous and null backgrounds. We observed that tumors from *K-ras*$^{LA2/+}$*;Rag2*$^{+/-}$ and *Kras*$^{LA2/+}$*;Rag2*$^{-/-}$ mice formed with similar morphologies (data not shown). We then proceeded to compare tumor burdens of littermates sacrificed at 4, 8, and 16 weeks of age. Analysis of animals at 4 and 8 weeks revealed no significant difference in the median ratio of tumor to lung areas. At 16 weeks of age, however, we noticed a substantially greater median tumor burden in *K-ras*$^{LA2/+}$*;Rag2*$^{+/-}$ mice compared to *Kras*$^{LA2/+}$*;Rag2*$^{-/-}$ littermates (Figure 1A). Examining these data kinetically over time, it is apparent that lung tumors overall in *Rag2*$^{-/-}$ mice exhibit stagnant tumor development.

We then attempted to determine if the cause of increased tumor burden in *Rag2*$^{+/-}$ mice was reflected by an increase in tumor number or average size. At both 8 and 16 weeks of age, we observed more tumors in immune-competent mice compared to immune-compromised mice (Figure 1B). Furthermore, average tumor size was not significantly different at either 8 or 16 weeks, although tumors tended to be larger at 8 weeks and smaller at 16 weeks in *Rag2*$^{-/-}$ mice versus *Rag2*$^{+/-}$ (Figure 1C). Therefore, animals with intact adaptive immune systems have a

greater lung tumor burden than immune-compromised mice and this may be a reflection of increased tumor initiation or progression at early stages of tumor devleopment.

These data indicate that the adaptive immune system is necessary to promote tumor development. As both B and T cells are absent in *Rag2*$^{-/-}$ mice, either cell type or both together could be responsible. Indeed, there is evidence for both (de Visser et al., 2005; Prehn and Prehn, 2008). It is possible that there are opposing effects on tumor development by different components of adaptive immunity. This notion is, perhaps, supported by the observation of biphasic tumor growth as judge by average tumor size at 8, then 16 weeks in *Rag2*$^{-/-}$ mice. Alternatively, it is formally possible that expression of Rag2 is required in a cell-autonomous manner for cancer growth. Although expression of Rag2 is restricted to lymphocytes in normal somatic tissues, it is possible that expression is activated in the context of malignant growth. In this context, recombinase activity could cause genomic changes that encourage cancer advancement. Thus, some component of adaptive immunity is required to promote lung tumor progression, but further studies will be necessary to elucidate the mechanism.

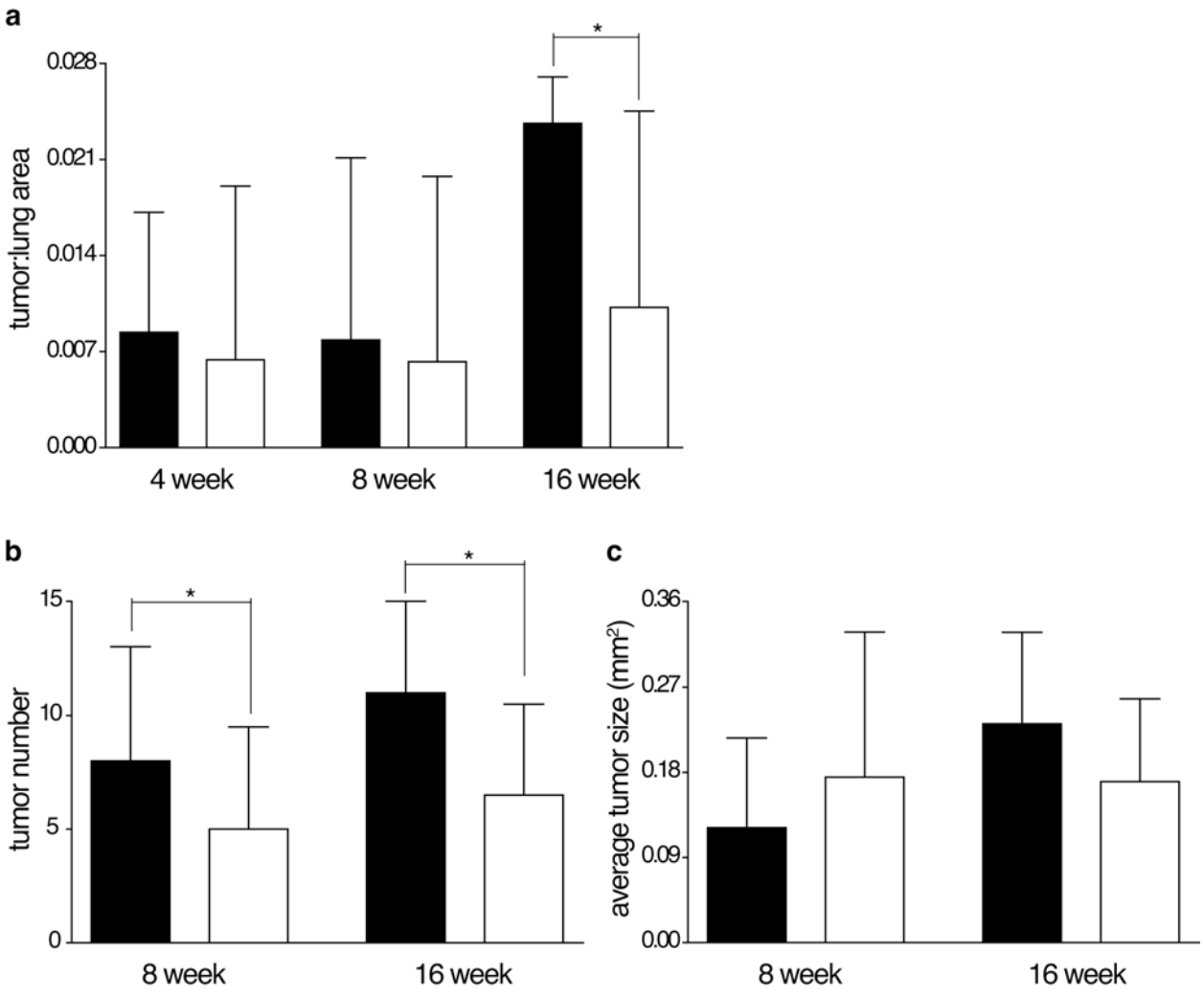

Figure 1. *K-ras*$^{LA2/+}$*;Rag2*$^{+/-}$ mice develop greater lung tumor burdens then *K-ras*$^{LA2/+}$*;Rag2*$^{-/-}$ littermates. Quantification of tumors in lungs on tissue sections. *A.* Median tumor to lung area ratios for *K-ras*$^{LA2/+}$*;Rag2*$^{+/-}$ (black bars) and *K-ras*$^{LA2/+}$*;Rag2*$^{-/-}$ (white bars) mice at 4, 8, and 16 weeks of age. *B.* Median tumor number in mice of the same genotypes as in *a* at 8 and 16 weeks of age. *C*. Median of average tumor sizes in mice of the same genotypes as in *a* at 8 and 16 weeks of age. *Bars*, median + interquartile range, *p <0.05 by Student's *t* test.

METHODS

Mice. *Rag2*- mice were purchased from Taconic. *K-ras*^{LA2} mice were generated in our laboratory (Johnson et al., 2001). Animals bearing *K-ras*^{LA2} *and Rag2*$^{-}$ alleles used for tumor quantification were on a mixed 129S4/S6 (SvJae/SvEv) genetic background and comparisons were performed among littermates. For tumor studies, lung histology was prepared and analyzed by Bioquant Image Analysis software as previously described (Jackson, 2005). Animal studies were approved by MIT's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

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APPENDIX 3:

Therapeutic vaccination against autochthonous lung tumors over-expressing a self-antigen breaks tolerance

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The author performed all of the experiments described. All experiments were performed in the laboratory of Tyler Jacks.

SUMMARY

Therapeutic vaccines are being used extensively in pre-clinical and clinical trials against various forms of cancer (Lollini et al., 2006; Purcell et al., 2007). These vaccines vary in form and may or may not be designed against specific tumor antigens. In addition, vaccine therapy may be used as a monotherapy or in combination with other therapies, including other immune-based therapies. We have demonstrated the ability of a live Influenza vaccine expressing SIY antigen (WSN-SIY) to break immune tolerance against a self-antigen SIY that is over-expressed by K-ras^{G12D}-driven lung tumors(Cheung et al., 2008). Here, we have pursued these initial observations further to determine whether WSN-SIY vaccine has therapeutic efficacy against autochthonous lung tumors.

Previously, we observed high cytotoxic activity against SIY-expressing target cells in WSN-SIY-infected recipients of naïve 2C cells, irrespective of genotype (*R26*$^{+/+}$ or *R26*$^{LSL\text{-}LSIY/+}$) and tumor burden(Cheung et al., 2008). In WSN-SIY-infected, tumor free *R26*$^{LSL\text{-}LSIY/+}$ animals, transferred 2C cells failed to be detectable and could not be boosted to proliferate a month after transfer (data not shown). In tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice, however, this was not the case. When we infected tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice with WSN-SIY concurrent with naïve 2C cells transfer, we could still detect 2C cells 3 weeks after transfer by flow cytometry. These 2C cells represented nearly 40% of all CD8$^+$ T cells in bronchoalveolar lavages (BALs) of these mice at this time (Figure 1A). In WSN-infected (control Influenza, not expressing SIY) *R26*$^{LSL\text{-}LSIY/+}$ mice, however, 2C cells were only 10% of CD8$^+$ T cells. In addition, lung draining lymph nodes of *R26*$^{LSL\text{-}LSIY/+}$ mice had comparable proportions of 2C cells whether WSN- or WSN-SIY-infected. These data indicate that 2C cells are better able to persist in the presence of lung tumors over-expressing SIY, despite peripheral tolerance. These conditions do not completely

overcome peripheral tolerance to SIY, however, as tumor-bearing *R26*$^{+/+}$ littermates are able to maintain higher proportions of 2C cells (about 70% of CD8^{+} T cells in BAL) when WSN-SIY-infected (Figure 1A).

To determine the influence of persisting 2C cells after WSN-SIY vaccination on tumors over-expressing SIY, we took tumor-bearing lungs from WSN- and WSN-SIY-infected mice for analysis. We assessed tumor burden by luciferase assay of whole lungs *ex vivo*. Remarkably, we observe a 90% decrease in luciferase activity in WSN-SIY vaccinated mice compared to WSN vaccinated mice (Figure 1B). Assuming that decreased luciferase represents death of tumor cells rather than decreased luciferase expression from live tumor cells, this decrease extrapolates to a 90% reduction in tumor burden in WSN-SIY vaccinated animals after just 3 weeks. As this assumption cannot be made with the available data, further analysis will be needed to determine the actual effect on SIY over-expressing lung tumors. This study can be followed up by longitudinal microCT analysis, histology, and luciferase assays on individual tumors remaining after treatment. Whether tumor cells die or decrease luciferase expression, however, it is apparent that WSN-SIY vaccination in addition to naïve 2C cell transfer influences tumors that over-express SIY self-antigen.

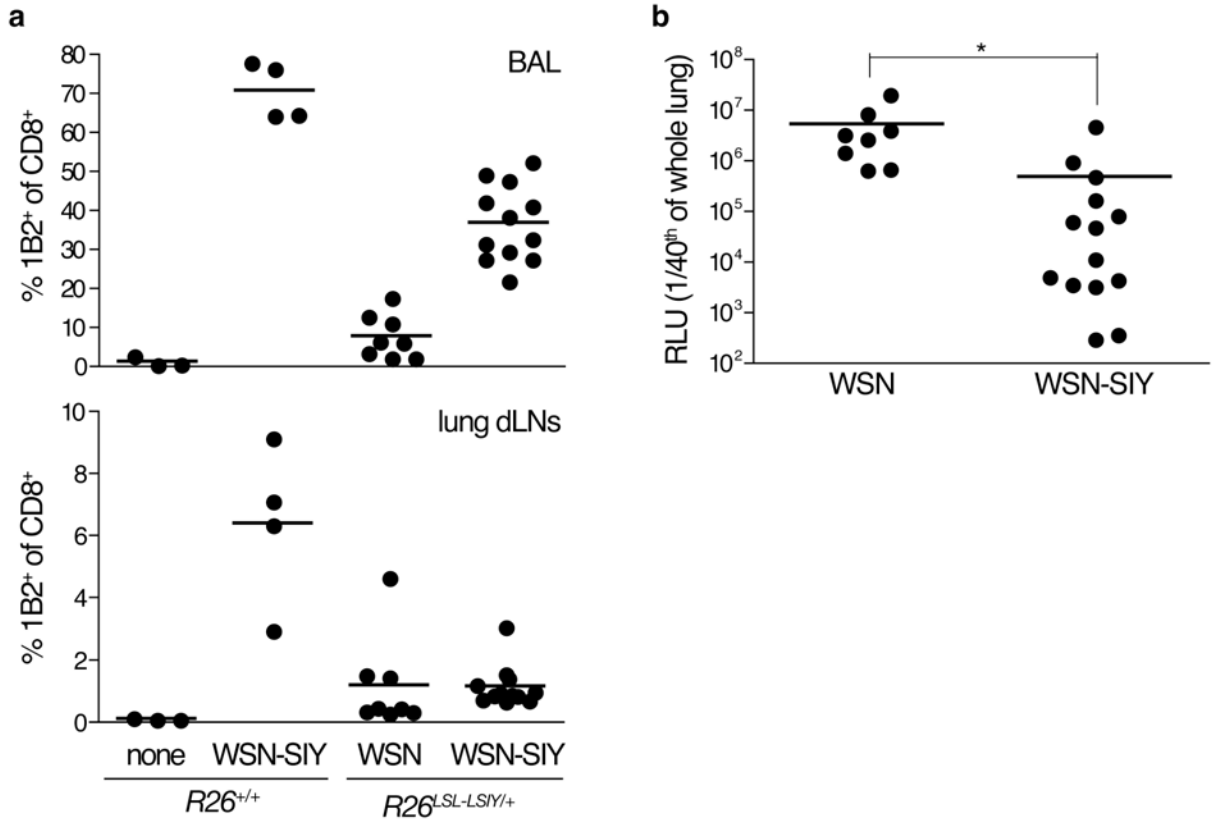

Figure 1. Therapeutic vaccination of *R26*$^{LSL\text{-}LSIY}$ mice bearing lung tumors that over-express SIY results in maintenance of transferred 2C cells and reduced luciferase reporter activity in lungs. *A.* Percentage of CD8$^+$ cells that stain positive for 1B2 in bronchoalveolar lavage *(top)* and lung draining lymph nodes *(bottom)* of untreated and WSN-SIY-treated *R26*$^{+/+}$ and WSN and WSN-SIY-treated *R26*$^{LSL\text{-}LSIY/+}$ mice bearing tumors 3 weeks after intravenous transfer of naive 2C cells concurrent with vaccination. *B.* Luciferase activity in 1/40th of whole lung from lung tumor-bearing *R26*$^{LSL\text{-}LSIY/+}$ mice 3 weeks after receiving naive 2C cells concurrent with either WSN or WSN-SIY vaccination. *Bars*, mean, *p <0.05 by Student's *t* test.

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APPENDIX 4:

Exploring combination chemotherapy and immunotherapy against autochthonous lung tumors

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The author performed all of the experiments described with help from Michel DuPage in lentiviral infections, from Trudy Oliver and James Burke in cisplatin treatments, and from Kamena Kostova in microCT data analysis. All experiments were performed in the laboratory of Tyler Jacks.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide with a five-year survival rate of 15%. A major obstacle to successful treatment is the resistance of cancer to chemotherapy. Cisplatin is a commonly used chemotherapeutic agent in multiple types of cancer including lung cancer. Chemotherapy with cisplatin has shown therapeutic efficacy in our mouse models of non-small cell lung cancer (NSCLC), yet some tumors are either inherently resistant or acquire resistance to therapy (Oliver et al, in preparation). The majority of K-ras^{G12D}-driven lung tumors respond to a single dose of cisplatin by inducing DNA damage signaling and undergoing cell cycle arrest or apoptosis. In fact, tumor regression and stasis were observed by micro-computed tomography (micro-CT) analysis of mice treated with just two doses of cisplatin. Despite reducing tumor growth, cisplatin treatment did not prolong survival of tumor-bearing *K-ras*$^{LSL\text{-}G12D/+}$ mice when animals received 4 sequential doses. Interestingly, tumors treated with cisplatin in this manner failed to show signs of DNA damage or reduced proliferation in response to a final dose of cisplatin in animals that had a month-long interruption in chemotherapy. These data indicate that lung tumors acquire resistance to cisplatin after prolonged therapy. Thus, while cisplatin initially improves tumor burden, it also promotes tumor progression and resistance in the long-term.

The primary goal of conventional anti-cancer therapies, such as chemotherapies and radiation therapy, is to directly kill tumor cells that exist in the presence of many sources of cellular stress (Luo et al., 2009). Upon killing tumor cells, however, chemotherapeutic agents also support anti-tumor immune responses. DNA damage induced by various chemotherapeutic drugs has been demonstrated to lead to the up-regulation of stress ligands on tumor cells that can stimulate the anti-tumor activity of NK cells and $CD8^+$ T cells (Diefenbach et al., 2001; Gasser et

al., 2005). Chemotherapy may also enhance tumor antigen presentation by improving the recruitment and activation of dendritic cells (DCs) that can engulf and cross-present tumor antigens to T cells (Zitvogel et al., 2008). Translocation of calreticulin to the surface of cancer cells after anthracyclin or γ-irradiation treatment has been found to govern DC engulfment and, hence, the immunogenicity of cell death in response to chemotherapy (Obeid et al., 2007). Furthermore, it has been observed for some time that dying tumor cells and their debris can induce DC maturation and the generation of anti-tumor cytotoxic T lymphocytes (Gallucci et al., 1999; Shi et al., 2000). Recently, a number of "danger" signals derived from dying and distressed cells after chemotherapeutic treatment have been found to be endogenous proteins, such as heat shock proteins, alarmin high-mobility-group box 1 protein, and uric acid (Apetoh et al., 2007; Binder et al., 2000; Obeid et al., 2007; Shi et al., 2003). These signals act by binding to pattern-recognition receptors on DCs, like Toll-like receptors (TLRs)-2 or -4, originally thought to only bind pathogen-associated molecules (Kono and Rock, 2008; Tesniere et al., 2008). Finally, chemotherapeutic agents have been shown to improve the trafficking of T cells to the tumor, perhaps by changing the inflammatory mediators in the tumor microenvironment or inducing the expression of attractive chemokines (Fujimori et al., 2005; Lake and Robinson, 2005; Matsumura et al., 2008).

Based on the immune-stimulatory properties of chemotherapy, one might postulate the ability to improve treatment of cancer by combining chemotherapy with immune therapy, particularly in light of acquired resistance to cisplatin after prolonged treatment. In fact, synergistic action between chemotherapy and immune therapy has been observed in several transplanted mouse cancer models. Combination peptide or DNA vaccines and cisplatin chemotherapy has been shown to be more effective than either treatment alone against

transplanted tumors expressing HPV antigens in mice (Bae et al., 2007; Tseng et al., 2008). The efficacy of cisplatin treatment was also increased with addition of chemotherapy-resistant dendritic cells in mice bearing injected melanoma cells (Perrotta et al., 2007). In addition, apoptosis of mesothelioma cells via gemcitabine treatment increased cross-presentation of tumor antigens and synergized with a viral vaccine against transplanted tumors in mice (Nowak et al., 2003). Furthermore, combination DC vaccination and paclitaxel treatment of Lewis lung carcinoma cells in mice augmented T cell infiltration of tumors and, hence, reduced tumor growth (Zhong et al., 2007). Thus, chemotherapy and immunotherapy are orthogonal treatments that will likely yield increased efficacy when used together in cancer treatment.

RESULTS and DISCUSSION

We explored the use of chemotherapy and immune therapy in combination to treat autochthonous lung tumors. For this investigation, we utilized a new lung cancer model that makes use of bi-functional lentiviruses expressing Cre and antigens from ovalbumin (Lv-OVA-Cre) to generate immunogenic tumors (DuPage et al, in preparation). Lv-OVA-Cre-induced tumors recruit OVA-specific T cells that are initially detectable by histology and flow cytometry. As tumors progress, however, immune infiltrates dissipate. Activated OT-I T cells, which are specific to an MHC class I-presented ovalbumin antigen, accumulate in lung tumors when adoptively transferred, even when endogenous tumor-infiltrating lymphocytes have dissipated. It is not yet known whether activated OT-I T cells kill tumor cells in this context.

For our experiment, we planned to treat animals with chemotherapy and adoptive T cell transfer, together or singly, and monitor tumor volume change by microCT scans before and after treatment. A cohort of C57BL/6 *K-ras*$^{LSL-G12D/+}$ animals infected intratracheally with

lentiviruses 7 months prior were scanned by microCT to assess tumor burden and inflammation. Of these animals, 22 were infected with Lv-OVA-Cre and 10 were infected with control Lv-Cre, which does not bear ovalbumin antigens producing non-immunogenic tumors. Mice that did not have discernible tumors, had too many tumors, and/or had obscuring inflammation by microCT analysis were excluded from the study. The 14 Lv-OVA-Cre-infected and 9 Lv-Cre-infected mice remaining were used for the experiment. These animals were divided into the following groups with at least 2 mice per group: 1) Lv-Cre mice left untreated, 2) Lv-OVA-Cre mice left untreated, 3) Lv-OVA-Cre mice treated with activated OT-I cells only, 4) Lv-Cre mice treated with cisplatin only, 5) Lv-OVA-Cre mice treated with cisplatin only, 6) Lv-Cre mice treated with both activated OT-I cells and cisplatin, and 7) Lv-OVA-Cre mice treated with both activated OT-I cells and cisplatin. A dose of 7μg/g was used for cisplatin treatments and $1.5x10^7$ activated OT-I cells were injected each time for adoptive T cell therapy. Animals received 3 doses of each appropriate treatment separated by 10 days. For combination therapy, OT-I transfer always followed cisplatin treatment by 4 days (see Figure 1A for treatment regimens). This time was chosen based on observing progressively increasing numbers of apoptotic tumor cells 1-3 days after cisplatin treatment (Oliver et al, in preparation). This experiment only serves as a pilot study due to the limited number of tumor-bearing animals available in this cohort.

MicroCT scans were taken a week before and a week after treatment, yielding 38-41 days between scans. Untreated lung tumors grew 2-2.5-fold on average within this timeframe. Tumors in Lv-OVA-Cre mice treated with transferred OT-I cells alone grew 3-fold on average, but this was not significantly different from untreated Lv-OVA-Cre tumors. Fold-change in tumor volumes varied greatly for this treatment group, but no individual tumor within this group regressed significantly (Figure 1B). Thus, these data indicate immune therapy alone via adoptive

transfer of activated OT-I cells does not substantially kill Lv-OVA-Cre-initated tumors. We do not know, however, if Lv-OVA-Cre tumor cells express/present sufficient levels of SIINFEKL (SIN), the ovalbumin antigen to which OT-I cells react, to result in significant cytolysis. Nevertheless, we do know that were is sufficient antigen presentation for T cell recognition of tumors as we observe lymphocytic infiltration in Lv-OVA-Cre tumors treated with OT-I cells, both alone and with cisplatin, when examined histologically (data not shown).

Cisplatin treatment alone had a significant effect on Lv-OVA-Cre tumors, yielding about 80% tumor regression (Figure 1B). Interestingly, Lv-Cre tumors on average failed to regress and instead demonstrated a 2-fold increase in average tumor volume similar to untreated Lv-Cre tumors. These data could indicate the induction of endogenous anti-tumor immunity when immunogenic tumors are treated with cisplatin, but not when non-immunogenic tumors are treated. Contradicting this idea, however, is that fact that we do not observe lymphocytic infiltration by histology in Lv-OVA-Cre tumors treated with cisplatin alone (data not shown). An alternative explanation for these data is that Lv-Cre mice received less cisplatin than Lv-OVA-Cre mice. Both Lv-Cre mice treated with cisplatin received only two-thirds of the dose Lv-OVA-Cre cisplatin-treated mice received because of their respective body weights (data not shown). In support of this is the effectiveness of cisplatin and OT-I transfer in causing regression of Lv-Cre tumors as these mice had body weights similar to Lv-OVA-Cre mice treated with cisplatin. These data indicate that cisplatin chemotherapy alone has a significant effect on tumor burden, but the effect may be modified by tumor immunogenicity and/or per animals dosage.

Combining adoptive OT-I T cell transfer with cisplatin chemotherapy did not appear to have an additive effect on killing tumor cells (Figure 1B). Lv-OVA-Cre mice treated with

combined therapy did demonstrate significant tumor regression by microCT analysis when compared to untreated mice, but tumor regression was not as great as with cisplatin treatment alone. The failure to see comparable levels of tumor regression could be due to an increase in apparent tumor volume when tumors are infiltrated by lymphocytes. Furthermore, the inability to see an additive effect on tumor regression with cisplatin and T cell therapy could be due to the unanticipated effectiveness with cisplatin treatment alone. The dose of cisplatin chosen for this experiment was used to treat animals with greater lung tumor burdens, as they are induced with higher dose Adenovirus-Cre (Oliver et al, in preparation). Tumor regression for Adenovirus-Cre-induced tumors was much more modest than the effect observed here for Lv-OVA-Cre-induced tumors. In future experiments, perhaps, a lower dose of cisplatin could be used.

Data from this experiment suggest that OT-I T cell transfer is ineffective, either alone or in combination with cisplatin, against Lv-OVA-Cre-induced tumors. In support of this is the comparable level of tumor regression observed between Lv-Cre and Lv-OVA-Cre tumors treated with combined chemotherapy and immune therapy (Figure 1B). It would be important to determine if the ineffectiveness of activated OT-I cells is due to the analysis of late stage tumors since the mice used in this study were aged 7 months after tumor initiation. Studies should be performed on younger mice and tumors should be analyzed at various time points after initiation for ovalbumin expression and presentation. Alternatively, tolerance mechanisms need to be investigated further in this model as OT-I T cell activity may be suppressed in such a manner that is not overcome by tumor cell death by cisplatin chemotherapy.

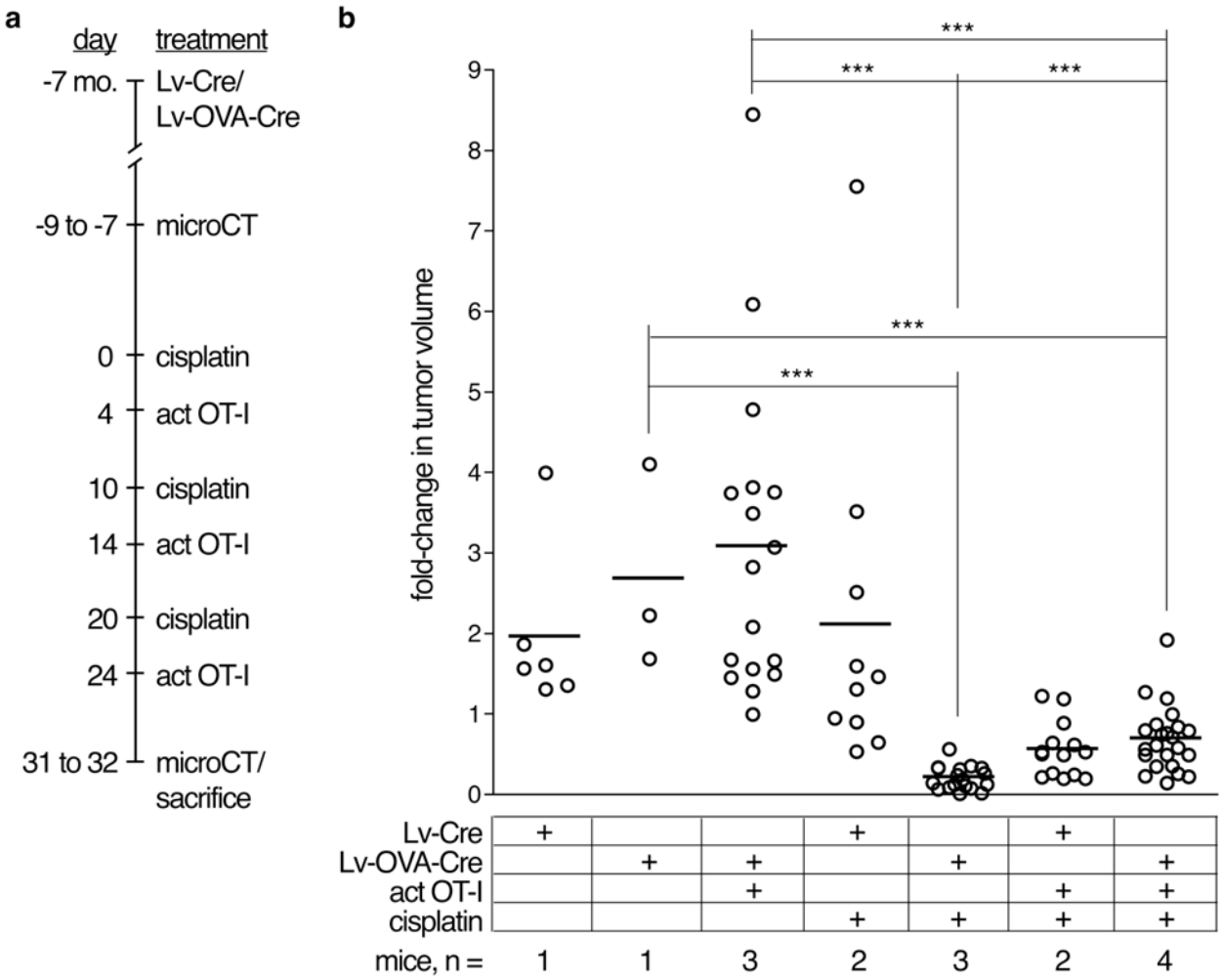

Figure 1. Cisplatin chemotherapy by itself greatly reduces volume of immunogenic tumors and no additive effect is observed with additional adoptive T cell transfer therapy. *A*. C57BL/6 *K-ras*$^{LSL\text{-}G12D/+}$ mice were infected with Lv-Cre or Lv-OVA-Cre intratracheally to induce non-immunogenic or immunogenic lung tumors, respectively. Seven months after tumor initiation animals were treated with *in vitro* activated OT-I T cell transfer intravenously, cisplatin intraperitoneally, both T cells and cisplatin, or neither (untreated). Lung of live mice were scanned via micro-computed tomography a week before and after treatment regimens were completed to determine the fold-change in individual lung tumor volumes. Treatments took place over a month with 3 doses each of $1.5x10^7$ activated OT-I cells and/or 7μg/g cisplatin. Doses of the same therapy were separated by 10 days with OT-I transfer following cisplatin by 4 days when used to treat mice in combination. *B*. The number of mice used in the analysis is indicated. *Bars*, mean, ***p <0.0005 by Student's *t* test.

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APPENDIX 5:

Complete loss of Apc promotes tumorigenesis and reduces β-catenin levels

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The author generated *Apc*$^{fle1\text{-}15}$ and *Apc*^{null} mice and was involved in all experiments performed. Alia Carter, an undergraduate student supervised by the author, assisted the author in generating survival curves and the quantification of tumor number and volumes in Figures 3, 4, and 5A,B. Kamena Kostova, an undergraduate student supervised by the author, assisted the author in Real-time PCR experiments in Figures 2A and 6 and staining and quantification of phospho-histone H3 in Figure 5C-E. Denise Crowley processed and cut sections. Roderick Bronson advised the author on histopathological analyses. Experiments were performed in the laboratory of Tyler Jacks.

ABSTRACT

The Adenomatous Polyposis Coli (APC) gene product is mutated in the vast majority of human colorectal cancer. Apc negatively regulates the Wnt pathway by aiding in the degradation of β-catenin, the transcriptional factor activated downstream of Wnt signaling. Apc mutation, thus, results in deregulation of β-catenin and constitutive Wnt activation leading to aberrant cellular proliferation. Biochemical and molecular studies have, however, revealed multiple protein domains within Apc and additional cellular processes in which Apc participates. These studies have predominantly been performed in cultured system with the use of ectopically expressed Apc protein fragments and, thus, bringing into question the *in vivo* significance of these findings. We have generated a novel allele of *Apc* in mice that results in complete loss of all Apc protein products. With this allele, we have identified a role for the N-terminal domains of Apc in intestinal tumor suppression. In addition, we have discovered a novel role for Apc in the regulation of β-catenin transcripts.

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer death in the Western world. Upwards of 80% of colorectal cancers have mutations in the *Adenomatous Polyposis Coli* (*APC*) gene. Both Familial Adenomatous Polyposis (FAP) and sporadic colon cancers tend to have *APC* alterations that result in truncated APC protein (Kinzler and Vogelstein, 1996). These mutations fall in a region of APC termed the mutation cluster region (MCR) and result in retained expression of N-terminal domains of the protein (Figure S1). Genotype-phenotype correlations of familial syndromes involving *APC* mutation suggest that different lengths and levels of APC expression can influence the number of polyps in the gut, the distribution of polyps, and extra-colonic manifestations of the disease (Soravia et al., 1998). Specifically, patients with Attenuated FAP (AFAP) tend to have fewer polyp counts and later age of colon cancer development. Interestingly, in AFAP, APC mutations tends to fall 5' and 3' of the MCR, presumably resulting in APC truncations bearing different protein domains than in classical FAP and/or hypomorphic APC expression. More thorough analysis of the phenotypic consequences of different heritable *APC* alleles is hampered by the low frequency of non-classical FAP and the existence of numerous genetic and environment modifiers of disease (de la Chapelle, 2004). Thus, many have turned to the mouse as a tool to examine the consequences of various *Apc* mutations.

The *Apc*^{Min} mouse was the first mutant allele of Apc developed in the mouse, discovered in an ethylnitrosourea mutagenesis screen (Su et al., 1992). *Apc*^{Min} mice developed multiple intestinal neoplasias (Min) that closely resembled human FAP, which had only concurrently been identified to be caused by *APC* mutation (Groden et al., 1991; Kinzler et al., 1991). In addition, the Apc^{Min} mutation is a nonsense mutation in codon 850 that results in a stable,

truncated Apc protein similar to that found in classical FAP patients. Since the development of *Apc*^{Min} mice, additional alleles of Apc have been generated, including those that result in shorter Apc products, as well as, longer polypeptides than Apc^{Min} (Colnot et al., 2004; Oshima et al., 1995; Smits et al., 1999). These alleles have yielded different phenotypic consequences relating to intestinal tumorigenesis (Fodde and Smits, 2001).

In the canonical Wnt pathway, Apc forms a complex with Axin and Gsk-3β to negatively regulate β-catenin by sequestering it in the cytoplasm and marking it for degradation. When Apc is lost, β-catenin is free to translocate into the nucleus where it binds to Tcf transcription factors to regulate target genes, such as *c-myc* (Clevers, 2006). Activated β-catenin itself is sufficient to initiate intestinal tumorigenesis in mice, resembling lesions caused by homozygous mutation of Apc (Harada et al., 1999). Some evidence suggests, however, that the role of Apc mutation in tumorigenesis reaches beyond deregulation of β-catenin. First, the vast majority of sporadic colon cancers have mutations in APC, whereas other cancers with deregulated WNT signaling have an equal or greater proportion of mutations in β-Catenin or other Wnt pathway components (Giles, 2003). Second, Apc is a relatively large protein, approximately 312 kDa, with a number of protein binding motifs (Figure S1). Consistent with this, Apc interacts with a variety of proteins outside of Wnt signaling and localizes to the nucleus, the plasma membrane, and microtubule ends, as well as, to the cytoplasm (Aoki and Taketo, 2007; Fodde et al., 2001; Mccartney and Nathke, 2008). Finally, the majority of APC mutations result in a stable, truncated polypeptide, missing most β-catenin and Axin binding domains, which lie in the middle portion of the protein. In these mutants, the microtubule-binding domain and EB1-binding site, at the C-terminus of APC, are also lost, but the N-terminal armadillo domain and oligomerization domains are retained (Nathke, 2004).

To dissect the pleiotropic roles of Apc in intestinal tumorigenesis, we have generated novel alleles of *Apc* in the mouse. Comparison of our new alleles to existing mutant *Apc* alleles has allowed us to identify a role for the N-terminal domains of Apc in tumor suppression. Furthermore, we have potentially gained insight into a novel function for Apc.

RESULTS

Generation of conditional $Apc^{fle1\text{-}15}$ and constitutive Apc^{null} alleles

We took a genetic approach to address the role of truncated Apc protein in intestinal tumorigenesis by constructing conditional and constitutive null alleles of Apc in mice. To generate a conditional null allele of *Apc*, $Apc^{fle1\text{-}15}$, we targeted the *Apc* locus in C57BL/6 embryonic stem cells with two sequential insertions to flank coding exons 1 through 15 with loxP sites (described in Figure 1). Targeting *Apc* in this manner placed loxP sites around a genomic region spanning ~90kb and would ensure the complete loss of the Apc gene product when Cre-mediated recombination of the locus was achieved. Furthermore, to generate a heritable constitutive Apc^{null} allele, we crossed $Apc^{fle1\text{-}15}$ to *Meox-Cre* transgenic animals, which express Cre in the germline (Figure 1) (Tallquist and Soriano, 2000).

To confirm the loss of Apc upon genomic deletion, we performed Real-time PCR to detect Apc with Taqman probes spanning exons 9-10 and 14-15. Analysis of cDNA generated from colons of 1 month old animals revealed approximately 50% reduction of Apc in $Apc^{+/null}$ mice compared to a $Apc^{+/+}$ littermate (Figure 2A). $Apc^{+/null}$ mice are predisposed to spontaneous intestinal tumor formation upon loss of heterozygosity (LOH) of *Apc* similar to $Apc^{+/Min}$ and $Apc^{+/del14}$ mice (Colnot et al., 2004; Su et al., 1992). We plucked colonic tumors from all aged mice from each of the three *Apc* mutant genotypes for analysis of tumor cDNA. Consistent with

a mechanism of LOH involving mitotic recombination resulting in tumors with $Apc^{null/null}$ genotype, we observe nearly complete loss of Apc transcript in colonic tumors from $Apc^{+/null}$ mice (Figure 2A). Residual Apc is likely derived from stroma within the lesions. Furthermore, in line with Cre-excised genomic regions, we observe retention of exons 9-10, but loss of exons 14-15 in colonic tumors from $Apc^{+/del14}$ mice, whereas $Apc^{+/Min}$ tumors maintain expression of both regions (Figure 2A; Figure S1).

Mice heterozygous for *Apc* develop lesions histologically similar to those in Apc^{Min} mice

We went on to compare the lesions that developed in the three mutant *Apc* genotypes. Upon histological examination, tumors varied in apparent grades and morphology, but did not differ significantly among the genotypes (data not shown). Heterozygous conditional $Apc^{+/fle14}$ and $Apc^{+/fle1\text{-}15}$ animals were generated that also bore a *Villin-Cre* transgene (El Marjou et al., 2004). Comparison of lesions that develop in mice with conditional *Apc* alleles revealed a similar result to constitutive mutants (Figure 2B-G). In addition, tumors from all Apc mutant mice stained positive for nuclear β-catenin by immunohistochemstry, although staining was heterogeneous (Figure 2H,I; data not shown).

Apc^{null} mice have reduced survival compared to other *Apc* mutant mice

We wanted to determine if Apc mutant mice differed in their survival. We aged cohorts of Apc mutants on pure C57BL/6 background since intestinal tumorigenesis is attenuated or exacerbated by numerous genetic modifiers. In addition, to reduce the influence of environmental modifiers on morbundity, we only utilized wild-type mothers and aging female mutants were kept virgin. We discovered that median survival was reduced by 23% in $Apc^{+/null}$ females compared to $Apc^{+/Min}$ females, at 119 and 154 days, respectively (Figure 3A). In males,

there was a similar reduction of 24% between the two genotypes, with median survival of 106.5 and 140.5, respectively for *Apc*$^{+/null}$ and *Apc*$^{+/Min}$ males (Figure 3B). Moribundity was supported by hematocrit measurements indicative of anemia in mice with extensive tumor burdens (Figure 3C).

Apc$^{+/del14}$ mice displayed an intermediate survival compared to mice with other mutant *Apc* alleles, but this differed somewhat between females and males (Figure 3). As the *Apc*^{fle14} allele, from which *Apc*^{del14} was derived, was generated on a non-C57BL/6 background, it is possible that these mice are influenced by genetic modifiers closely linked to the *Apc* locus (Colnot et al., 2004). Because of this variable, we will not consider their phenotype in our descriptions further, although additional analyses did include these mice.

Apc^{null} mice are predisposed to increased number of tumors and with larger size

We went on to investigate the cause of reduced survival in *Apc*$^{+/null}$ mice. Aside from a few animals developing mammary lesions, no other extra-intestinal lesion appeared to influence animal survival (data not shown). Therefore, the simplest explanation for altered survival would be a difference in tumor development. We sacrificed additional cohorts of animals at 3 months of age to examine tumor multiplicity and size. Upon counting tumor number, we noticed more tumors in both small intestines and colons of *Apc*$^{+/null}$ mice compared to *Apc*$^{+/Min}$ mice (Figure 4). This result was apparent in both females and males, but only statistically significant in females. Female *Apc*$^{+/null}$ mice had 50% more small intestinal tumors and greater than twice the number of colonic tumors on average than *Apc*$^{+/Min}$ mice (Figure 4C,D). The distribution of lesions through the intestinal tract, however, did not differ between the Apc mutants (Figure 4A,B).

From these same animals, we plucked colonic tumors and measured these with calipers. In female *Apc*$^{+/null}$ mice, tumors were more than twice the volume of those in female *Apc*$^{+/Min}$ mice (Figure 5A). There was no distinction, however, among colonic tumor volumes in males (Figure 5B). We reasoned that increased tumor size could be due to either reduced cell death or increased proliferation. We examined cell death by cleaved-caspase 3 staining, but observed no apparent difference (data not shown). In addition, we counted mitotic cells stained positive by immunohistochemistry for phospho-histone H3 as a surrogate marker of proliferation, but again failed to detect a significant difference between *Apc*$^{+/null}$ and *Apc*$^{+/Min}$ lesions (Figure 5C,D,E).

It is possible that our methods are not sensitive enough to detect changes in these cell fate decisions, particularly considering the enormous variability in mitotic cell counts. Alternatively, larger lesions can be the result of earlier tumor initiation. Bearing in mind the greater number of tumors counted in *Apc*$^{+/null}$ colons compared to *Apc*$^{+/Min}$, increased volume could simply be a reflection of a longer time for tumors to progress, assuming a lower limit of detection (Figure 4D; Figure 5A). Thus, truncated Apc acts as a tumor suppressor leading to reduced survival when lost. Additional experiments will need to be conducted to parse out how this function is manifested.

Complete loss of APC in colon tumors results in reduced β-catenin levels and activity

During analysis of tumor samples stained for β-catenin by immunohistochemistry, we consistently observed reduced staining in tumors from *Apc*$^{+/null}$ mice compared to *Apc*$^{+/Min}$ mice (Figure 2H; data not shown). As both mutations in *Apc* result in loss of domains that mediate β-catenin destruction, we found this to be rather unexpected (Figure S1). We investigated this further via Real-time PCR for β-catenin transcript levels. Consistent with immunohistochemical staining, colonic tumors from *Apc*$^{+/null}$ mice had lower levels of β-catenin than those from

Apc$^{+/Min}$ mice (Figure 6A). We proceeded to measure levels of β-catenin activity by using mRNA levels of its direct transcriptional target Axin2 as a surrogate marker. In proportion to β-catenin levels, Axin2 was reduced in tumors from *Apc*$^{+/null}$ compared *Apc*$^{+/Min}$ mice (Figure 6B).

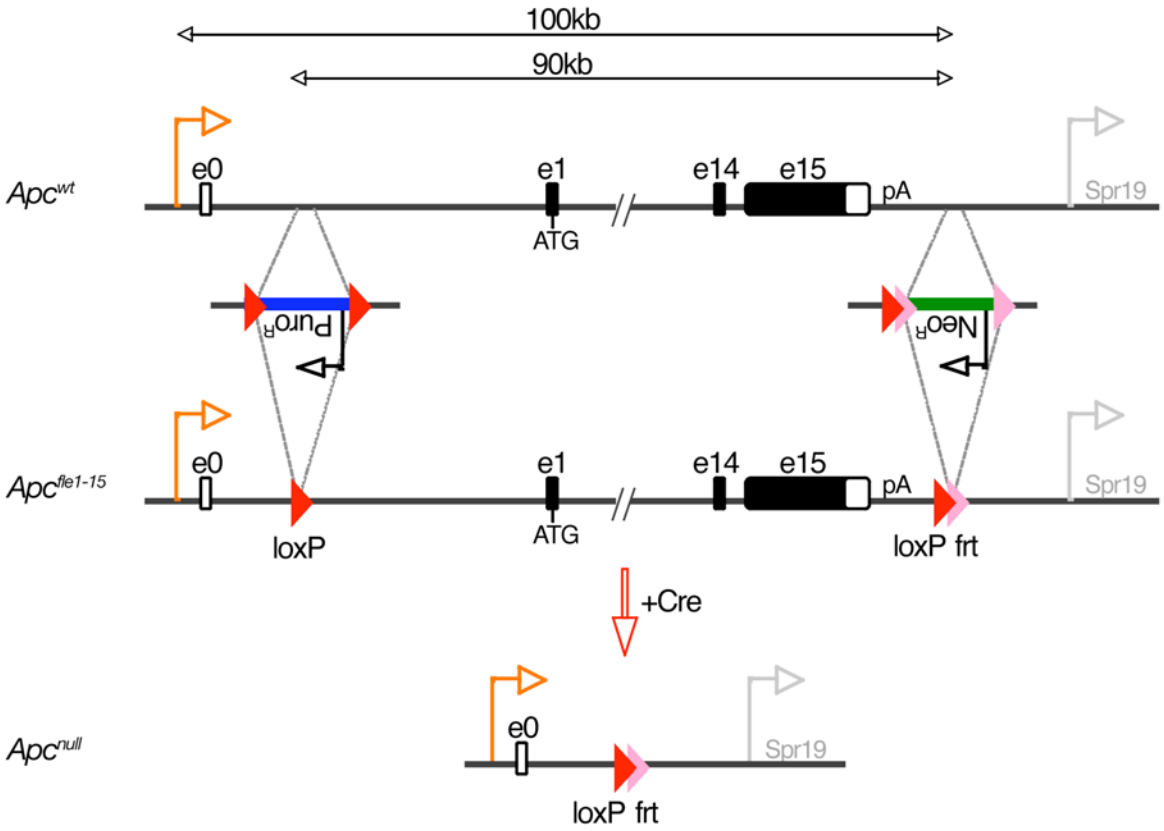


Figure 1. Generation of the *Apc*$^{fle1-15}$ and *Apc*^{null} alleles. Schematic of the wild-type *Apc* genomic locus locus showing non-coding exon 0 and coding exons 1, 14, and 15. A floxed puromycin resistance gene was targeted to intron 1 of *Apc* in C57BL/6 embryonic stem (ES) cells. ES cells were selected on puromycin and screened by Southern blot for homologous recombination. Targeted clones were then transfected with a Cre expression plasmid to excise the Puro^{R} cassette leaving behind a single loxP site. After confirming successful removal of Puro^{R}, the intergenic region 3' to *Apc* was targeted with a construct containing a neomycin resistance gene flanked proximally by frt sites on both sides and a distal loxP site on one side (loxP-frt-Neo^{R}-frt cassette). ES cells were selected by neomycin and again by Southern blot for homologous recombination. Correctly targeted ES cells were injected into FVB blastocystes to generate chimeric mice. Chimeras were mated to wild-type C57BL/6 mice and pups were screened by coat color and PCR for germline transmission of the targeted allele. The Neo^{R} cassette was removed *in vivo* by germline FlpE expression leaving a loxP and a frt site 3' to *Apc*. This strategy yielded the conditional null allele *Apc*$^{fle1-15}$. Germline expression of Cre resulted in excision of the genomic region flanked by targeted loxP sites yielding a constitutive null allele *Apc*^{null}.

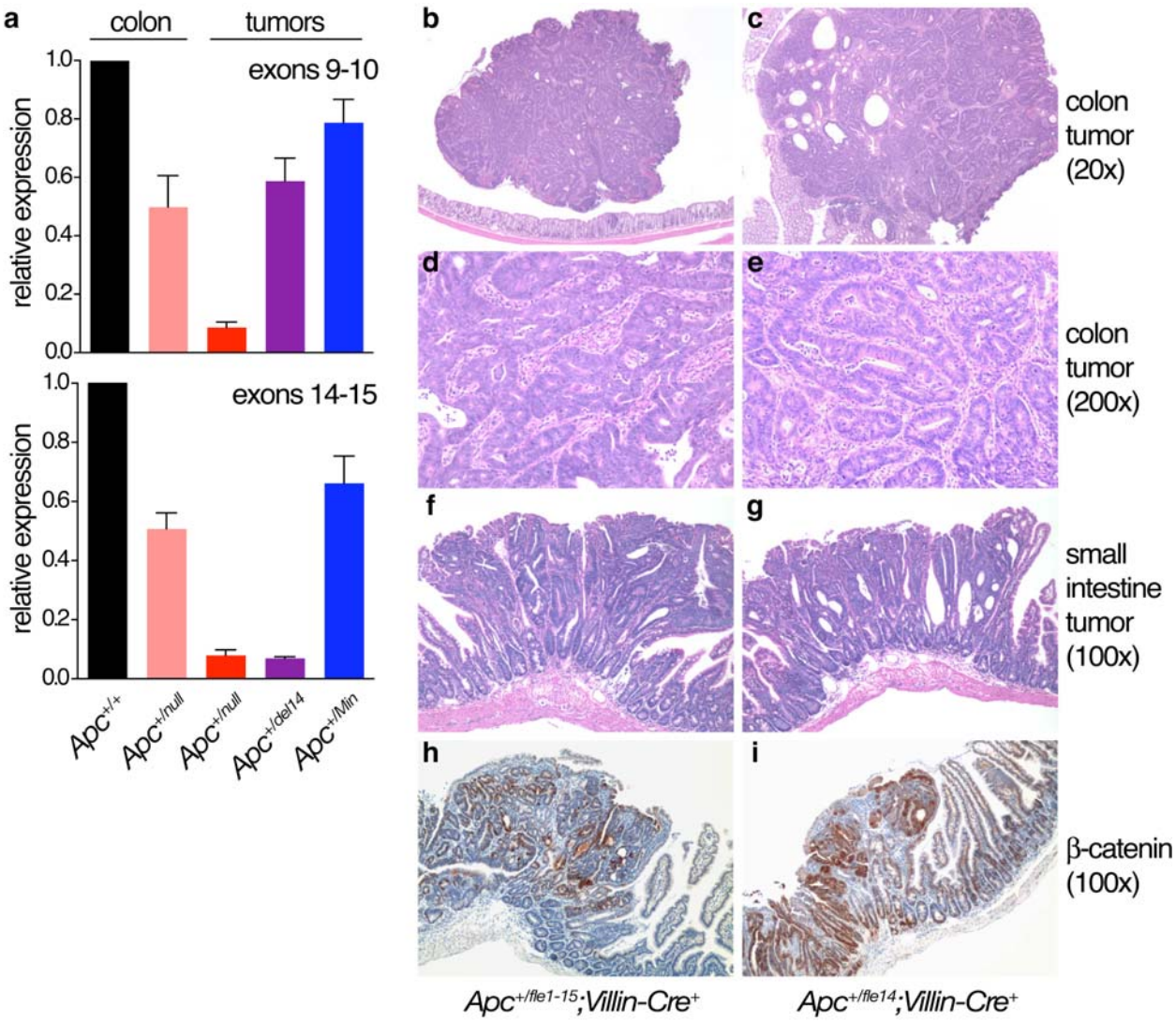


Figure 2. *Apc*$^{+/null}$ mice develop tumors that lack Apc expression, but resemble tumors from other APC mutant mice histologically. *A.* Real-time PCR of cDNA from colons of 1 month old wild-type *(black bars)* and *Apc*$^{+/null}$ *(pink bars)* mice and colon tumors from aged *Apc*$^{+/null}$ *(red bars), Apc*$^{+/del14}$ *(purple bars),* and *Apc*$^{+/Min}$ *(blue bars)* mice. Taqman probes spanning exons 9 and 10 *(top)* and exons 14 and 15 *(bottom)* of Apc transcript were used and C_t values were normalized to GAPDH for each sample. Expression of Apc is shown relative to *APC*$^{+/+}$ colon. *B-G*. Hematoxylin and eosin stained sections of colon tumors *(b-e)* and small intestine tumors *(f,g)* from aged *Apc*$^{+/fle1-15}$*;Villin-Cre*$^{+}$ *(b,d,f)* and *Apc*$^{+/fle14}$*;Villin-Cre*$^{+}$ *(b,d,f)* mice. *H-I*. Immunohistochemistry for β-catenin in small intestinal tumors from *Apc*$^{+/fle1-15}$*;Villin-Cre*$^{+}$ *(h)* and *Apc*$^{+/fle14}$*;Villin-Cre*$^{+}$ *(i)* mice.

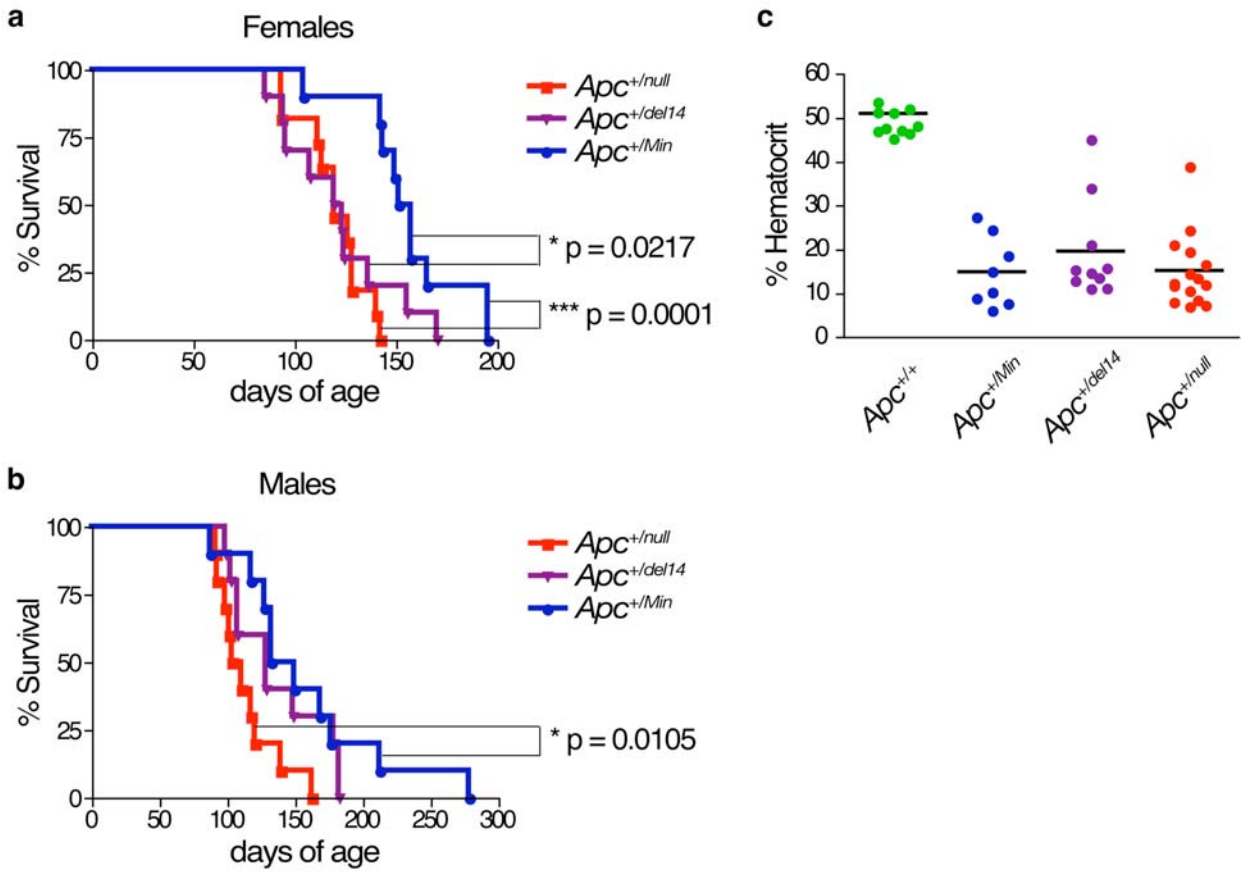


Figure 3. *Apc*$^{+/null}$ mice have reduced survival compared to *Apc*$^{+/Min}$ mice. *A.* Survival curves for *Apc*$^{+/null}$ *(red squares)*, *Apc*$^{+/del14}$ *(purple triangles)*, and *Apc*$^{+/Min}$ *(blue circles)* female mice. Median survival values are 119, 121, and 154 days for the 3 alleles, respectively. P values by chi-square statisitic is indicated for significantly different comparisons. *B.* Survival curves for male mice of the same genotypes as in *a*. Median survival values are 106.5, 128, and 140.5 days for the 3 alleles, respectively. P values by chi-square statisitic is indicated for significantly different comparisons. *C*. Percent hematocrit for Apc mutant mice compared to wild-type Apc controls *(green)* at time of sacrifice. *Bars*, mean values.

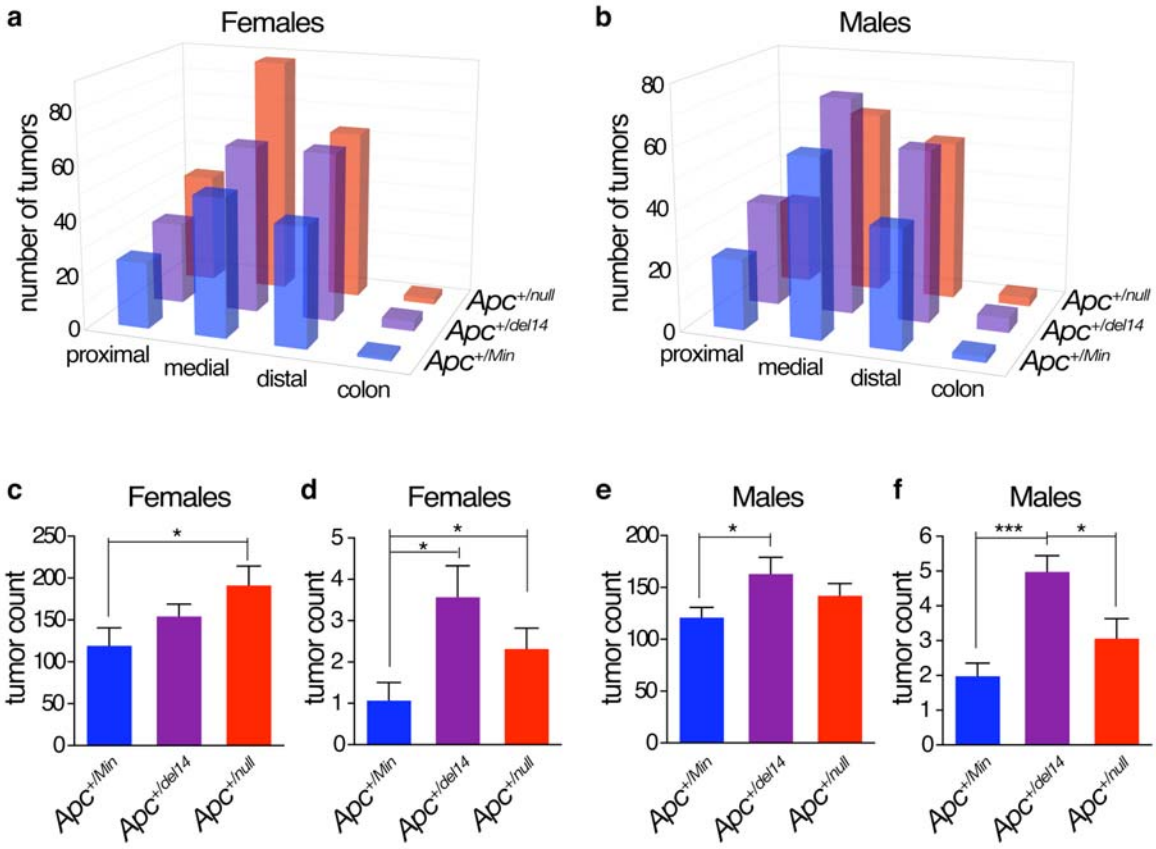


Figure 4. *Apc*$^{+/null}$ mice have increased tumor multiplicity compared to *Apc*$^{+/Min}$ mice. *A, B.* Distribution of tumors along intestinal tracts of *Apc*$^{+/null}$ *(*n=12 for each gender*, red bars)*, *Apc*$^{+/del14}$ *(*n=12 for females and n=9 for males, *purple bars)*, and *Apc*$^{+/Min}$ *(*n=12 for each gender, *blue bars)* female *(a)* and male mice *(b)* at 3 months of age. *C-F*. Total number of tumors in small intestines *(c,e)* and colons *(d,f)* of Apc mutant female *(c,d)* and male *(e,f)* mice in *a,b*. *Bars*, mean + s.e.m., *p <0.05, ***p <<0.001 by rank sum test.

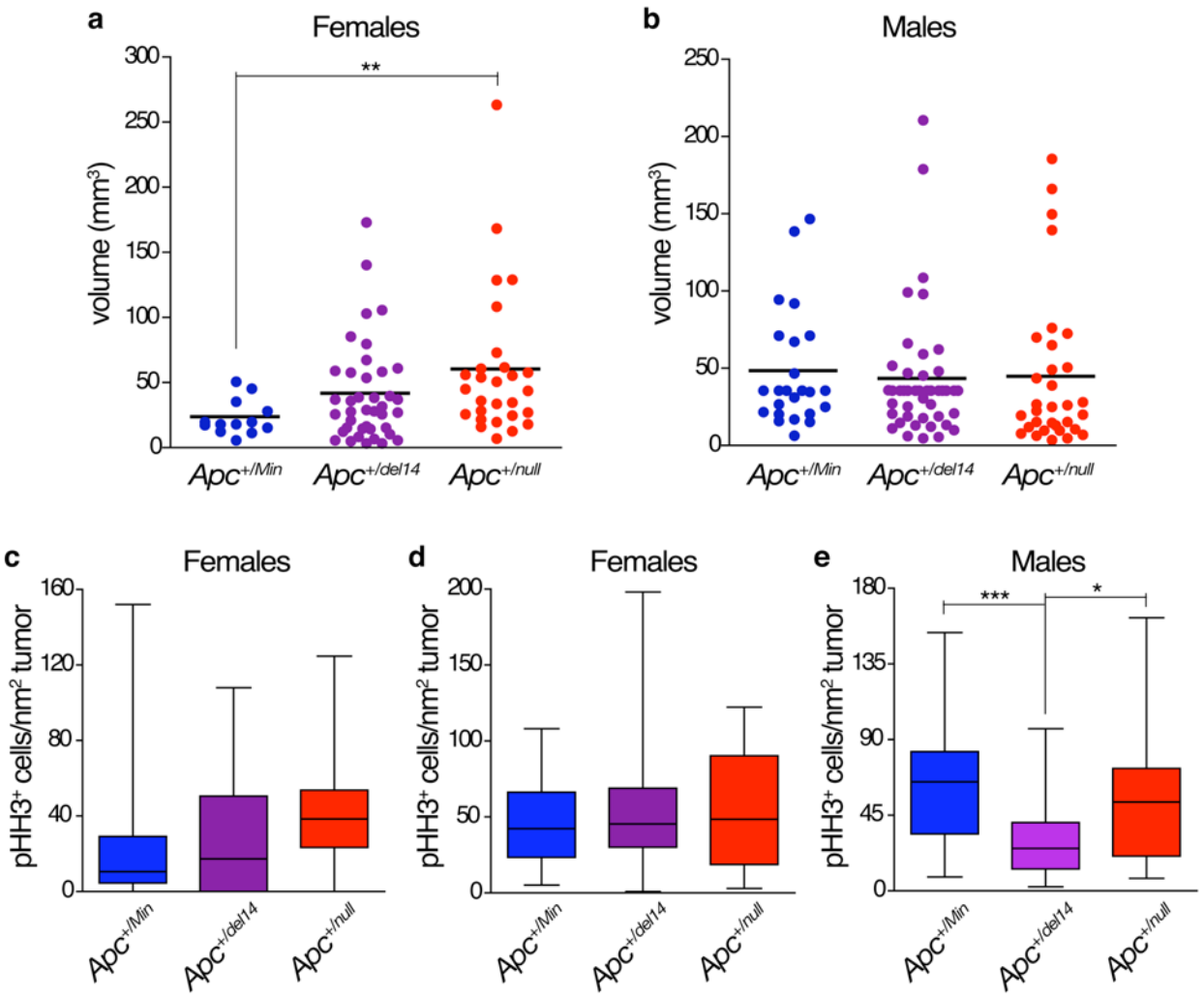


Figure 5. Female *Apc*$^{+/null}$ mice have larger colonic tumors than to *Apc*$^{+/Min}$ females. *A, B.* Volumes of colonic tumors from *Apc*$^{+/null}$ *(*n=28 for females and n=32 for males, *red circles)*, *Apc*$^{+/del14}$ *(*n=40 for females and n=44 for males, *purple circles)*, and *Apc*$^{+/Min}$ *(*n=13 for females and n=24 for males, *blue circles)* female *(a)* and male mice *(b)* at 3 months of age. *Bars*, mean + s.e.m.,**p <0.005 by rank sum test. C,D,E. Quantification of phospho-histone H3^{+} cells per nm^{2} small intestine *(c)* and colon *(d,e)* tumor area by immunohistochemistry of tissue sections from female *(c,d)* and male *(e) Apc*$^{+/Min}$ *(blue bars), Apc*$^{+/fle14}$ *(purple bars)*, and *Apc*$^{+/null}$ *(red bars)*. *Bars*, mean + s.e.m.,*p <0.05, ***p <0.0005 by Student's *t* test.

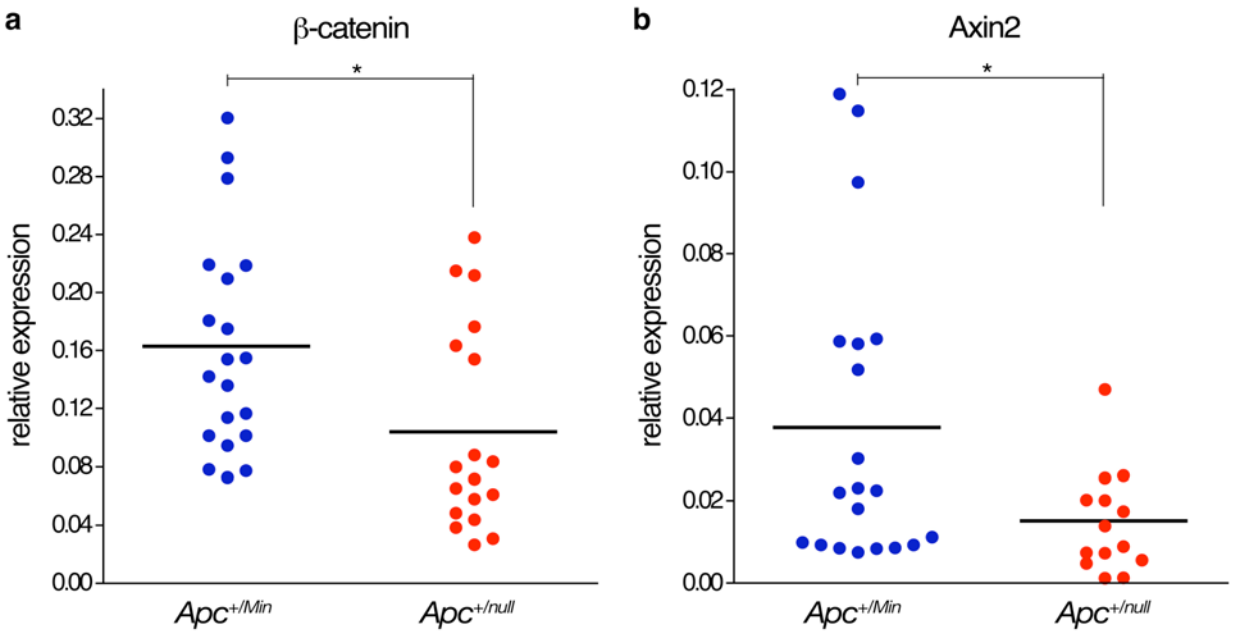
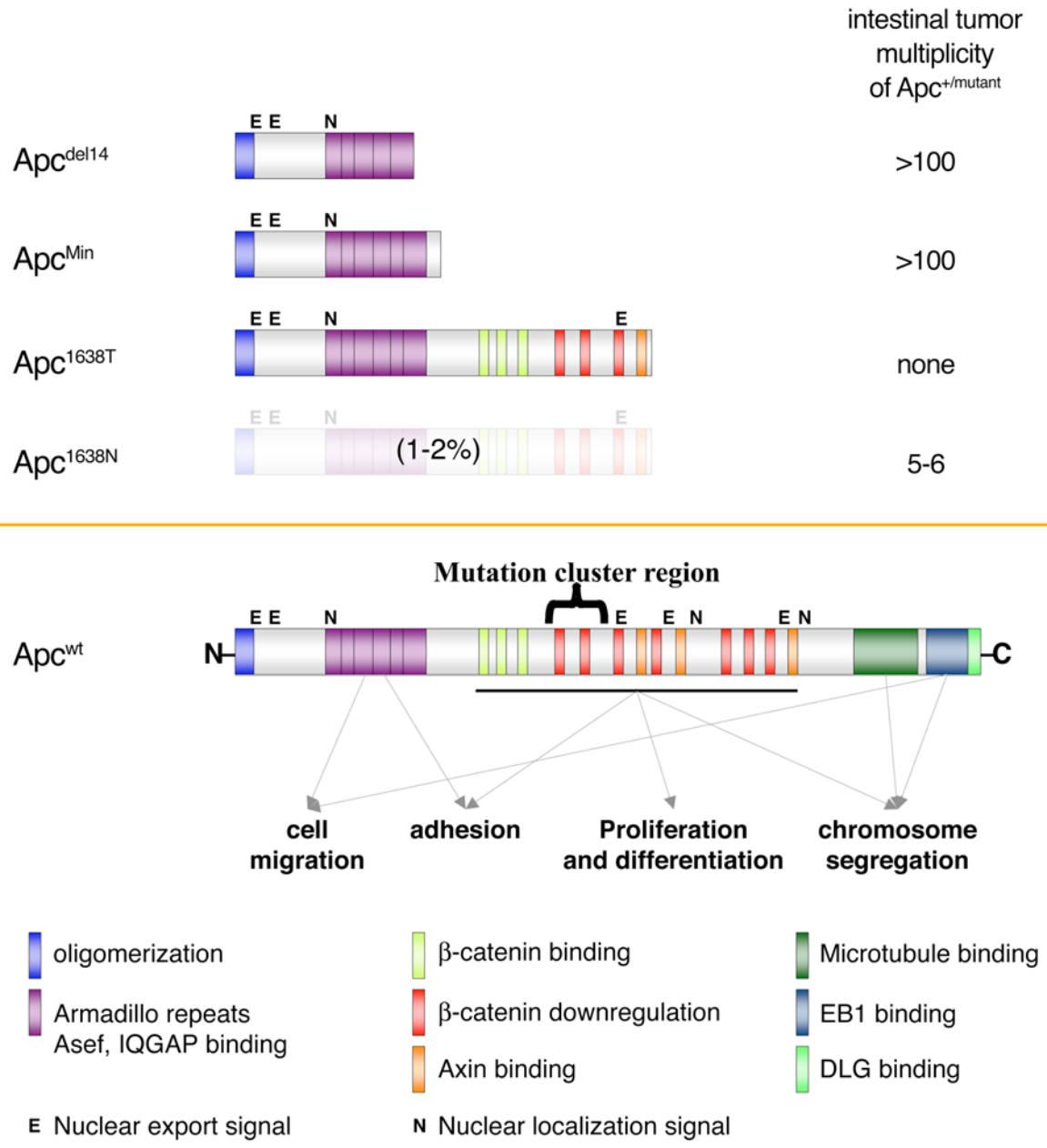


Figure 6. Colon tumors from *Apc*$^{+/null}$ mice have lower levels of β-catenin and decreased expression of β-catenin's transcriptional target Axin2 compared to tumors from *Apc*$^{+/Min}$ mice. *A,B.* Real-time PCR of cDNA from colon tumors from aged *Apc*$^{+/null}$ *(red circles)* and *Apc*$^{+/Min}$ *(blue circles)* mice. Taqman probes for β-catenin *(a)* and Axin2 *(b)* transcripts were used and C_t values were normalized to GAPDH for each sample. *Bars*, mean,*p <0.05 by Student's *t* test.

DISCUSSION

We have generated two novel alleles of the *Adenomatous polyposis coli (Apc)* gene, conditional and constitutive null alleles, in mice. These alleles result in complete loss of all Apc protein products allowing us to dissect the role of Apc truncation in intestinal tumorigenesis. Our studies indicate that truncated Apc, derived from the *Min* allele of *Apc*, has tumor suppressor function in the gut, such that mice with heterozygous deletion of Apc have reduced survival and are predisposed to an increased tumor burden compared to Apc^{Min} mice. We have not yet determined, however, whether greater tumor burden is a reflection of higher rates of tumor initiation or faster tumor progression. In addition, we observe reduced levels of β-catenin transcript and protein, which, in turn, results in lower levels of its transcriptional target Axin2. To our knowledge, this is the first report of Apc mutation yielding changes in β-catenin transcript levels. We do not know how direct this effect on β-catenin is or whether this indicates a change in β-catenin transcription and/or mRNA stability. Furthermore, it is not clear if β-catenin activity drives the differences we observe in tumor phenotype between truncated and deleted alleles of *Apc*.



Supplementary Figure 1. Protein domain structures and intestinal phenotypes of published alleles of mutant *Apc* in mice.

METHODS

Mice. *Apc*^{Min} and *Meox-Cre* mice were purchased from Jackson Laboratories. *Apc*^{fle14} mice were a gift from Dr. M. Giovannini, and *Apc*^{del14} animals were derived by crossing to *Meox-Cre*. *Villin-Cre* mice were a gift from Dr. S. Robine. *Apc*$^{fle1\text{-}15}$ mice were generated from C57BL/6 embryonic stem cells and maintained on C57BL/6 background, even with the derivation of the *Apc*^{null} germline allele. All animals used in the survival study and for quantified measurements were on C57BL/6 background. Animals in the survival study were progeny of *Apc* mutant fathers and wild-type *Apc* mothers. Females were kept virgin as they aged and animals were checked for morbundity on a daily basis. Animals for tumor multiplicity and colon tumor measurements were sacrificed at 3 months of age. Intestines were flushed with PBS, cut open longitudinally, and fixed flat on bibulous paper in 4 segments. Fixed intestine were stained with 1:10 dilution in water of 1% methylene blue in 95% ethanol and destained in 70% ethanol before counting tumors on a dissection microscope. Colonic tumor volumes were determined by plucking lesions from surface of intestine and measuring tumor masses by calipers. Volumes were calculated based on an ellipsoid shape; volume = (4/3)*pi*x*y*z, with x, y, and z being the widths of each perpendicular axis. Animal studies were approved by MIT's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

Real-time PCR. Samples were prepared by flushing fresh colons with cold PBS or by plucking colonic tumors from intestinal surface. Tissues were then minced with a razor blade before

Trizol extraction for RNA. RNA was quantified by spectrophotomter, and the quality was assessed by agarose gel electrophoresis and visualization of 28S and 18S ribosomal RNA bands. Non-degraded samples were subjected to cDNA synthesis using random primers and Superscript III reverse transcriptase enzyme. Samples were treated with RNase H to degrade template RNA after cDNA synthesis was complete. Taqman probes and reagents were purchased from Applied Biosystems. Relative expression of Apc, β-catenin and Axin2 were determined by normalization to GAPDH.

Immunohistochemistry. α–β-catenin (cat#9582), α-cleaved caspase-3 (cat#9661), and α-phospho-histone H3 (cat#9701) antibodies were purchased from Cell Signaling Technology. Immunohistochemistry was performed on paraffin sections as previously described (Haigis et al., 2008).

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