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CD4+ T Cells Contribute to the Remodeling of the Microenvironment Required for Sustained Tumor Regression upon Oncogene Inactivation

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SUMMARY

Oncogene addiction is thought to occur cell autonomously. Immune effectors are implicated in the initiation and restraint of tumorigenesis, but their role in oncogene inactivation-mediated tumor regression is unclear. Here, we show that an intact immune system, specifically CD4+ T cells, is required for the induction of cellular senescence, shutdown of angiogenesis, and chemokine expression resulting in sustained tumor regression upon inactivation of the *MYC* or *BCR-ABL* oncogenes in mouse models of T cell acute lymphoblastic lymphoma and pro-B cell leukemia, respectively. Moreover, immune effectors knocked out for thrombospondins failed to induce sustained tumor regression. Hence, CD4+ T cells are required for the remodeling of the tumor microenvironment through the expression of chemokines, such as thrombospondins, in order to elicit oncogene addiction.

INTRODUCTION

The inactivation of a single oncogene is sufficient to induce sustained tumor regression in vivo through the phenomenon of oncogene addiction, as has been demonstrated experimentally in many conditional transgenic mouse model systems (Felsher, 2008; Sharma and Settleman, 2007; Weinstein and Joe, 2008) and through the development of targeted therapeutics such as Gleevec (Weinstein and Joe, 2006). Oncogene addiction is associated with proliferative arrest, apoptosis, differentiation, and cellular senescence as well as the shutdown of host programs such as angiogenesis (Felsher, 2003; Felsher and Bishop, 1999; Giuriato et al., 2006; Jain et al., 2002; Shachaf et al., 2004; Wu et al., 2007). To date, it has been presumed that oncogene inactivation induces tumor regression through cell autonomous mechanisms, independent of host effector cells.

The host immune system plays an important role in tumorigenesis. Both antigen-dependent and -independent mechanisms are implicated through multiple cellular effectors and effects on inflammation and the tumor microenvironment (Crowe et al., 2002; Shankaran et al., 2001). Indeed, it is well documented that CD8+ T cells contribute to antigen-dependent and NK cell-mediated tumor elimination (Shanker et al., 2007; van der Bruggen et al., 1991). Additionally, CD4+ T cells may also contribute to

Significance

Through transgenic mouse models of conditional oncogene inactivation, we show that the absence of an intact immune system results in a 10- to 1000-fold reduction in the rate, extent, and duration of tumor regression upon oncogene inactivation. We uncovered an unanticipated role for CD4+ T cell effectors in mediating cellular senescence and the shutdown of tumor angiogenesis and discovered a critical role for the expression of thrombospondin-1 in immune effectors. Most strategies to identify therapeutic agents utilize in vitro models or in vivo xenograft models overlooking the effect of the immune system. Our results argue for the necessity of models that include an intact host immune system to properly evaluate the potential efficacy of targeted therapeutics for maximum clinical impact.

tumor regression (Corthay et al., 2005; Qin and Blankenstein, 2000). Chemokines produced by the immune system have been shown to play an important role during tumor evolution and therapeutic response (Rossi and Zlotnik, 2000; Smyth et al., 2004).

In general, tumors coevolve with host immune effectors and chemokines through a process that has been described as immune editing (Dougan and Dranoff, 2009; Dunn et al., 2002; Dunn et al., 2006; Reiman et al., 2007; Swann et al., 2008). Immune editing has been dramatically illustrated in several models of carcinogenesis (Bui et al., 2006; Shankaran et al., 2001; Willimsky and Blankenstein, 2005). Host immune effectors also contribute to the initiation of tumorigenesis through profound effects on the tumor microenvironment (Coussens and Werb, 2002; de Visser et al., 2006). Thus, the immune system appears to play a complex role in both the initiation and restraint of tumorigenesis.

The role of the immune system in mediating tumor regression upon targeted oncogene inactivation is not known. Hosts that are immune compromised have a markedly increased incidence of many different types of cancers (Birkeland et al., 1995; Dunn et al., 2002; Pham et al., 1995). The host immune system is intimately involved not only in the promotion and prevention of neoplasia but also in determining the therapeutic response to treatment of cancer (Andreu et al., 2010; Boshoff and Weiss, 2002; Dave et al., 2004; Galon et al., 2006; Gatti and Good, 1971; Kohrt et al., 2005; Zitvogel et al., 2008). However, experimental study of therapeutics for cancer is usually performed in vitro or in vivo in immune compromised hosts, circumstances in which immune effectors are necessarily absent. These models do not account for the role of host-tumor interactions and the role of the immune system.

The *MYC* oncogene has been implicated in the pathogenesis of many human tumors (Meyer and Penn, 2008). *MYC* is involved in the etiology of many types of lymphoma including Burkitt's large cell and T cell acute lymphoblastic lymphoma (T-ALL) (Boxer and Dang, 2001; Pelengaris et al., 2002). We have previously described our conditional transgenic mouse models of *MYC*-induced T cell acute lymphoblastic lymphoma (T-ALL) (Felsher and Bishop, 1999). The inactivation of *MYC* is sufficient to induce sustained tumor regression associated with proliferative arrest, apoptosis, differentiation, cellular senescence and the shutdown of angiogenesis (Felsher and Bishop, 1999; Giuriato et al., 2006; Wu et al., 2007). Here, we show that defects in the host immune system have a profound influence on the ability of oncogene inactivation to elicit oncogene addiction.

RESULTS

Immune System Is Required for Rapid, Complete, and Sustained Tumor Regression

To interrogate if the immune system is required to elicit oncogene addiction upon *MYC* inactivation, we transplanted luciferase labeled tumors from our conditional transgenic mouse model of *MYC*-induced hematopoietic tumorigenesis into wild-type hosts and in hosts with specific defects in immune compartments: SCID, RAG2$^{-/-}$cγc$^{-/-}$, RAG2$^{-/-}$, CD4$^{-/-}$CD8$^{-/-}$, CD4$^{-/-}$CD8$^{+/+}$, CD4$^{+/+}$CD8$^{-/-}$ (Figures 1A and 1B). By using bioluminescence imaging, we could measure the kinetics of tumor regression upon *MYC* inactivation.

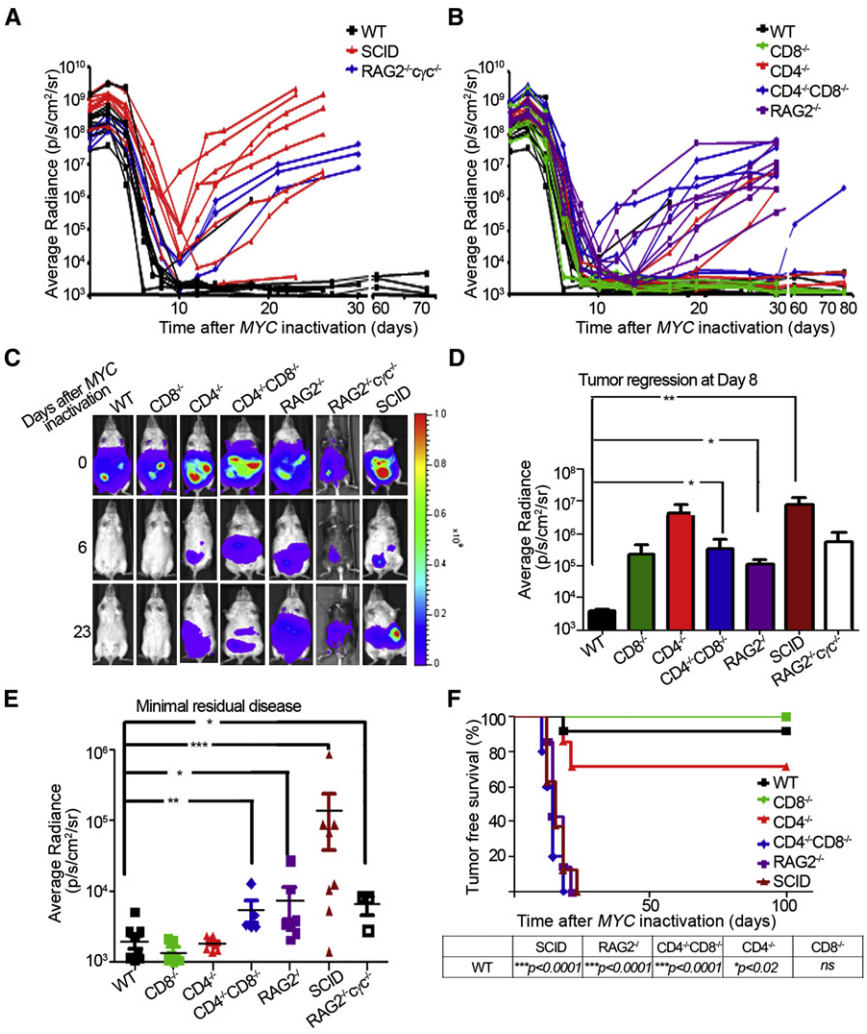
Tumors initially exhibited regression regardless of the host immune status (Figures 1A, 1B, and 1C). However, severely immune compromised hosts (SCID and RAG2$^{-/-}$cγc$^{-/-}$ mice deficient in the adaptive immune system and NK cells) demonstrated significantly delayed kinetics of tumor regression upon *MYC* inactivation compared with wild-type (WT) hosts (Figure 1D, SCID versus WT, $p < 0.001$) and failed to execute complete tumor elimination with up to 1000-fold more minimal residual disease (MRD) after *MYC* inactivation (Figure 1E, SCID versus WT, $p < 0.0001$; RAG2$^{-/-}$ cγc$^{-/-}$ versus WT, $p = 0.01$ at the nadir of luciferase activity upon *MYC* inactivation). Similarly, less severely immune compromised hosts also exhibited delayed kinetics (Figures 1B and 1D, RAG2$^{-/-}$ versus WT, $p = 0.02$; CD4$^{-/-}$CD8$^{-/-}$ versus WT, $p = 0.02$) and a significantly increased MRD (Figure 1E, RAG2$^{-/-}$ versus WT, $p = 0.01$; CD4$^{-/-}$CD8$^{-/-}$ versus WT, $p < 0.01$). Hence, an intact immune system is required for rapid and complete tumor regression.

To determine if host immune status influenced the frequency of tumor recurrence, we continued to observe mice for 80 days after *MYC* inactivation noting that tumors recurred at a statistically significant higher frequency in SCID, RAG2$^{-/-}$cγc$^{-/-}$, RAG2$^{-/-}$, and CD4$^{-/-}$CD8$^{-/-}$ hosts (87.5%, 100%, 100%, and 80%, respectively) compared with WT hosts (9%) (immune compromised hosts versus WT, $p < 0.0001$, also see Figure 1F). CD4$^{-/-}$ but not CD8$^{-/-}$ deficient hosts exhibited a significant influence on tumor recurrence (28.5%, 0%, respectively) (Figure 1F). Correspondingly, CD4^{+}, but not CD8^{+} T cell deficiency alone was sufficient to impede sustained tumor regression compared to WT mice (Figure 1F, WT versus CD4$^{-/-}$, $p = 0.02$). Similar results could be obtained using nonluciferase labeled tumors (Figure S1A). By qPCR analysis it was confirmed that doxycycline treatment resulted in similar suppression of transgenic *MYC* expression regardless of host immune status (Figure S1B). Hence, defects in the host immune system prevented sustained tumor regression upon *MYC* inactivation.

Immune System Is Not Required to Induce Proliferative Arrest or Apoptosis

Previously, we have shown that upon *MYC* inactivation in a transgenic model of T-ALL, tumor cells undergo proliferative arrest and apoptosis (Felsher and Bishop, 1999). We determined if the mechanism by which immune cells were contributing to the process of tumor regression was through effects on proliferation and apoptosis of tumor cells before and after *MYC* inactivation (Figures 2A and 2B). After 4 days of *MYC* inactivation, tumors from wild-type and immunodeficient hosts exhibited an overall loss of pleomorphic characteristics evidenced by a similarly marked reduction in cell size and nuclear to cytoplasmic ratio in both cohorts. Importantly, upon *MYC* inactivation, we observed marked changes in the total number of cells per field and carefully controlled for these changes in our quantification of TUNEL and Ki67 staining.

To measure apoptosis, TUNEL staining was performed. Apoptosis occurred similarly upon *MYC* inactivation regardless of host immune status (Figure 2A), suggesting that initial tumor regression occurs similarly regardless of the presence or



	SCID	RAG2-/-	CD4-/-CD8-/-	CD4-/-	CD8-/-
WT	***p<0.0001	***p<0.0001	***p<0.0001	*p<0.02	ns

Figure 1. An Intact Immune System Is Required for Sustained Tumor Regression

(A and B) Graphical representation of tumor regression and relapse kinetics as measured by bioluminescence imaging. Luciferase-labeled tumor cell lines from our conditional mouse T-ALL model were injected s.c. into different cohorts of mice (WT, n = 11; $CD8^{-/-}$, n = 7; $CD4^{-/-}$, n = 6; $CD4^{-/-}CD8^{-/-}$, n = 5; SCID, n = 8; $RAG2^{-/-}$, n = 7; $RAG2^{-/-}c\gamma c^{-/-}$, n = 3). *MYC* was inactivated by administering doxycycline (dox) to the mice when tumors reached a comparable bioluminescence signal (10^8p/s/sr/cm^2).

(C) Bioluminescence images of tumors regressing in the different immunodeficient hosts. Data are representative of three experiments.

(D) Quantitative analysis of tumor regression in the indicated hosts, 8 days after *MYC* inactivation.

(E) Quantification of minimum residual disease in the indicated hosts. Data are presented as the minimum bioluminescence signal after *MYC* inactivation.

(F) Kaplan Meier curves of tumor-free survival in the various immunodeficient genotypes. A mouse was scored as a relapse when its tumor bioluminescence signal first began to increase after tumor regression. The log-rank test was used to compare survival curves. Data are representative of three experiments using two cell lines and one primary tumor. Statistical significance was analyzed by pooling data from all experiments (WT, n = 43; $CD8^{-/-}$, n = 20; $CD4^{-/-}$, n = 24; $CD4^{-/-}CD8^{-/-}$, n = 15; SCID, n = 15; $RAG2^{-/-}$, n = 46) (p value evaluated by unpaired Student's t test) is shown.

*p < 0.01, **p < 0.001, ***p < 0.0001. Error bars are ± SEM. See also Figure S1.

absence of an immune system. Quantification of TUNEL staining revealed a 2-fold increase in the extent of apoptosis upon *MYC* inactivation in tumors from WT hosts (Figure 2B, WT *MYC* On versus Off, p = 0.05). Moreover, the apoptosis in regressing tumors from WT hosts was not significantly different from that of regressing tumors in either $RAG2^{-/-}$ or $CD4^{-/-}$ hosts (Figure 2B, WT versus $RAG2^{-/-}$, $CD4^{-/-}$ *MYC* Off, p = 0.3 and 0.3, respectively). Finally there was a small but statistically insignificant increase in the levels of apoptosis upon *MYC* inactivation in $RAG2^{-/-}$ or $CD4^{-/-}$ hosts (Figure 2B, $RAG2^{-/-}$, $CD4^{-/-}$ *MYC* On versus Off, p = 0.07 and 0.09, respectively). Hence, the absence of the immune system may slightly impede apoptosis of tumor cells upon *MYC* inactivation.

Next, changes in cellular proliferation upon *MYC* inactivation were measured by Ki67 staining. *MYC* inactivation in tumors from WT and immunodeficient hosts resulted in a significant reduction in Ki67 staining (Figures 2A and 2B, WT, $RAG2^{-/-}$, $CD4^{-/-}$ *MYC* On *versus* *MYC* Off, p < 0.01). Interestingly, in comparison to WT hosts, $RAG2^{-/-}$ but not $CD4^{-/-}$ hosts, underwent a statistically significant further decrease in Ki67 staining upon *MYC* inactivation (WT versus $RAG2^{-/-}$ or $CD4^{-/-}$ *MYC* Off, p = 0.02 or p < 0.05, respectively). Thus, the absence of the host immune system either has no effect or modestly enhances the effect of *MYC* inactivation in inducing proliferative arrest.

Immune System Is Required to Induce Cellular Senescence and the Shutdown of Angiogenesis

We have reported that upon *MYC* inactivation tumor cells undergo cellular senescence (Wu et al., 2007) and the shutdown of angiogenesis (Giuriato et al., 2006). We examined the role of both processes. Tumors from WT hosts expressed a 20-fold increase in senescence-associated acidic β-gal (SA-β-Gal) activity upon *MYC* inactivation and demonstrated a 26- and 6-fold increase in senescence-associated markers, p16INK4a and p21, respectively, upon *MYC* inactivation (Figures 3A and 3B). In contrast, *MYC* inactivation in tumors in $RAG2^{-/-}$ and $CD4^{-/-}$ mice did not result in increased SA-β-Gal or the induction of p16INK4a or p21 (Figures 3A and 3B, WT versus $RAG2^{-/-}$ *MYC* Off SA-β-Gal, p = 0.01, p16 staining p = 0.002, p21 staining p = 0.01; WT versus $CD4^{-/-}$, *MYC* Off SA- β-Gal, p = 0.009, p16 staining, p = 0.0005, p21 staining, p = 0.004).

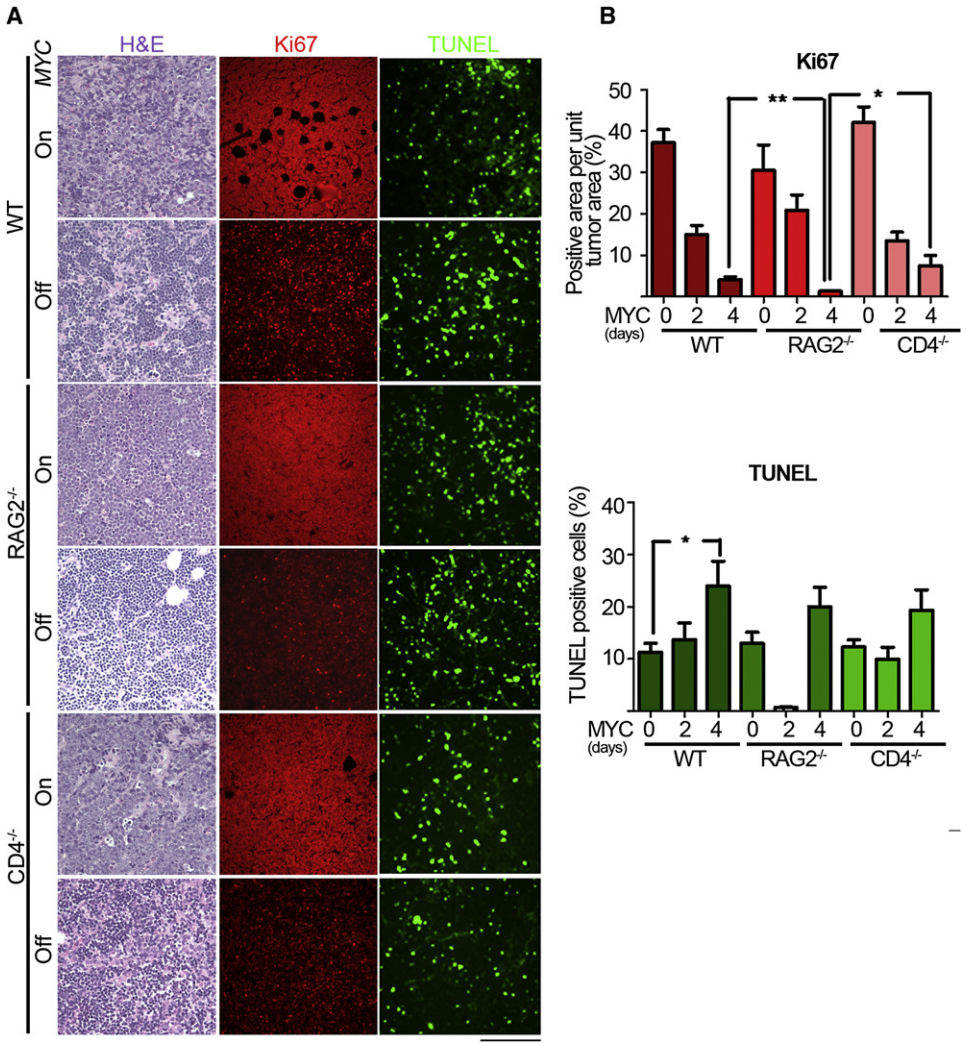


Figure 2. The Immune System Does Not Influence Apoptosis or Proliferative Arrest upon *MYC* Inactivation

(A) Micrographs of hematoxylin and eosin staining (left panel), TUNEL (middle panel), and Ki67 (right panel) immunostaining of tumors derived from untreated (*MYC* On) and 4 day dox-treated mice (*MYC* Off) from WT (top panel), RAG2$^{-/-}$ (middle panel), and CD4$^{-/-}$ (bottom panel) hosts. Scale bar = 100 μm.

(B) Quantitative representation of Ki67 (top panel) and TUNEL (bottom panel) immunostaining shown in (A) for 0, 2, and 4 days after *MYC* inactivation. Quantification of TUNEL and Ki67 immunostaining is presented as the average percentage of TUNEL-positive cells and area of Ki67-positive regions, respectively, within the tumors. At least five different fields from three different tumors injected with at least two different tumor cell lines for each different condition. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. Error bars are represented as ± SEM.

Thus, in immune deficient mice, *MYC* inactivation is impeded from inducing cellular senescence in tumor cells. Notably, CD4$^+$ T cells specifically appeared to be required.

We determined if an intact immune system was required for *MYC* inactivation to induce the shutdown of angiogenesis associated with the secretion of TSP-1, a potent antiangiogenic protein (Giuriato et al., 2006; Kazerounian et al., 2008; Lawler, 2000). Upon *MYC* inactivation there was a 3.5-fold induction of TSP-1 in tumors from WT hosts but not in RAG2$^{-/-}$ or CD4$^{-/-}$ hosts (Figure 4A, WT versus RAG2$^{-/-}$, CD4$^{-/-}$ *MYC* Off, $p = 0.001$). Furthermore, while tumors in WT mice demonstrated very little change in mean vascular density (MVD) as measured by CD31 staining upon *MYC* inactivation (Figure 4B), RAG2$^{-/-}$ and CD4$^{-/-}$ mice exhibited a 5- and 12-fold, respectively, increase in tumor MVD upon *MYC* inactivation (Figures 4A and 4B, RAG2$^{-/-}$ *MYC* On versus Off, $p < 0.0001$; CD4$^{-/-}$ *MYC* On versus Off, $p = 0.07$). Thus, the absence of CD4$^+$ T cells impairs the ability of *MYC* inactivation to induce cellular senescence as well as shutdown angiogenesis.

Finally, TSP-1 expression requires host immune cells and specifically CD4$^+$ T cells. Indeed, we found that TSP-1 protein expression is markedly decreased in spleens of immune compromised versus wild-type hosts (Figure S2A). Further, we show that activated CD4$^+$ T cells express TSP-1 (Figure S2B).

CD4$^+$ T Cells Home to the Tumor and Are Sufficient to Restore Sustained Tumor Regression

We examined if CD4$^+$ T cells were homing to the tumor site upon oncogene inactivation. Upon adoptive transfer into RAG2$^{-/-}$ hosts, luciferase$^+$ CD4$^+$ T cells rapidly localized to the tumor

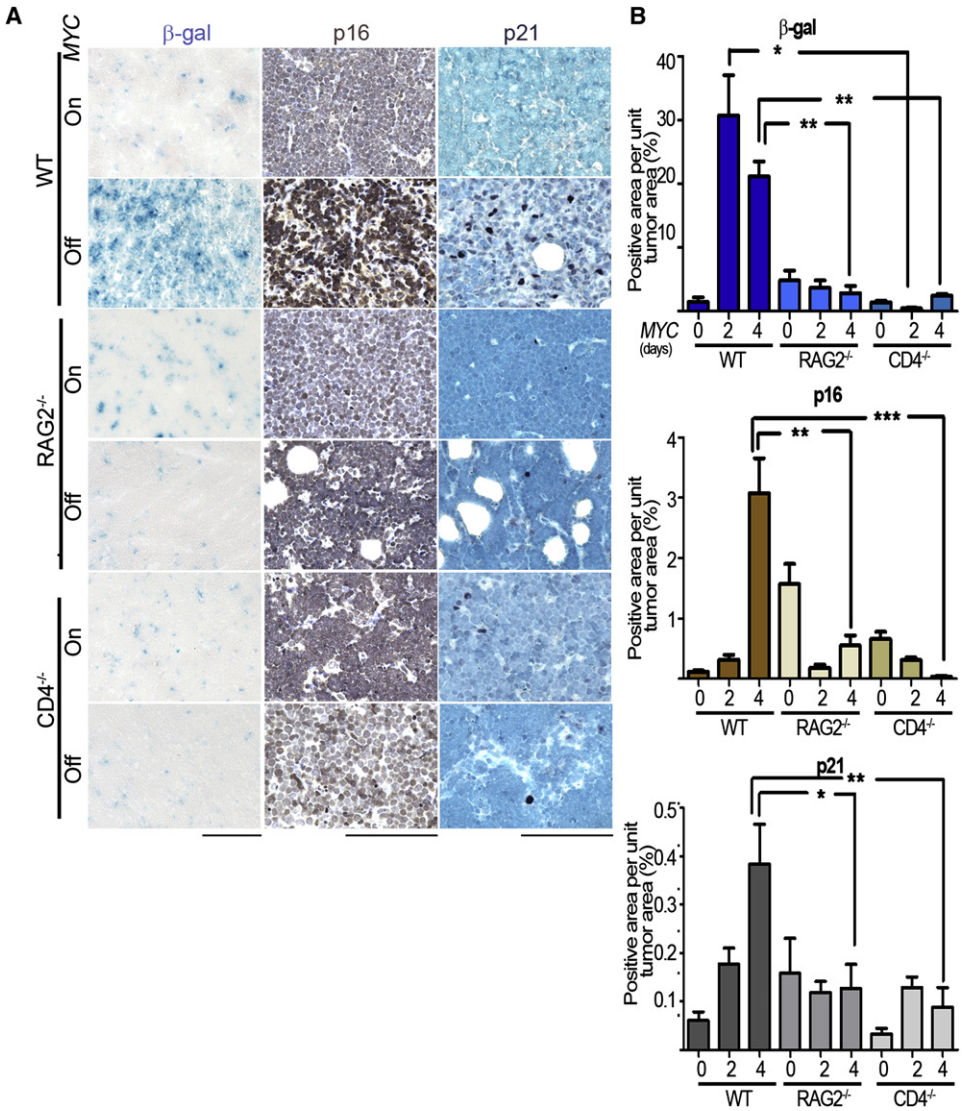


Figure 3. An Intact Immune System Is Required for the Induction of Cellular Senescence upon *MYC* Inactivation

(A) Micrographs of senescence associated β-galactosidease (SA β-gal, left panel), p16 (middle panel), and p21 (right panel) immunostaining of tumors derived from untreated (*MYC* On) and 4 day dox-treated (*MYC* Off) mice of the indicated genotypes. Scale bar = 100 μm.

(B) Quantification of SA-β-gal (top panel), p16 (middle panel), and p21 (bottom panel) staining shown in (A) for 0, 2, and 4 days after *MYC* inactivation. Quantification is presented as the average percentage of positively stained regions within the tumors. At least five different fields from three different tumors injected with at least two different tumor cell lines were analyzed for each different condition. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. Error bars are ± SEM.

site upon *MYC* inactivation as seen by bioluminescence imaging of these tumors before and after *MYC* inactivation (Figure 5A). Inactivating this oncogene causes $CD4^+$ T cells to localize at the tumor site as early as 4 days after oncogene inactivation, peak at day 12 and persist up to 3 weeks after *MYC* inactivation. Thus, *MYC* inactivation is associated with trafficking of $CD4^+$ T cells to sites of tumor involvement. Notably, $CD4^+$ T cell-depleted luciferase$^+$ splenocytes also localized to the site of the tumor upon *MYC* inactivation, suggesting the recruitment of additional host immune effector populations (Figure S3A).

Next, we evaluated if we could restore the ability of *MYC* inactivation to induce sustained tumor regression in immune compromised hosts by adoptively transferring specific lymphocyte populations into $RAG2^{-/-}$ mice. By FACS analysis, we confirmed reconstitution of effector cells (Figure S3B). As expected, $RAG2^{-/-}$ mice adoptively transferred with splenic lymphocytes exhibited sustained regression (Figure 6B). $RAG2^{-/-}$ hosts demonstrated a significant amount of MRD after *MYC* inactivation compared with WT hosts (Figures 5B and 5C, $RAG2^{-/-}$ versus WT, $p = 0.007$). Reconstitution of immunodeficient hosts with naive $CD8^+$ T cells continued to have a significant burden of MRD (Figure 5C, $RAG2^{-/-}CD8^+$ versus WT, $p = 0.03$), whereas reconstitution of $RAG2^{-/-}$ hosts with naive $CD4^+$ T cells completely eliminated MRD, similar to WT hosts upon *MYC* inactivation (Figure 5C, $RAG2^{-/-}CD4^+$ versus WT, $p = 0.09$). Moreover, $RAG2^{-/-}$ hosts adoptively transferred with $CD4^+$ T cells

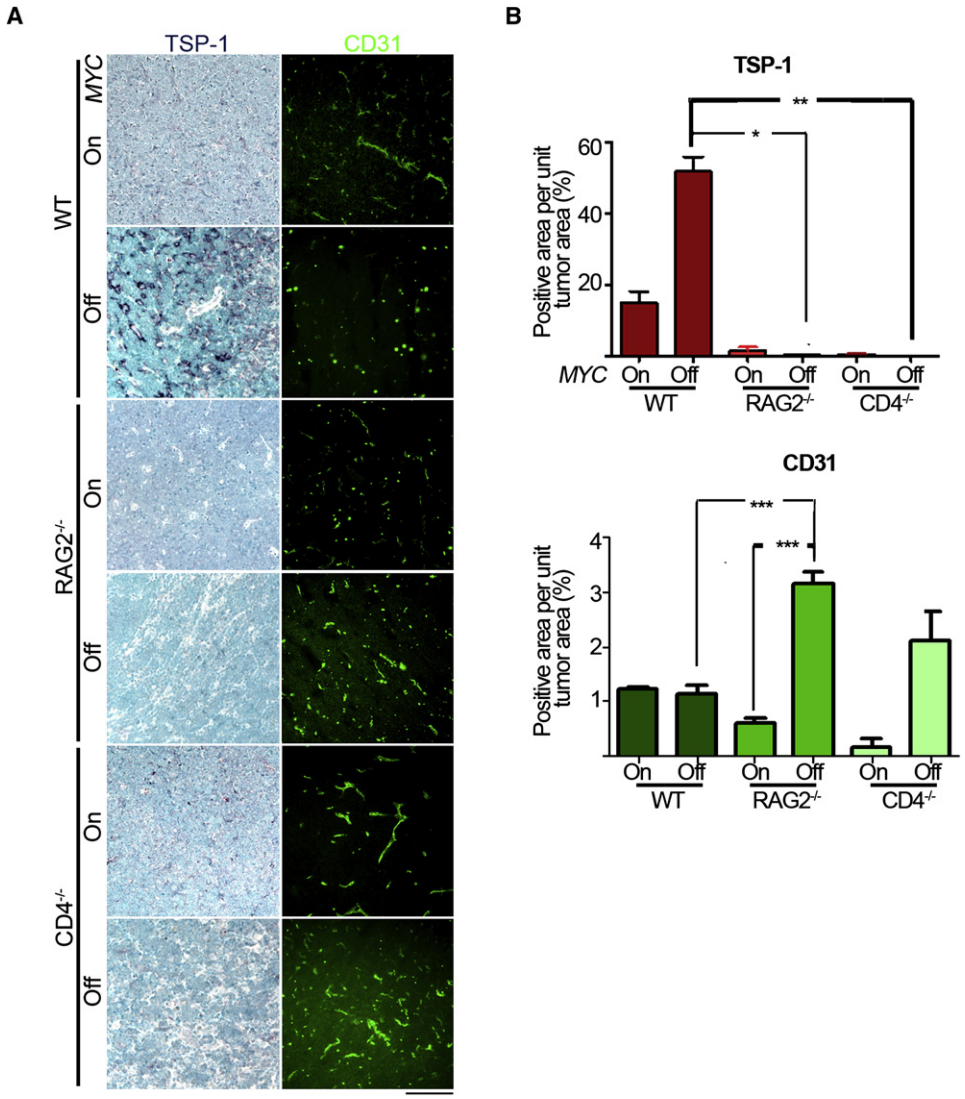


Figure 4. An Intact Immune System Is Required for the Inhibition of Angiogenesis upon *MYC* Inactivation

(A) Micrographs of TSP-1 (left panel) and CD31 (right panel) immunohistochemical and immunofluorescence staining of tumors derived from untreated (*MYC* On) and 4 day dox-treated (*MYC* Off) mice of the indicated genotypes. Scale bar = 100 μm.

(B) Quantification of TSP-1 (top panel) and CD31 (bottom panel) staining shown in (A). Quantification is presented as the average percentage of positively stained regions within the tumors. At least five different fields from two different tumors were analyzed for each different condition. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. Error bars are ± SEM.

See also Figure S2.

exhibited statistically significant prolonged tumor-free survival compared with RAG2$^{-/-}$ or RAG2$^{-/-}$ hosts reconstituted with CD8^{+} T cells (Figures 5B and 5D, RAG2$^{-/-}$ versus RAG2$^{-/-}$ CD4^{+}, p = 0.007, RAG2$^{-/-}$CD4^{+} versus RAG2$^{-/-}$CD8^{+}, p = 0.03). Hence, restoration of CD4^{+} T cells alone was sufficient for the ability of *MYC* inactivation to eliminate MRD and induce sustained tumor regression.

Host Immune System Is Required to Elicit Changes in Chemokine Expression

We measured relative fold changes in cytokine production in tumors growing in WT or RAG2$^{-/-}$ hosts after *MYC* inactivation (Figure 6A). *MYC* inactivation in tumors from WT compared with RAG2$^{-/-}$ hosts revealed an upregulation of antiproliferative and antiangiogenic ("antitumor") cytokines that suggest potential involvement by other immune effectors. Eotaxin-1 and IL-5 (Figure 6A, WT versus RAG2$^{-/-}$ fold change upon *MYC* inactivation p = 0.02 and p = 0.003, respectively) are potent T_H2 cytokines that have been implicated in the recruitment of an eosinophil-mediated antitumor inflammatory response (Simson et al., 2007). IFN-γ was observed to increase over 4-fold upon *MYC* inactivation in the WT hosts with virtually no change in the absence of the host immune system (WT versus RAG2$^{-/-}$ fold change upon *MYC* inactivation p = 0.03) while TNF-α was significantly downregulated in RAG2$^{-/-}$ hosts (RAG2$^{-/-}$ *MYC* On versus Off, p = 0.02); its upregulation was close to statistical

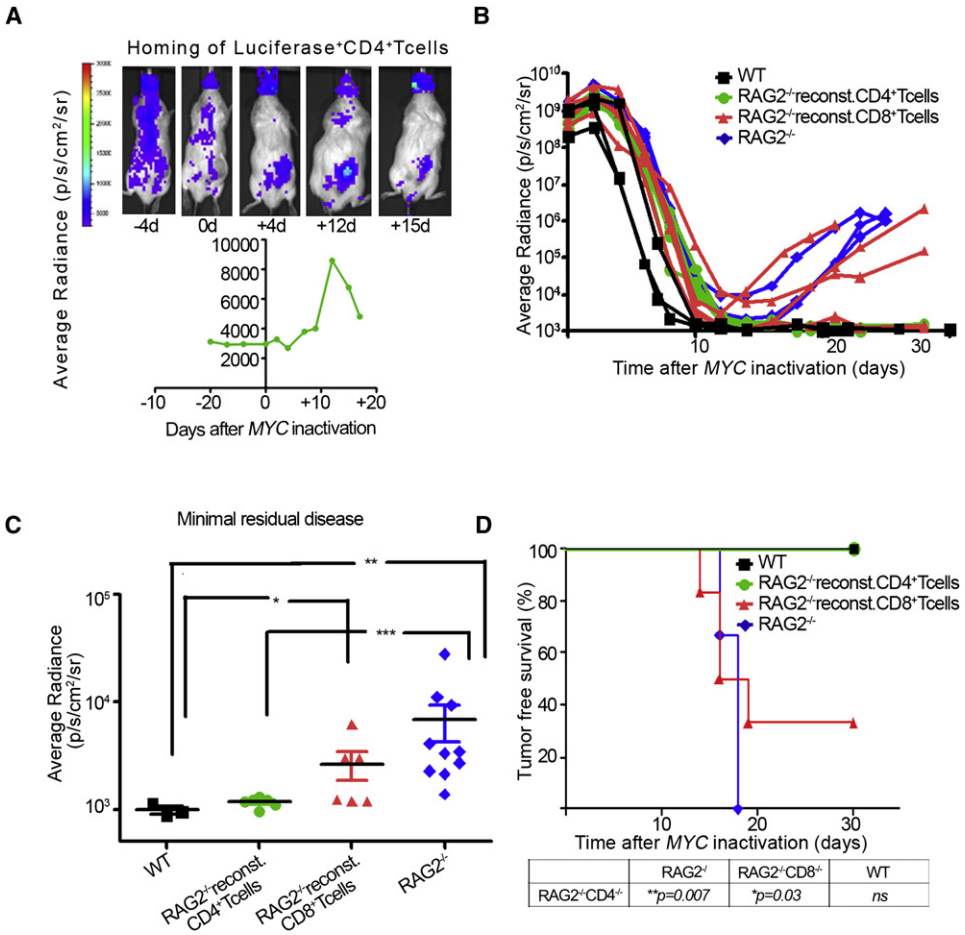


Figure 5. CD4$^+$ T Cells Home to the Tumor and Are Sufficient to Induce Sustained Tumor Regression upon *MYC* Inactivation

(A) Bioluminescence signal of luciferase$^+$CD4$^+$ T cells that home to the tumor microenvironment. RAG2$^{-/-}$ mice were reconstituted with luciferase$^+$ CD4$^+$ T cells and unlabeled tumor cell lines were injected s.c. 8 days post reconstitution. *MYC* was inactivated when tumors grew to a size of 1000 mm^3. Data are represented as bioluminescence signal (average radiance) plotted against time after *MYC* inactivation (n = 3).

(B) Tumor regression and relapse kinetics measured by bioluminescence imaging. RAG2$^{-/-}$ mice were reconstituted with CD4$^+$ (RAG2$^{-/-}$reconst.CD4$^+$T cells, n = 5) or CD8$^+$ (RAG2$^{-/-}$reconst. CD8$^+$T cells, n = 6) T cells from WT mice. Eight days after reconstitution, luciferase$^+$ tumor cell lines were injected s.c. *MYC* was inactivated when tumors in all hosts reached a comparable bioluminescence signal. Data are presented as bioluminescence signal (average radiance) plotted against time after *MYC* inactivation. WT (n = 3) and RAG2$^{-/-}$ (n = 3) mice were used as positive and negative controls.

(C) Quantification of minimum residual disease. Bioluminescence signals of tumors at their maximally regressed state are plotted against genotype. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *p < 0.01, **p < 0.001, ***p < 0.0001. Error bars are ± SEM.

(D) Kaplan Meier curves of tumor-free survival in the reconstituted RAG2$^{-/-}$, RAG2$^{-/-}$, and WT mice. Log-rank test was used compare the survival curves. Data are representative of three experiments. Statistics were performed including all data: n = 14, RAG reconstituted with CD8$^+$ T cells: n = 12. reconst. = reconstituted with.

See also Figure S3.

significance in the WT hosts (WT *MYC* On versus Off, p = 0.07). Both cytokines have been shown by many to be critical mediators of potent CD4$^+$ antitumor activity (Qin and Blankenstein, 2000; Thomas and Hersey, 1998). Interestingly, MCP-1, a potent chemoattractant of inflammatory tumor-associated macrophages (TAMs), specifically, tumor-promoting M2 macrophages (Allavena et al., 2008; Hu et al., 2009), was significantly downregulated in the tumors from immunodeficient hosts compared with WT (WT versus RAG2$^{-/-}$ fold change upon *MYC* inactivation p = 0.008).

Also, the downregulation of "protumor" cytokines was measured in tumors from WT and RAG2$^{-/-}$ hosts. Vascular endothelial growth factor (VEGF) was downregulated almost 4-fold in WT hosts (WT *MYC* On versus Off, p = 0.01), whereas no change in its expression could be detected in tumors from immunodeficient hosts. IL-β decreased significantly close to 2-fold (WT versus RAG2$^{-/-}$ fold change upon *MYC* inactivation, p = 0.02); downregulation of these two cytokines suggests enhanced suppression of angiogenesis in the presence of an intact host immune system upon *MYC* inactivation (Kowanetz and Ferrara, 2006; Shchors et al., 2006).

Finally, RAG2$^{-/-}$ hosts that had been reconstituted with CD4$^+$ T cells exhibited similar changes in chemokine expression to WT hosts upon *MYC* inactivation (Figure S4B). The antitumor

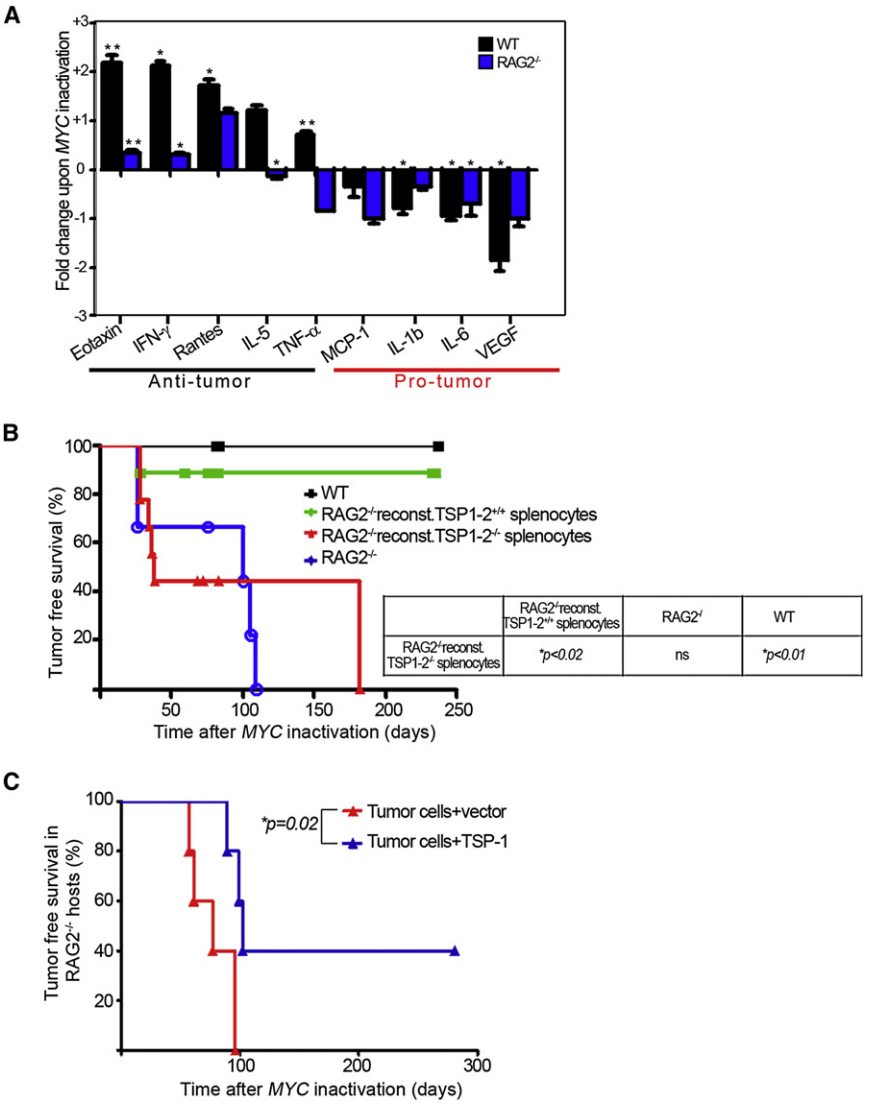


Figure 6. Cytokines Produced by the Immune System Contribute to Sustained Tumor Regression upon *MYC* Inactivation

(A) Graphical representation of fold change of indicated cytokines upon *MYC* inactivation in tumors from WT and RAG2$^{-/-}$ hosts. Tumors from WT and RAG2$^{-/-}$ mice were harvested at tumor onset and 4 days after *MYC* inactivation and run on a luminex platform to check for protein expression of 21 different cytokines. The significant fold changes in the various cytokines upon *MYC* inactivation were $\log_2$ transformed and plotted for various pro- and antitumor cytokines. *p < 0.01, **p < 0.001. Asterisks above the bars represent significance in cytokine expression upon *MYC* inactivation in the indicated host. Error bars are ± SEM.

(B) Kaplan-Meier curves of tumor-free survival of reconstituted RAG2$^{-/-}$, RAG2$^{-/-}$, and WT mice. RAG2$^{-/-}$ mice were reconstituted with splenocytes from WT (n = 18) or TSP-1,2$^{-/-}$ (n = 16) mice i.v. Eight days post reconstitution, mice were transplanted with lymphoma cells s.c. *MYC* was inactivated when tumors were 1000 mm^3. WT (n = 8) and RAG2$^{-/-}$ (n = 11). Log-rank test was used to analyze survival of indicated genotypes. Data are representative three experiments.

(C) Kaplan-Meier curves of tumor-free survival of RAG2$^{-/-}$ mice injected with TSP-1 transfected tumor cell lines (n = 5) or vector transfected control tumor cell lines (n = 5). A p53$^{-/-}$ conditional *MYC* lymphoma cell line was used.

See also Figure S4.

cytokines (eotaxin, IFN-γ and RANTES) increased, while the protumor cytokine, VEGF decreased in protein expression (Figure S4B). Thus, the host immune status is responsible for the regulation of changes in cytokine expression.

TSP Expression Is Required for Sustained Tumor Regression upon *MYC* Inactivation

Our results suggested to us the possibility that specific cytokines may be critical to the remodeling of the tumor and the tumor microenvironment upon *MYC* inactivation. We used two approaches to investigate the role of TSP-1. First, we reconstituted RAG2$^{-/-}$ mice with splenocytes from either TSP-1,2$^{+/+}$ (WT) or TSP-1,2$^{-/-}$ mice. Both TSP-1 and 2 have been implicated in the inhibition of angiogenesis and have similar structural domains (Kazerounian et al., 2008; Lawler, 2000). By FACS analysis, we verified equivalent immune reconstitution (Figure S4A). Indeed, RAG2$^{-/-}$ mice reconstituted with TSP-1,2$^{-/-}$ splenocytes completely failed to protect from sustained tumor regression upon *MYC* inactivation compared to RAG2$^{-/-}$ mice reconstituted with WT splenocytes (Figure 6B, relapse rate WT versus TSP-1,2$^{-/-}$, 10% versus 100%, p = 0.02). We conclude that TSP expression in immune effectors is important for sustained tumor regression upon *MYC* inactivation.

Next, we addressed whether we could bypass the requirement for TSP-1 expression from host immune cells by artificially introducing TSP-1 into tumor cells. We compared tumor recurrence upon *MYC* inactivation in RAG2$^{-/-}$ hosts of tumors infected with a vector control versus tumors infected with a TSP-1 expression vector. TSP-1 overexpressing tumors exhibited a delay in the kinetics (mean latency 80 versus 102 days) and a decreased frequency of tumor recurrence (40% versus 100%) resulting in a statistically significant survival advantage (Figure 6C, RAG2$^{-/-}$ TSP-1^{+} versus RAG2$^{-/-}$, p = 0.02). Thus, TSP-1 overexpression of tumor cells is sufficient to increase the duration and frequency of sustained tumor regression upon *MYC* inactivation in immune compromised hosts.

Cyclosporine A Treatment of Primary Tumors Impedes Senescence and Shutdown of Angiogenesis

To examine if similar results would be observed in primary transgenic tumors, we determined the influence of the pharmacological suppression of the host immune system with cyclosporine A (Shevach, 1985) on the consequences of *MYC* inactivation.

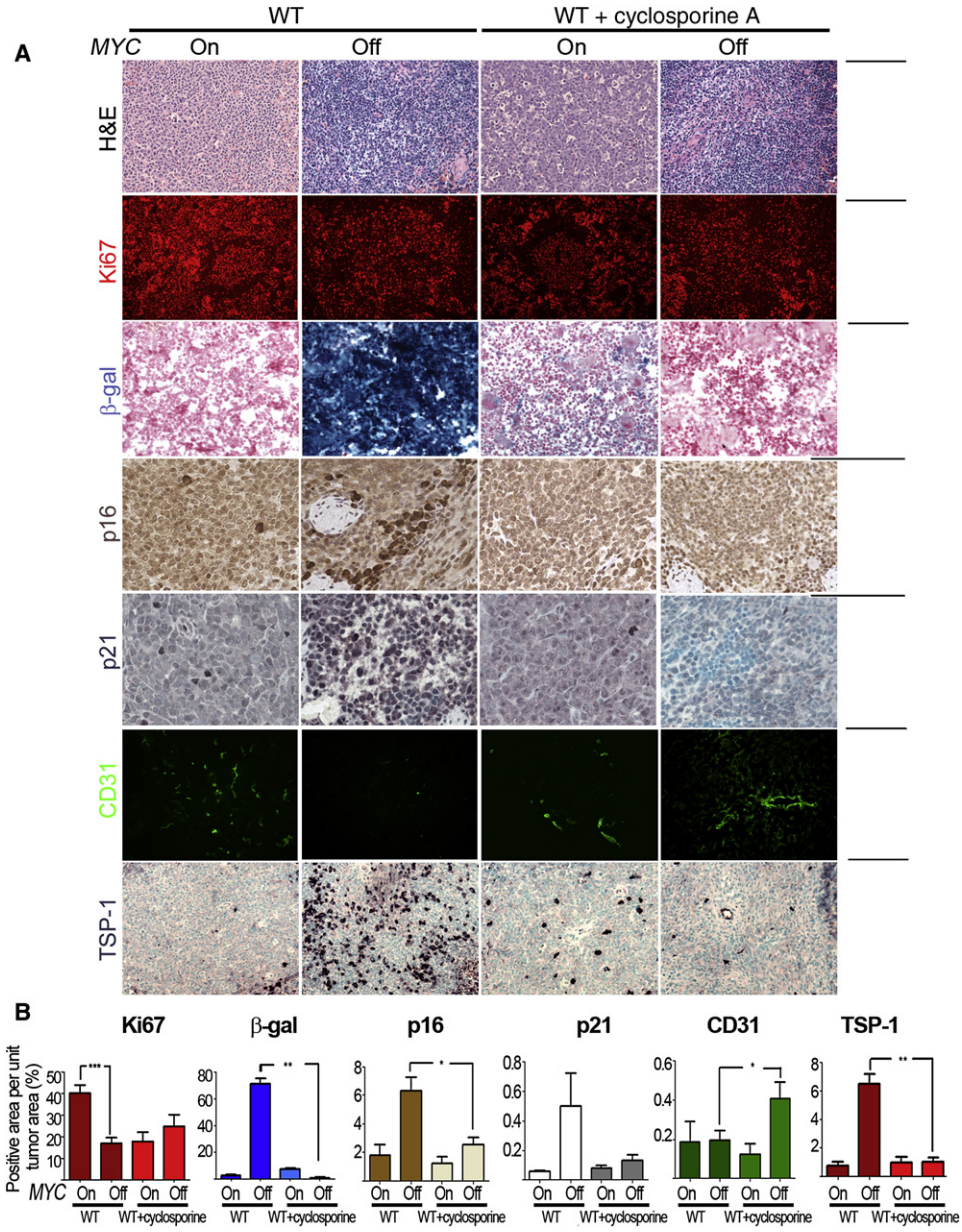


Figure 7. Cyclosporine A Treatment Inhibits Induction of Senescence and Inhibition of Angiogenesis in Tumors from Primary *MYC*-Induced T-ALL

(A) Micrographs of hematoxylin and eosin, Ki67, SA-β-gal, p16, p21, CD31, and TSP-1 immunostaining (ordered from top to bottom) of tumors derived from untreated and cyclosporine A-treated primary tumor bearing mice (*MYC* On and 4 day dox-treated *MYC* Off). Scale bar = 100 μm.

(B) Quantification of immunostaining shown in (A). Ordered from left to right, the graphs represent quantification of Ki67, SA-β-gal, p16, p21, CD31, and TSP-1 expression. Quantification is the average percentage of positively stained regions within the tumors. At least five different fields from two different tumors were analyzed. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. Error bars are ± SEM.

See also Figure S5.

Cyclosporine A did not have any direct effects on the proliferation of tumor cells in vitro (Figure S5). Cyclosporine A treated primary transgenic mice illustrated a marked inhibition on the ability of *MYC* inactivation to induce both cellular senescence as measured by staining for SA β-galactosidase (70% versus 1%; $p < 0.01$), p16 (6% versus 2%; $p < 0.05$), and p21 (0.5% versus 0.1%, $p < 0.01$) as well as the suppression of angiogenesis as measured by decrease in staining for CD31 (0.2% versus 0.4%, $p = 0.05$) and the induction of TSP-1 (6% versus 1%, $p = 0.0006$) (Figures 7A and 7B). Thus, cyclosporine A blocked the ability of *MYC* inactivation to induce senescence and shut down angiogenesis. We observed no effects on apoptosis as measured by TUNEL staining (data not shown). However, cyclosporine A treatment may suppress the ability of *MYC* inactivation

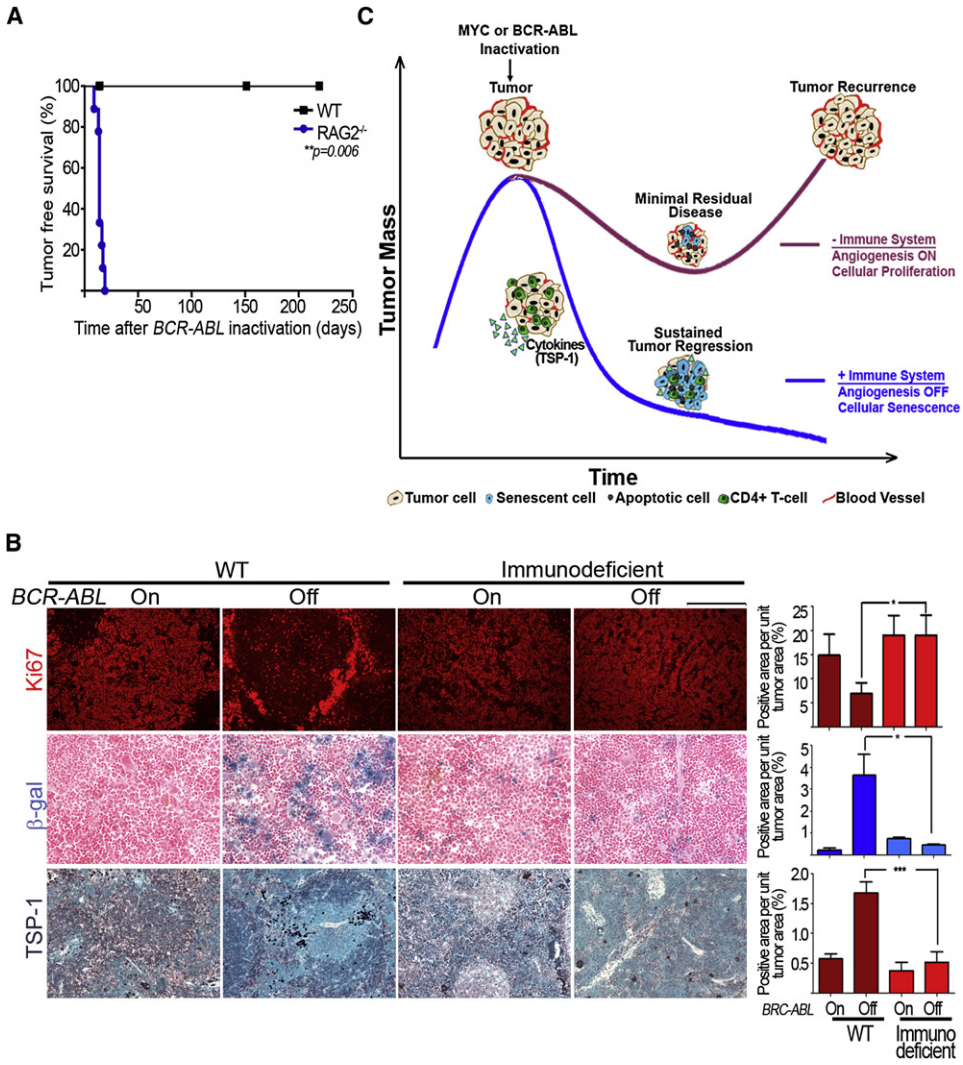


Figure 8. An Intact Immune System Is Required for Sustained Regression of Tumors in a Conditional Mouse Model of *BCR-ABL*-Induced B-ALL

(A) Kaplan-Meier curves of tumor-free survival of RAG2$^{-/-}$ (n = 9) and WT (n = 4) mice transplanted with unlabelled leukemia cells i.p. When mice were moribund with tumor, *BCR-ABL* was inactivated, and mice were scored for relapse.

(B) Micrographs and quantification of Ki67, SA-β-gal, and TSP-1 immunostaining (ordered from top to bottom) of tumors derived from untreated (*BCR-ABL* On) and doxycycline-treated (*BCR-ABL* Off) wild-type and immunodeficient tumor-bearing mice. Scale bar = 100 μm. Quantification is average percentage of positively stained regions. At least five different fields from two different tumors were analyzed for each different condition. Statistical significance (p value evaluated by unpaired Student's t test) is shown. *p < 0.01, **p < 0.001, ***p < 0.0001.

(C) Model for role of immune system in eliciting oncogene addiction.

See also Figure S6.

to induce proliferative arrest. Interestingly, cyclosporine A seemed to inhibit proliferation during tumor progression when *MYC* was still activated. This suggests, that when *MYC* is on, T cells might promote tumor formation indicating the dual nature of the immune response in cancer (de Visser et al., 2006).

Immune System Is Required for Sustained Regression of *BCR-ABL*-Induced B Cell Acute Lymphocytic Leukemia (B-ALL)

To determine if our results would generalize to another model of hematopoietic tumorigenesis, we used a conditional transgenic model of *BCR-ABL*-induced pro-B cell lymphocytic leukemia (B-ALL) (Huettner et al., 2000). First, we determined if host immune status influenced the ability of *BCR-ABL* inactivation to induce sustained tumor regression. Similar to *MYC* inactivation, tumors upon *BCR-ABL* inactivation underwent sustained tumor regression in wild-type hosts while 100% of the immunodeficient hosts relapsed within 14 days of *BCR-ABL* inactivation (Figure 8A, WT versus RAG2$^{-/-}$, p = 0.006). Hence, *BCR-ABL* inactivation also induces sustained tumor regression only in immune intact hosts.

Upon *BCR-ABL* inactivation, Ki67 expression showed a non-significant decrease in tumors transplanted into wild-type hosts but no change in tumors transplanted into immunodeficient

hosts. Ki67 expression was higher in tumors transplanted into immunodeficient hosts compared with those transplanted into immune intact hosts (Figure 8B, WT *BCR-ABL* off versus immunodeficient *BCR-ABL* off $p = 0.03$). Cellular senescence increased upon *BCR-ABL* inactivation in tumors from wild-type hosts versus immunodeficient hosts as measured by increased SA-β-gal staining (4% versus 0.4%, $p = 0.05$). Finally, there was a 3-fold increase in TSP-1 upon *BCR-ABL* inactivation in tumors from immunocompetent hosts while TSP-1 expression did not change upon *BCR-ABL* inactivation in immunodeficient hosts (Figure 8B, TSP-1 panel, WT *BCR-ABL* on *versus BCR-ABL* off, $p < 0.0001$; WT *BCR-ABL* Off versus immunodeficient *BCR-ABL* Off, $p = 0.0001$). We were unable to measure any significant CD31 expression in any of the tumors. Hence, *BCR-ABL* inactivation also induces sustained tumor regression only in immune intact hosts.

DISCUSSION

Oncogene addiction has been presumed to be a cell autonomous process. Here, we have shown that interactions between the tumor microenvironment and the immune system are essential for sustained tumor regression upon oncogene inactivation. In the absence of an intact immune system, we see a 10- to 1000-fold reduction in the rate, extent, and duration of tumor regression upon *MYC* inactivation. The absence of $CD4^+$ T cells alone was sufficient to markedly impede sustained tumor regression. Thus, oncogene addiction is not necessarily cell autonomous. $CD4^+$ T cells may play a critical role in enabling *MYC* inactivation to elicit changes in the microenvironment and in cytokine expression that appear to be required for cellular senescence and the shutdown of angiogenesis. TSP-1 must be expressed by immune effectors to cooperate with *MYC* inactivation to induce tumor regression. Our results generalized to primary tumors from *MYC*-induced T-ALL bearing hosts that had been treated with the immunosuppressive agent cyclosporine A and a conditional transgenic model of *BCR-ABL*-induced B-ALL. Oncogene inactivation generally may induce tumor regression through immune cell-dependent mechanisms.

Our observations are consistent with a multitude of reports that document the role of the immune system in neoplasia (de Visser et al., 2005, 2006; Dunn et al., 2002; Soucek et al., 2007). Tumors coevolve in the context of an intact immune system through the process of immune editing, resulting in tumor elimination, dormancy, or evolving to escape the immune system and progress to full malignancy (Dunn et al., 2002; Guerra et al., 2008; Teng et al., 2008). Upon *MYC* inactivation, a massive recruitment of $CD4^+$ T cells occurs that is associated with marked changes in cytokine production in the tumor microenvironment leading to cellular senescence and the shutdown of angiogenesis. TSP-1 is one of the critical chemokines. Interestingly, immune effector recruitment and associated changes in chemokines occur upon restoration of the tumor suppressor p53 in both liver cancer (Xue et al., 2007) and upon *MYC* inactivation in lymphoma (Giuriato et al., 2006).

Provocatively, $CD4^+$ T cells emerged as the critical host effector population for sustained tumor regression upon *MYC* inactivation. Notably, hosts deficient in $CD4^+$ T cells exhibited impaired kinetics, degree and durability of tumor regression as well as reduced senescence and suppression of angiogenesis upon *MYC* inactivation. The reconstitution of $CD4^+$ T cells into $RAG2^{-/-}$ hosts alone was capable of restoring the ability of *MYC* inactivation to induce sustained tumor regression. The reconstitution of $CD4^+$ T cells into $RAG2^{-/-}$ hosts had more potent effects on tumor regression compared with the depletion of these cells, perhaps reflecting that in hosts that are congenitally defective for a specific immune compartment there may be compensation from other immune effectors (Xing et al., 1998).

$CD4^+$ T cells have been previously implicated in the restraint of tumor growth through regulation of antigen dependent mechanisms involving either macrophages or cytotoxic T cells (Corthay et al., 2005; Dranoff et al., 1993; Qin and Blankenstein, 2000). Intriguingly, host $CD4^+$ T cells sculpted the tumor's response to *MYC* inactivation, likely not by their modest influence upon apoptosis or proliferation, but by dramatically inducing cellular senescence and the shutdown of angiogenesis, processes previously suggested by us to be integral to the ability of *MYC* inactivation to effect sustained tumor regression. Two of the hallmarks of oncogene addiction, both the induction of cellular senescence and the suppression of angiogenesis, have been linked to the expression of cytokines known to be expressed by $CD4^+$ T cells (Acosta et al., 2008; Beatty and Paterson, 2001; Kuilman et al., 2008; Muller-Hermelink et al., 2008).

Thus, $CD4^+$ T cells are one important component of the mechanism of tumor regression upon oncogene inactivation. Other host immune effectors are likely to contribute, and we recognize that other innate and adaptive immune compartments are also likely to be involved including macrophages, NK cells, mast cells, and B cells. Recent work suggests that mast cells and macrophages both may be critical (Soucek et al., 2007; Xue et al., 2007). Indeed, it is possible that $CD4^+$ T cells are mediating part of the effects we have observed by recruiting these effector populations.

TSP-1 is critical for the mechanism by which host immune effectors mediate tumor regression upon *MYC* inactivation. TSP-1 is a potent cytokine that has been implicated in the regulation of many cellular processes including the regulation of angiogenesis (Jimenez et al., 2000; Kazerounian et al., 2008; Lawler, 2000; Short et al., 2005; Zaslavsky et al., 2010). Furthermore, TSP-1 also has been suggested to regulate lymphocyte homing and function (Li et al., 2006) and appears to be required for the ability of $CD4^+$ T cells to contribute to sustained regression upon oncogene inactivation.

Additionally, other cytokines including eotaxin-1, IL-5, IFN-γ and TNF-α are possible candidates for mediating the changes in cellular senescence and angiogenesis upon *MYC* inactivation, consistent with reports that these chemokines may be involved in these processes (Beatty and Paterson, 2001; Beyne-Rauzy et al., 2004). The downregulation of other cytokines such as VEGF, IL-1β, and MCP-1 could also contribute (Kowanetz and Ferrara, 2006; Shchors et al., 2006; Su et al., 2010). IFN-γ and TNF-α have been previously implicated in the regulation of cellular quiescence and angiogenesis (Beatty and Paterson, 2001; Beyne-Rauzy et al., 2004; Kuilman et al., 2008; Muller-Hermelink et al., 2008), and eotaxin-1 and IL-5 have demonstrated potent antitumor activity in numerous mouse models of cancer

(Simson et al., 2007). Notably, tumor regression induced by the restoration of p53 expression was also associated with marked changes in chemokine expression (Xue et al., 2007).

In primary transgenic tumor hosts, an immune compromised state induced via treatment with cyclosporine A greatly impeded the consequences of oncogene inactivation. Therefore, our results generalize in the case when endogenous tumor-host interactions evolved throughout tumorigenesis. Cyclosporine A treatment is well known to increase the frequency of hematological malignancies in patients (Cockburn and Krupp, 1989; Opelz and Dohler, 2004). Hence, this agent may impede sensitivity to oncogene-directed therapies.

An immune intact host was also found to be required for *BCR-ABL* inactivation to induce sustained tumor regression in B-ALL. Similar to *MYC* inactivation, inactivation of the *BCR-ABL* oncogene resulted in the induction of cellular senescence, the shutdown of tumor angiogenesis, and ultimately sustained tumor regression only in the presence of the host immune system. However, different from *MYC* inactivation, *BCR-ABL* inactivation appeared to be less capable of suppressing cellular proliferation. Hence, the host immune system appears to be generally important in mediating the consequences of oncogene inactivation.

Thus, oncogene addiction is a consequence of both cell autonomous processes such as proliferative arrest and apoptosis as well as host-immune-dependent mechanisms such as cellular senescence and angiogenesis (Figure 8C). Upon oncogene inactivation, tumor cells are eliminated primarily in a cell autonomous manner. However, the kinetics of tumor cell elimination and the extent of tumor elimination, or minimal residual disease, as well as the durability of sustained tumor regression are all dictated by the presence of an immune system and appear to be strongly associated with its ability to elicit cellular senescence and shut down angiogenesis. These processes may contribute to the constraint of minimal residual disease (Aguirre-Ghiso, 2007). $CD4^+$ T cells are a critical component to this phenomenon and TSP-1 emerges as a possible cytokine regulating these processes. Other immune effectors and chemokines/cytokines (including IFN-γ, eotaxin-1, IL-5, TNF-α, and MCP-1) are likely to be involved. Immune cells and inflammation can be important to the pathogenesis of cancer through many effects on the tumor microenvironment (Coussens and Werb, 2002; Greten and Karin, 2004; Xue et al., 2007).

In general, the deficiency in $CD4^+$ T cells may render the treatment of tumors in patients less efficacious and impede the complete elimination of tumor cells. Indeed, AIDS patients exhibit not only a more than 100-fold increased frequency of lymphomas often associated with *MYC* overexpression but are much less responsive to therapy (Boshoff and Weiss, 2002; Carbone, 2003). Hence, $CD4^+$ T cells may contribute to the efficacy of therapeutic agents.

Our results suggest that screening methods used to identify therapies that rely on the in vitro study of cell lines or in vivo analysis of xenograft models in immune compromised hosts may underestimate the efficacy of a therapy by failing to faithfully recapitulate tumor-host interactions (Ronnov-Jessen and Bissell, 2009; Weigelt and Bissell, 2008). Moreover, the active modulation of $CD4^+$ T cell function may enhance the efficacy of therapeutics for cancer (Gattinoni et al., 2006; Lake and Robinson, 2005). Thus, a combination of targeted oncogene inactivation with immunotherapy may be a particularly efficacious anticancer therapy.

EXPERIMENTAL PROCEDURES

Transgenic Mice

The generation and characterization of Tet system transgenic lines for conditional expression of MYC, have been described (Felsher and Bishop, 1999). $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-/-}CD8^{-/-}$, and $RAG2^{-/-}$ mice were generously provided in the FVB/N background by Lisa Coussens (University of California, San Francisco). TSP-$1,2^{-/-}$ mice were generously provided by Ben Barres (Stanford University). $Luciferase^+$L2G85 mice were generously provided by Robert Negrin (Stanford University). Tet-o-BCR-ABL mice were generously provided by Daniel Tenen (Harvard University). Genotyping was performed by PCR on genomic DNA from tails. All animal experiments were approved by Stanford's Administrative Panel on Laboratory Animal Care (APLAC) and in accordance with institutional and national guidelines.

Tumor Surveillance and Tumorigenicity Assays

Mice that were moribund with tumor were either humanely euthanized or treated with doxycycline in their drinking water (100 μg/ml) to follow tumor regression and relapse. Statistical comparison of Kaplan-Meier curves is based on the log-rank test. Further details can be found in Supplementary Experiemental Procedures.

Reconstitution of $RAG2^{-/-}$ mice

$RAG2^{-/-}$ mice were injected intravenously (i.v.) with either (1) 20×10^6 splenocytes from WT or TSP-$1,2^{-/-}$ mice or (2) $4X10^6$ $CD4^+$ or $CD8^+$ T cells isolated from spleens and lymph nodes of WT mice using magnetically activated cell sorting (MACS). Eight days post reconstitution, mice were bled from the tail vein and $CD4^+$ and $CD8^+$ T cell reconstitution was verified using FACS.

In Vivo Bioluminescence Imaging

Mice with tumors were anesthetized with a combination of inhaled isoflorane/oxygen delivered by the Xenogen XGI-8 5-port Gas Anaesthesia System. The substrate **d**-luciferin (150 mg/kg) was injected into the animal's peritoneal cavity 10 min before imaging. Animals were then placed into a light-tight chamber and imaged with an IVIS-200 cooled CCD camera (Xenogen, Alameda, CA) (Contag et al., 1997). Living Image was used to collect, archive, and analyze photon fluxes and transform them into pseudocolor images by using Living Image software (Xenogen). At least five mice per group were injected with tumors expressing luciferase.

Luminex Cytokine Assay

The concentration of 21 cytokines was measured from tumor tissue lysates from WT and $RAG2^{-/-}$ mice at tumor onset and 4 days after *MYC* inactivation. Concentrations were measured using Luminex xMAP technology. Data were obtained as mean fluorescence intensity based on a standard curve generated for each cytokine. Further details can be found in Supplementary Experiemental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found online at doi:10.1016/j.ccr.2010.10.002.

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