Endogenous T Cell Responses to Antigens Expressed in Lung Adenocarcinomas Delay Malignant Tumor Progression

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SUMMARY

Neoantigens derived from somatic mutations in tumors may provide a critical link between the adaptive immune system and cancer. Here, we describe a system to introduce exogenous antigens into genetically engineered mouse lung cancers to mimic tumor neoantigens. We show that endogenous T cells respond to and infiltrate tumors, significantly delaying malignant progression. Despite continued antigen expression, T cell infiltration does not persist and tumors ultimately escape immune attack. Transplantation of cell lines derived from these lung tumors or prophylactic vaccination against the autochthonous tumors, however, results in rapid tumor eradication or selection of tumors that lose antigen expression. These results provide insight into the dynamic nature of the immune response to naturally arising tumors.

INTRODUCTION

The potential for functionally important interactions between a developing tumor and the immune system has been appreciated for over a century. The association of tumor cells and lymphocytes has led researchers to postulate that the immune system actively inhibits the formation and progression of transformed cells and ultimately "shapes" nascent tumors by forcing the selective evolution of tumor cells that can evade the immune response, a phenomenon called tumor immunoediting (Dunn et al., 2002). Significant numbers of T cells specific to mutated tumor proteins have been identified in cancer patients (Finn, 2008; Novellino et al., 2005; Parmiani et al., 2007; Rubio et al., 2003), and several studies have correlated immune infiltration in tumors with improved prognosis (Buckanovich et al., 2008; Budhu et al., 2006; Finak et al., 2008; Galon et al., 2006; Mollard et al., 2000; Pages et al., 2005; Piersma et al., 2007). However, the persistence of malignant disease despite immune recognition presents an important medical and therapeutic question: how do tumors escape immune surveillance?

Several mouse models have been used to gain insights into the mechanisms by which tumors may subvert immune responses, but each of these has critical limitations. Transplantation of primary or cultured tumor cells is commonly used, but these models are limited because they ectopically introduce large numbers of fully developed tumor cells that grow rapidly, and transplantation can initiate proinflammatory responses or traffic tumor cells directly to lymphoid organs (Khong and Restifo, 2002; Ochsenbein et al., 2001; Spiotto et al., 2002). Carcinogen-induced models have focused predominantly on sarcomas rather than cancers of epithelial origin and these tumors are expected to harbor large numbers of mutations that may lead to artificially robust immune responses (Dunn et al., 2002; Khong and Restifo, 2002). Transgenic mouse models

Significance

Our immune system may naturally protect us from cancer by recognizing and eliminating nascent tumors. However, the study of immune-tumor interactions has relied upon T cell responses to transplanted tumors or tumor-associated antigens expressed from transgenic mouse cancer models. Here, we report a system to assess how T cells respond and adapt to endogenously arising lung tumors expressing exogenous antigens that model tumor neoantigens. We uncover critical differences in the way that the immune system responds to tumors that originate from transformation of somatic cells versus transplanted tumor cell lines. Rather than eliminate tumors, T cells suppress tumor progression, reflecting data in cancer patients. This model provides a valuable system for the optimization of immune therapies to eradicate human cancers.
of cancer that develop tumors spontaneously and express model tumor antigens throughout targeted organs fail to fully recapitulate immune responses against the human disease because antigen expression in normal tissues likely alters the T cell response, and thymic deletion of antigen-specific T cells typically prevents the study of endogenous T cell responses to tumors (Bai et al., 2008; Drake et al., 2005; Getnet et al., 2009; Lyman et al., 2004; Muller-Hermelink et al., 2008; Nguyen et al., 2002; Savage et al., 2008; Smith et al., 1997; Speiser et al., 1997). Furthermore, as transgenic models rely on sporadic tumor-initiating events that can vary widely from tumor to tumor and mouse to mouse (Frese and Tuveson, 2007), it is difficult to follow the dynamics of the T cell response over time.

In contrast, genetically engineered mouse models of many human cancers accurately recapitulate both the genetic and histopathologic progression of the human disease from its earliest lesions to metastasis and can provide spatiotemporal control of tumor onset (Frese and Tuveson, 2007). However, few studies have employed these models to elucidate the interplay between tumors and the immune system (Cheung et al., 2008; Clark et al., 2007; Huijbers et al., 2006; Willimsky and Blankenstein, 2005). This is especially true in the context of lung cancer, which is responsible for more than one million deaths each year worldwide. In an effort to investigate the effect of T cell responses against tumor-specific antigens on tumor progression, we developed a system to induce potentially immunogenic autochthonous lung adenocarcinomas in a genetically engineered mouse model of the disease.

RESULTS

Autochthonous Lung Tumors Expressing Tumor Antigens Are Infiltrated by Lymphocytes Early during Tumor Development

To generate autochthonous lung tumors in a spatiotemporally controlled fashion, we utilized a model of human lung adenocarcinoma driven by the conditional expression of oncogenic K-rasG12D in combination with the loss of p53 (DuPage et al., 2009; Jackson et al., 2001, 2005). Tumor formation was initiated in K-rasLSL-G12D/+;p53fl/fl mice by inhalation of lentiviral vectors expressing Cre recombinase. To induce tumors that lack expression of model antigens, we used a lentiviral vector expressing Cre alone (Lenti-x, Figure 1A). These tumors exhibited little to no lymphocyte infiltration throughout their development as assessed by hematoxylin and eosin (H&E) staining and immunohistochemistry (Figures 1B–1E). To induce tumors that express model tumor antigens, we engineered lentiviral vectors that, in addition to Cre, express the T cell antigens SIYRYGL (SIY) and LucOS (Lenti-LucOS) (Figure 1A). This system allows for the controlled induction of tumors expressing model antigens and provides a means to study the dynamics of T cell infiltration over time.

Figure 1. Induction of Lung Tumors Using Lentiviral Vectors

(A) Design of Lenti-x and Lenti-LucOS (SIN-LTR: self-inactivating long terminal repeat, ψ: HIV packaging signal, cPPT: central polypurine tract, PGK: phosphoglycerate kinase promoter, WRE: woodchuck posttranscriptional regulatory element, Ubc: ubiquitin C promoter). (B) Lung tumors induced with Lenti-x or Lenti-LucOS were stained with H&E, anti-CD3, or anti-B220, 8 weeks after tumor initiation. Scale = 50 μm (inset 10 μm). (C and D) Quantification of immune infiltrates by IHC for CD3 and B220 8 and 16 weeks post tumor initiation. p values are \(10^{-15}\) and \(10^{-15}\) and 0.02 for B220 at 8 and 16 weeks, respectively. n = 2–5 mice, 16–100 tumors, per group. (E) Percentage of lung tumors/mouse containing infiltrating lymphocytes at 8, 16, and 24 weeks after tumor initiation. n = 9–22 mice, 49–1594 tumors, per group. Data are mean ± SEM. See also Figure S1.
and two antigens from ovalbumin – SIINFEKL (SIN, OVA257-264) and OVA233-239 – fused to the C terminus of luciferase, enabling us to monitor antigen expression in tumors (Lenti-LucOS, Figure 1A). As shown in Figure 1, Lenti-LucOS-induced tumors had large numbers of infiltrating lymphocytes including both T and B cells at 8 weeks after tumor initiation (Figures 1B–1E). There were no differences in innate immune cell infiltrates between tumor types (see Figure S1 available online).

**T Cell Responses against Tumor Antigens Are Generated but Not Maintained**

To examine the T cell response to LucOS expression in tumors over time, we assessed the degree of T cell infiltration at 16 and 24 weeks after tumor initiation. In contrast to the robust lymphocytic infiltration of Lenti-LucOS tumors 8 weeks after tumor initiation, there was a dramatic reduction in tumor-infiltrating lymphocytes at later time points, indicating that the immune response was not sustained or tumors had escaped (Figures S2C–S2H). This indicates that while antigen expression is largely maintained and presented in the lung DLNs early during tumor development. To further investigate the early response, we analyzed the T cell response after Lenti-LucOS introduction into the lungs of mice that lacked the K-rasLSL-G12D allele and could not form tumors (Figures S2C–S2H). We found that SIN-specific T cells were generated in the absence of tumor formation, presumably due to antigen expression in normal cells of the lung (Figures S2I–S2K). This indicates that while antigen expression is largely restricted to tumors in this system, the lentiviral infection can stimulate antigen-specific T cells that may contribute to the ensuing antitumor T cell response. Importantly, however, the T cell response in the absence of transformation was weak and short-lived when compared with mice that generated tumors (Figures S2C–S2H). This may indicate that the immune system responds differently to transformed cells or that tumor proliferation within the first several days following K-rasLSL-G12D activation leads to greater antigen production and, hence, more T cell activation.

**Endogenous Tumor-Reactive T Cells Are Not Fully Functional and Display Traits of Terminally Differentiated Effector Cells**

To determine whether T cells specific to the tumor antigens were functional, we measured their capacity to produce IFN-γ and TNF-α at multiple time points after tumor initiation (Figures 2D and 2E; Figures S2L–S2M). Very few tumor-reactive T cells in the lungs had the capacity to produce both IFN-γ and TNF-α, and this activity was almost completely lost within the first 5 weeks of tumor development, whereas T cells maintained production of IFN-γ alone (Figure 2D). This pattern of cytokine production during tumor progression contrasted with the productive T cell response against WSN-SIY and may indicate an increase in terminally differentiated effector T cells (IFN-γ+TNF-αNEG) that is accompanied by a progressive loss of high-quality, memory-like T cells (IFN-γ+TNF-α) (Figures 2D and 2E; Figure S2N) (Seder et al., 2008; Wherry et al., 2007). It is possible that persistent, nonproductive T cell engagement with tumor antigens drives T cells into an exhausted state (Bucks et al., 2009; Mueller and Ahmed, 2009; Redmond and Sherman, 2005). Consistent with this, as tumors progressed, tumor-reactive T cells showed increased expression of PD-1, a marker of T cell exhaustion, and low expression of CD127 (the IL-7Rα chain), a marker of long-lived memory cells (Figure 2F; not shown).

**Adoptively Transferred T Cells Primed by Tumors Rapidly Lose Activity**

To develop a more detailed view of the dynamics of the T cell response to established tumors from activation to effector function, we transferred naive 2C or OT-I TCR transgenic T cells (which recognize SIY or SIN, respectively) into mice bearing Lenti-x or Lenti-LucOS tumors. Three days after transfer, 2C and OT-I T cells were activated and proliferated in the lung-draining lymph nodes and they could be detected in the lungs 12–14 days after transfer (Figures 3A–3C). However, the activity of these T cells was significantly impaired in the DLN and lungs compared with T cells responding to the same antigens expressed in the context of influenza infection (Figure 3D; Figure S3). Therefore, although T cells can be stimulated to proliferate and migrate to tumors, few T cells retain the capacity to produce effector cytokines in response to tumors.

Two potential mechanisms may explain the dysfunction of naive T cells responding to antigens expressed by the Lenti-LucOS tumors: the presence of an immunosuppressive environment in tumor-bearing mice or the suboptimal priming of T cells by antigens expressed in tumors. To test whether the priming of T cells in the DLN is impaired, we assayed whether T cell activity in the lungs could be rescued by transferring effector T cells that were activated in vitro. Indeed, in vitro activated tumorspecific T cells immediately had the capacity to produce both IFN-γ and TNF-α and maintained this activity in the lungs of Lenti-LucOS tumour-bearing mice (Figures 3D and 3E). Furthermore, in vitro activated T cells had comparable function in DLNs to T cells primed in vivo by WSN-SIN (Figure 3E). T cell activity may be exacerbated by interactions with cells or secreted factors in the tumor microenvironment. Foxp3+ T regulatory cells were found to specifically infiltrate Lenti-LucOS tumors (Figure S1). Nevertheless, T cell priming by tumor antigens is clearly suboptimal and contributes to the weak antitumor T cell response.

**Antigen Expression and Presentation Is Maintained in Lenti-LucOS Tumors**

The loss of T cell infiltrates in tumors during tumor progression could also be due to the selection of tumors that lose antigen
expression or antigen presentation to escape the immune response (Dunn et al., 2002; Khong and Restifo, 2002; Uyttenhove et al., 1983; Ward et al., 1990; Zhou et al., 2004). To determine whether the T cell response was driving the outgrowth of tumors lacking antigen, we compared luciferase activity in Lenti-LucOS tumors generated in immune-competent and Rag-2−/− mice. At 16 and 20 weeks post tumor initiation, time points associated with the dissipation of immune infiltrates in tumors, luciferase levels were comparable between normal and immune-compromised mice (Figure 4A). Tumor antigen presentation in the DLN was maintained during the latest stages of disease because tumor-specific 2C T cells still proliferated specifically in the DLN of Lenti-LucOS tumor-bearing mice (Figure 4B). Lenti-LucOS tumors also maintained higher expression

Figure 2. T Cell Responses to Tumor Antigens Are Generated but Not Sustained
(A) Representative analysis of SIY and SIN specific CD8+ T cells in the lung and the draining mediastinal lymph node (DLN) of Lenti-x or Lenti-LucOS tumor-bearing mice or WSN-SIY infected mice using peptide-loaded Kb reagents. FACS plots are gated on PI-negative, CD8+ cells.

(B and C) Percentage of lymphocytes specific for SIN or SIY during Lenti-LucOS tumor progression in the lung or DLN. The percentage of anti-SIN or anti-SIY reactive cells (of total lymphocytes) was determined as shown in Figure S2A. n = 2–5 mice per time point.

(D) IFN-γ and TNF-α cytokine production in SIY and SIN-reactive T cells from the lungs of Lenti-LucOS tumor-bearing mice at several time points after tumor initiation. The percentage of SIY and SIN-specific T cells from the lungs that were IFN-γ+TNF-α+ or IFN-γ+TNF-α− in the absence (no peptide) or in the presence of SIY and SIN peptides (+ peptide) is shown. Percentages were determined by staining duplicate samples for either DimerX or cytokine production as described in Figure S2L. n = 2–7 mice per time point.

(E) The percentage of SIY and SIN-specific T cells in the DLN that were IFN-γ+TNF-α+ 16 weeks after tumor initiation with Lenti-LucOS or WSN-SIY infection. n = 4 mice per group.

(F) PD-1 surface expression on anti-SIY/Kb+ and anti-SIN/Kb+ CD8+ cells (open histograms) or nonspecific CD8+ cells (filled histograms) from the lungs of Lenti-LucOS tumor-bearing mice at several time points after tumor initiation. n = 2–3 mice per group.

Data are mean ± SEM. See also Figure S2.
of MHC class I compared with Lenti-LucOS tumors that arose in Rag-2−/− mice or Lenti-x tumors (Figures 4C and 4D). However, MHC class I expression decreased on Lenti-LucOS tumors between 16 and 24 weeks after tumor initiation (Figures 4D and 4E). Reduced MHC class I expression could contribute to tumor escape and may result from waning antitumor T cell activity and infiltration into tumors. In support of this hypothesis, we found that MHC class I expression and SIN presentation on Lenti-LucOS tumor cell lines were regulated by IFN-γ and that antigen-specific T cell activity in vitro was sufficient to upregulate MHC class I and allow for the specific killing of Lenti-LucOS tumor cells (Figure S4). Therefore, the rapid decline in T cell infiltration into tumors may result from the combined effect of reduced antitumor T cell activity that lowers MHC class I expression on tumor cells, ultimately decreasing the potential for T cells to recognize tumors. This model was supported in vivo by the antigen-specific retention in Lenti-LucOS tumors of fully functional in vitro-activated 2C T cells transferred into tumor-bearing mice 16 weeks after tumor initiation, a time when few tumors retain endogenous lymphocytic infiltrates (Figure 4F). Furthermore, by staining tumor sections with the epithelial marker cytokeratin 8, we could observe tumor-infiltrating 2C T cells making direct contacts with the epithelial cancer cells themselves rather than cytokeratin 8-negative stromal cells (Figures 4G and 4H). Thus, the dissipating T cell response to Lenti-LucOS tumors during the later stages of tumor progression is not due to the escape of tumors that lose the capacity to present the tumor antigens.
Immunogenic Tumors Exhibit Delayed Tumor Progression

Despite the suboptimal antitumor T cell response, it remained possible that the immune response might still affect the course of the disease. To understand the impact of the T cell response on tumor progression, we first compared the size of “immunogenic” Lenti-LucOS tumors to “nonimmunogenic” Lenti-x tumors over the lifespan of tumor-bearing mice. As shown in Figure 5, at each time point examined, immunogenic tumors were smaller than nonimmunogenic tumors, with the differences in size being most significant at 8 and 16 weeks after tumor initiation, coincident with T cell infiltration into tumors (Figure 5A).

Analysis of tumors 8 weeks after initiation revealed an increase in apoptosis in Lenti-LucOS compared with Lenti-x tumors but no difference in proliferation (Figures S5A and S5B). Importantly, the difference in size between Lenti-LucOS and Lenti-x tumors required the presence of the adaptive immune system, because the expression of the LucOS antigens had no effect on tumor size when induced in Rag-2−/− mice (Figure 5B). We also followed tumor progression by examining the histological tumor grades at each time point. At 8 and 16 weeks after tumor initiation, Lenti-LucOS tumors were of lower grade compared with Lenti-x tumors (Figure 5C). By 24 weeks after tumor initiation, the primary lung tumor grades were similar; however, far fewer
mice with immunogenic tumors had metastatic disease (Figure 5D). Thus, early antitumor T cell responses can have long-lasting effects on tumor progression that delay the most deadly phase of the disease. Analysis of human lung adenocarcinoma gene expression datasets indicated that high expression of CD3 genes in tumor samples, as well as other genes expressed specifically in T and B cells, was predictive of better patient outcomes (Figure 5E; Figures S5C–S5F).

Loss of Antigen Expression in Transplantable Models of Lung Cancer

Tumor antigen loss has been reported as a mechanism of tumor immune evasion in transplantable models of cancer (Spiotto et al., 2004; Uyttenhove et al., 1983; Ward et al., 1990; Zhou et al., 2004). Since the endogenously arising lung tumors did not lose antigen expression or presentation in the face of an antitumor immune response, we hypothesized that this may be the consequence of a weaker immune response against endogenous tumors compared with transplanted tumors. To compare immune responses in the context of transplantable or endogenously arising models of cancer, we transduced cell lines from the K-rasG12D-driven lung tumor model with lentiviral vectors to introduce either SIY fused to luciferase (LKR10-LucS) or ovalbumin (containing SIN and OVA 323–339) and SIY fused to luciferase (LKR13-LucOS), and transplanted the cell lines subcutaneously into syngeneic immune-competent mice (Figure S4A). While the parental LKR10 and LKR13 cell lines produced tumors upon transplantation, the antigen-expressing LKR10-LucS and LKR13-LucOS were rejected or exhibited delayed growth and generated CD8+ T cells specific to SIN and/or SIY (Figures S6A–S6D). In stark contrast to the situation with autochthonous tumors, all the tumors that grew in immune-competent mice lost expression of the antigens (Figure 6C). Transferring naive 2C or OT-I T cells into immune-compromised mice bearing established LKR10-LucS or LKR13-LucOS tumors led to reductions in tumor volume and antigen expression in an antigen-specific manner (Figures 6D and 6E). Thus, T cell responses to
a single antigen expressed in transplanted lung tumor cell lines are sufficient to elicit tumor rejection or induce the selective outgrowth of tumors that lose antigen expression.

It has been reported that spontaneous tumors induce a state of systemic tolerance such that T cells can no longer respond to transplanted tumors (Willimsky and Blankenstein, 2005). In this model, however, mice bearing autochthonous lung tumors expressing model tumor antigens still produced effective T cell responses against transplanted tumors expressing the same antigens (Figure S6E). We suspect that leaky expression of tumor antigens in normal tissues, rather than tumor-specific expression, may be responsible for the systemic tolerance to tumor antigens in these transgenic models (Bai et al., 2008; Cheung et al., 2008; Drake et al., 2005; Willimsky and Blankenstein, 2005).
a T cell-dependent manner when transplanted subcutaneously into immune-competent mice (Figures 6F and 6G). These cell lines also lost antigen expression after orthotopic transplantation into the lung, indicating that transplantation, even into a tumor’s native site, can induce T cell responses that drive tumor antigen loss (Figure 6H).

Enhancing the Antitumor T Cell Response with Vaccination Causes Tumor Elimination and Tumor Antigen Loss

Enhanced T cell priming in the context of transplanted tumors is a likely explanation for tumor elimination and tumor antigen loss in this setting. Therefore, priming by vaccination might elicit similar antitumor T cell responses against autochthonous tumors. To examine this possibility, we vaccinated mice prophylactically with a dendritic cell line engineered to express the LucOS antigens (DC2.4-LucOS) and subsequently induced Lenti-LucOS or Lenti-x tumors. Vaccination did not affect the number of Lenti-x tumors, but dramatically reduced the number of Lenti-LucOS tumors and antigen expression in tumors that formed (Figures 7A–7C). This was likely due to an enlarged T cell response early during tumor development, as well as a functionally enhanced response characterized by increased secretion of effector proteins and the maintenance of CD127+ memory-like cells (Figures 7D–7F).

Despite the dramatic reduction of antigen expression in Lenti-LucOS tumors from vaccinated mice, there were still significant numbers of tumor-infiltrating lymphocytes 8 weeks after tumor initiation. Interestingly, in vitro-activated T cells specific to the model antigens could still recognize Lenti-LucOS tumors in DC2.4-LucOS vaccinated mice after adoptive transfer (Figure 7G). The maintenance of a T cell response to the tumor antigens in vaccinated mice led us to ask whether the progression of Lenti-LucOS tumors was altered in vaccinated compared with unvaccinated mice. Although Lenti-LucOS tumors in vaccinated mice appeared only modestly smaller 16 weeks after tumor initiation, the differences in tumor size between vaccinated and unvaccinated mice increased at 24 and 30 weeks after tumor initiation (Figure 7H; Figure S7A). The delayed tumor growth in vaccinated mice correlated with a prolonged retention of lymphocytic infiltrates into tumors (Figure 7I; Figure S7B), again highlighting the potential for tumor-infiltrating lymphocytes to delay tumor progression.

DISCUSSION

Several important conclusions regarding immune-tumor interactions and their role in regulating tumor progression are evident from our study that could not be directly addressed using previous cancer models. Thus far, using genetically engineered mouse models, it has not been possible to compare tumors expressing or lacking tumor-specific antigens (Clark et al., 2007; Huijbers et al., 2006; Willinsky and Blankenstein, 2005). With the recent sequencing of several cancer genomes, including lung adenocarcinomas, it is clear that human tumors contain hundreds of mutated proteins and many of these have the potential to be presented on MHC molecules, thus providing a means for T cells to recognize tumors as distinct from their surrounding normal tissues (Ding et al., 2008; Segal et al., 2008; Sjoblom et al., 2006). Our ability to specifically control the induction of tumor formation and tumor antigen expression has allowed us to closely recapitulate the effects of T cell responses to tumor-specific antigens from inception to malignant transformation.

In contrast to transgenic models of tumor-associated antigen expression, here we followed endogenous T cell responses against antigens expressed in autochthonous lung tumors in an attempt to model T cell responses to tumor neoantigens. By tracking the response of endogenous T cells as well as transferred T cells, we find that suboptimal T cell responses against tumors are not necessarily due to immune ignorance (Nguyen et al., 2002; Ochsenbein et al., 2001; Speiser et al., 1997; Spiotto et al., 2002). However, the expansion of T cells specific to the tumor antigens was markedly delayed, potentially allowing tumor escape (Hanson et al., 2000; Ochsenbein et al., 2001), and the T cell response eventually declined in both magnitude and quality as tumors progressed. Despite their ability to proliferate and migrate to tumors, T cells had an altered phenotypic state that was exemplified by a poor capacity to produce and secrete effector cytokines and a rapid loss of TNF-α production in the lung. The reduced capacity of T cells to produce effector cytokines such as IFN-γ and TNF-α as tumors progressed may account for their inefficient migration to tumors over time (Calzascia et al., 2007; Muller-Hermelink et al., 2008). The ability to precisely time tumor initiation revealed a dynamic relationship between antitumor T cells and tumor cells. Loss of T cell activity can contribute to the rapid decline in tumor-infiltrating lymphocytes by allowing for reduced MHC class I expression on tumor cells. Tumor-reactive T cells were also found to express high levels of PD-1 and low levels of CD127, features that resemble T cell responses during chronic infections (Bucks et al., 2009; Wherry et al., 2007). Indeed, the T cell response to lung tumors was quite distinct from the response to the same antigens expressed by recombinant influenza virus after infection of the lung. Signaling through the inhibitory PD-1/PD-L1 pathway alone was not responsible for the loss of T cell reactivity against tumors in this model because blocking the function of this pathway with the use of antibodies or PD-1-deficient T cells did not rescue the activity of tumor-reactive T cells (not shown). Instead, T cell exhaustion might be exacerbated by an ineffective antitumor immune response that allows prolonged T cell exposure to antigens expressed from tumors. Chronic T cell stimulation with cognate antigen has been shown to be sufficient to drive exhaustion in several settings (Bucks et al., 2009; Drake et al., 2005; Mueller and Ahmed, 2009; Redmond and Sherman, 2005). We also found that T regulatory cells were recruited specifically to tumors expressing the LucOS antigens, and T regulatory cells have been shown to suppress antitumor responses in several cancer models (Clark et al., 2007; Ercolini et al., 2005; Pellegrini et al., 2009). Despite the evidence of an incomplete antitumor T cell response, the immune response still delayed tumor progression, indicating that suboptimal T cell responses that do not eliminate tumors are capable of slowing cancer progression (Koebel et al., 2007). This mirrors observations made here and in several retrospective studies of human cancer patients in which immune infiltration into tumors significantly correlated with improved patient outcomes (Buckanovich et al., 2008; Budhu et al., 2006; Dunn et al., 2002; Finak et al., 2008; Sjoblom et al., 2006).
Figure 7. Vaccination in Autochthonous Lung Tumor Model Reduces Tumor Burden and Promotes Loss of Antigen Expression

(A and B) Tumor number on the lungs of mice with Lenti-x or Lenti-LucOS tumors 16 weeks after tumor initiation with or without prior DC2.4-LucOS vaccination. p values are ~0.50 and ~0.0008, respectively.

(C) Freshly explanted Lenti-LucOS tumors were assayed for luciferase activity and the percentage of tumors that were luciferase positive (>50 RLU/μg protein) ± DC2.4-LucOS vaccination is plotted. n = 5–6 mice, 27–34 tumors, per group.

(D) Representative FACS plots of SIY/Kb+-specific T cells (gated on CD8) from the lung and DLN ± DC2.4-LucOS vaccination, 9 days after tumor initiation with Lenti-LucOS. n ≥ 2 mice per group.

(E) FACS for surface-exposed CD107α and captured IFN-γ (gated on CD8) after stimulation of DLN cells with SIY and SIN from Lenti-LucOS or Lenti-x tumor-bearing mice ± DC2.4-LucOS vaccination (16 weeks post tumor initiation). Mean fluorescent intensities of CD107α and IFN-γ on reactive T cells from Lenti-LucOS tumor-bearing mice is plotted. n = 2–4 mice per group.

(F) FACS analysis of CD8+ anti-SIY/Kb+ and anti-SIN/Kb+-specific T cells for CD127 and CD44 surface expression from the lungs or DLN of Lenti-LucOS or Lenti-x tumor-bearing mice ± DC2.4-LucOS vaccination (8 weeks post tumor initiation). The ratio of CD44+CD127+ (memory-like) to CD44+CD127- (effector-like) is shown below each FACS plot.

(G) Lenti-LucOS tumors from mice 8 weeks after tumor initiation and 24 hr after transfer of SIY-reactive, CFSE-labeled activated T cells (green) ± DC2.4-LucOS vaccination. DAPI counter stained. Tumors are outlined. Scale = 50 μm.

(H) Mean tumor area of Lenti-LucOS lung tumors at 16, 24, and 30 weeks after tumor initiation ± DC2.4-LucOS vaccination. p values are ~0.050, ~0.038, and ~0.027, respectively, from n = 3–10 mice, 70–506 tumors, per group at 16 and 24 weeks, and n = 1–2 mice, 15–46 tumors, per group at 30 weeks.

(I) Percentage of Lenti-LucOS tumors/mouse containing infiltrating lymphocytes at 16, 24, and 30 weeks after tumor initiation ± DC2.4-LucOS vaccination (analysis of mice from [H]). Data are mean ± SEM. See also Figure S7.
2008; Galon et al., 2006; Molidrem et al., 2000; Pages et al., 2005; Piersma et al., 2007).

Prophylactic vaccination provided substantial protection from autochthonous tumors, but it also led to prolonged immune infiltration into the Lenti-LucOS tumors, further delaying tumor progression even though antigen expression was dramatically reduced. Delayed tumor progression despite antigen loss may occur through direct targeting of tumor cells by T cells or via indirect elimination of tumor cells during T cell-mediated destruction of stromal cells that cross-present tumor antigens in the tumor microenvironment (Spiotto et al., 2004). This result has important implications for immunotherapy because it indicates that generating large numbers of highly functional, tumor-reactive T cells that persist long-term can effectively inhibit tumor progression even after the selective outgrowth of tumors that express very low levels of the targeted antigen.

The majority of studies investigating immunity to cancer have focused on transplantable models of cancer (Dunn et al., 2002; Hanson et al., 2000; Khong and Restifo, 2002; Koebel et al., 2007; Ochsenbein et al., 2001; Schreiber, 2003; Shankaran et al., 2001; Spiotto et al., 2002; Street et al., 2002; Thomas and Massague, 2005). These studies have led to the discovery of several important mechanisms in both T cells and tumor cells that can modulate the effectiveness of antitumor T cell responses or allow for tumor escape. However, these models may not accurately reflect the human disease (Khong and Restifo, 2002). Here, we have used comparable models of transplantable and autochthonous lung cancer to show that transplantation induces antitumor T cell responses capable of driving tumor immunoediting. Even orthotopic transplantation of cell lines derived from Lenti-LucOS-induced tumors that had escaped immune surveillance in their autochthonous setting led to an adaptive immune response that forced the outgrowth of tumors with reduced antigen expression, a phenomenon that never occurred with autochthonous tumors expressing these antigens. This was due in part to suboptimal priming of T cells in the context of autochthonous tumors. T cells activated in vitro had enhanced activity compared with T cells stimulated in vivo and priming of T cells by vaccination led to responses against autochthonous tumors that were comparable to responses against transplanted tumors. From our comparison of autochthonous and transplanted tumors, we conclude that the immune system recognizes tumors that originate from transformation of somatic cells in an inherently different way than transplanted tumor cell lines.

Future experiments should address the diversity of antitumor immune responses that likely stem from subtle differences in the models used for the investigations. The development of models that link the tumor antigen to the driving oncogenic event will be useful for investigating whether T cell responses to oncogenic antigens, whose expression in tumors cannot easily be downregulated to evade an immune response, instigate alternative mechanisms of tumor escape (Willimsky and Blankenstein, 2005). Models in which tumor initiation and expression of tumor-specific antigens are temporally separable may discern whether T cell responses and tumor development change depending on the context of the disease.

### EXPERIMENTAL PROCEDURES

**Mice and Tumor Induction**

129S/SvJae strains backcrossed 8 generations were used for all tumor transplant experiments and most autochthonous tumor studies. C57/BL6 mice backcrossed five generations were used when transgenic T cells from C57/BL6 mice were adoptively transferred (Figures 3 and 4B). Tp53+/− mice were provided by A. Berns, K-rasLSL-G12D+/− were generated in our laboratory, and Rag-2−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lung tumors were induced in K-rasLSL-G12D+/−;p53+/− mice by intratracheal instillation and inhalation of viruses expressing Cre recombinase as reported (DuPage et al., 2009). J. Chen provided the 2C, OT-I, and OT-II TCR transgenic mice that were C57/BL6 and crossed into Rag-2−/−. All animal studies and procedures were approved by the Massachusetts Institute of Technology’s Committee for Animal Care.

**Lentiviral Production**

Lentiviruses were produced by transfection of 293T cells with and CMV-VSV-G as described (DuPage et al., 2009).

**Histology, Tumor Size, Tumor Grading**

Mice were killed at designated time points and lung tissues were preserved by fixation with 3.6% formaldehyde solution or frozen in O.C.T. Biocuane Image Analysis software was used to measure the area of individual tumors in histological sections. Grading was performed by R.B. as described (DuPage et al., 2009). Metastasis was monitored by gross examination of mice at necropsy and histological examination of lung DLNs.

**Immunohistochemistry**

We performed immunohistochemical analysis on formalin-fixed paraffin sections after heat-mediated antigen retrieval in sodium citrate (see Abcam protocol). We used α-CD3 (1:100, Dako), α-B220 (1:50, RA3-82B), α-Mac-3 (1:10, M3/84) (BD Pharmingen), α-Foxp3 (1:25, FJK-16s) (eBioscience), α-cytokeratin 8 (1:25) primary antibodies and Vectastain ABC secondary reagents followed by DAB substrate detection (Vector Laboratories).

**Flow Cytometry**

Cell suspensions from lymphoid organs were prepared by mechanical disruption between frosted slides or from lungs by mincing and digesting the tissues for −1 hr at 37°C in 125 U/ml Collagenase Type I (Gibco) and 60 U/ml Hyaluronidase (Sigma). Cells were stained with antibodies for 20–30 min after
treatment with FcBlock (BD PharMingen), α-CD8α (53-6.7), α-CD45.1 (A20), α-mouse IgG3, (X56), α-PD-1 (J43), α-IFN-γ (XMG1.2), α-TNF-α (MP6-XT22), α-CD107α (ID48), α-CD44 (IM7), α-CD62L (MEL-14), α-CD69 (H1.2F3), and DimerX I (Dimeric Mouse H-2Kb/Ig) were from BD PharMingen. Immune-competent and Rag-2 utilizing the log rank test. Correction, Figure 5D utilized the Fischer exact probability test, and Figure 5E comparisons with the following exceptions: Figure 7F utilized Welch's 20 pfu of virus by intratracheal inhalation.

Cytokine Production
Cells were resuspended in the presence or absence of SIVRYYGL and/or SIINFEKL peptides in OPTI-MEM I (GIBCO) supplemented with GolgiPlug (BD PharMingen) for 4–5 hr at 37°C, 5% CO2. Cells were then fixed and stained for intracellular cytokines using the Cytofix/Cytoperm kit (BD Biosciences). For IFN-γ capture, cells were stained with the capture and detection antibodies as directed (Miltenyi Biotec).

Luciferase Detection
Freshly explanted tumors or cell lines were lysed in Cell Culture Lysis Reagent, mixed with Luciferase Assay Reagent (Promega), and relative light units (RLU) were detected using the Opticon I luminometer (MM Instruments). RLUs were standardized based on the total number of cells or total protein (Bio-Rad Protein Assay). In vivo bioluminescence images were obtained using the NightOwlII LB983 (Berthold Technologies) after intraperitoneal injection of 1.5 mg Beetle Luciferin (Promega).

CFSE Labeling and Adoptive Transfer
Naive and activated T cells were labeled with 5 μM CFSE (Molecular Probes) for 10 min at 37°C. Naive TCR transgenic T cells were harvested from lymphoid organs, stained, and 5 × 105–5 × 106 CD8+ T cells were transferred intravenously. Activated TCR transgenic cells were stimulated in vitro with cognate peptide for 16–20 hr and then cultured for 6–7 days in complete RPMI supplemented with 5 ng/ml IL-2 (R&D Systems). 10^5 in vitro-activated T cells were transferred intravenously.

Tumor Cell Lines and Transplantation
LKR10 and LKR13 cell lines were derived from K-rasG12D-driven mouse lung tumors from 129Sv/SvJae mice. LKR10-LucS and LKR13-LucOS were generated by lentiviral infection with LucOS or LucOS constructs (no Cre). KP-LucOS and KP-x cell lines were derived from Lent-LucOS- or Lent-x-induced lung tumors, respectively, from 129Sv/SvJae mice. Cells (10^5–10^6) were transplanted subcutaneously (sc) or intravenously (for orthotopic). 129Sv/SvJae immune-competent and Rag-2–/– transplanted recipients came from the same mouse strains used to generate the autochthonous tumors. Sc tumor volumes were calculated by multiplying length × width × height of each tumor.

DC2.4-LucOS Vaccination
DC2.4-LucOS cells were generated from the dendritic cell line (DC2.4) after infection with lentivirus that expresses LucOS (no Cre). Mice were vaccinated by intraperitoneal injection of 5 × 10^6 cells 1 month prior to tumor initiation.

Influenza
WSN-SIY and WSN-SIN were provided by J. Chen. Mice were infected with 20 pfu of virus by intratracheal inhalation.

Statistical Analyses
P values from unpaired two-tailed Student’s t tests were used for statistical comparisons with the following exceptions: Figure 7F utilized Welch's correction, Figure 5D utilized the Fischer exact probability test, and Figure 5E utilized the log rank test.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.ccr.2010.11.011.

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REFERENCES


