

SUPPLEMENTAL INFORMATION:

Endogenous T cell responses to antigens expressed in lung adenocarcinomas delay malignant tumor progression

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Supplemental Figures:

Figure S1 (related to Figure 1)
Figure S2 (related to Figure 2)
Figure S3 (related to Figure 3)
Figure S4 (related to Figure 4)
Figure S5 (related to Figure 5)
Figure S6 (related to Figure 6)
Figure S7 (related to Figure 7)

Supplemental Experimental Procedures.

Supplemental References.

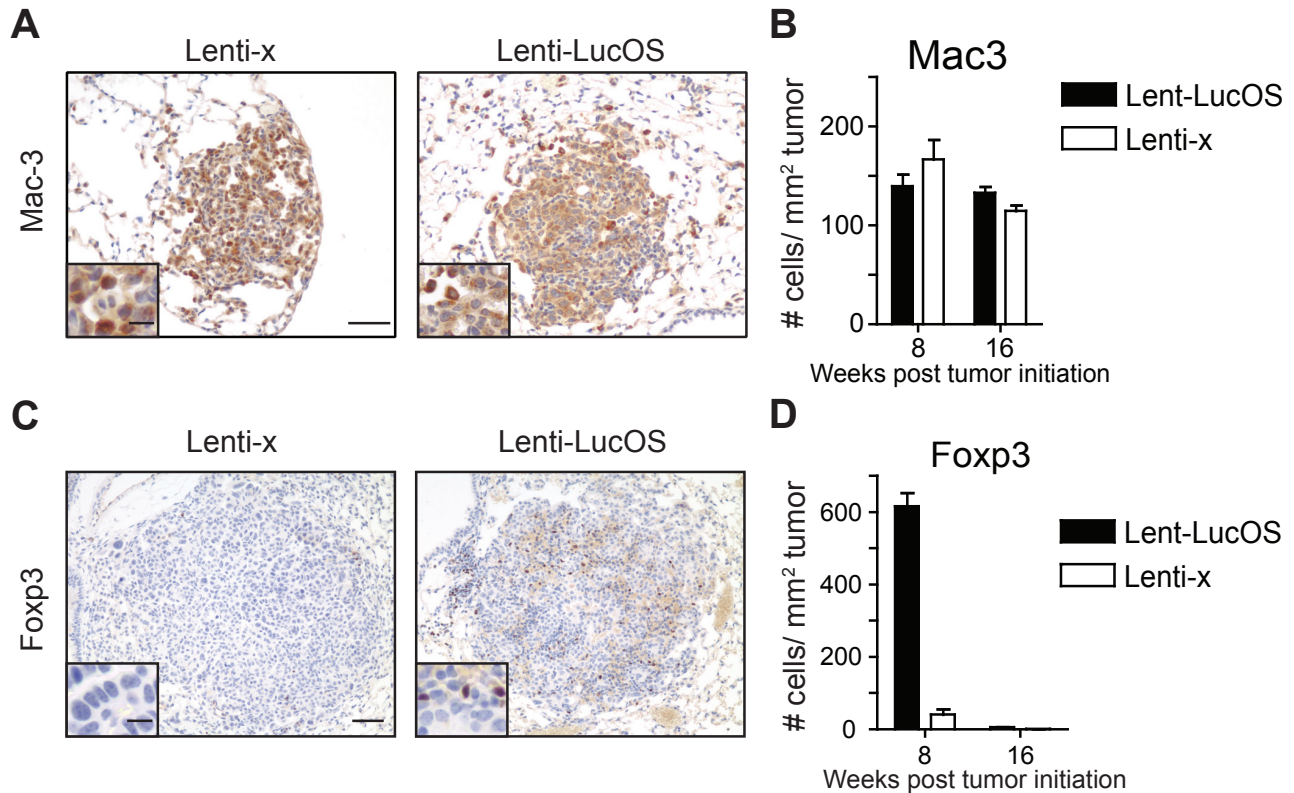


Figure S1 (related to Figure 1). Infiltration of additional immune cells subsets in immunogenic Lenti-LucOS tumors

(A) Lung tumors induced with Lenti-x or Lenti-LucOS in *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} mice stained with anti-Mac3 eight weeks after tumor initiation. Scale = 50µm (inset 10µm).

(B) Quantification of the macrophage infiltrates by IHC 8 and 16 weeks after tumor initiation (n= 2-5 mice, 11-79 tumors, per group). Mean ± SEM.

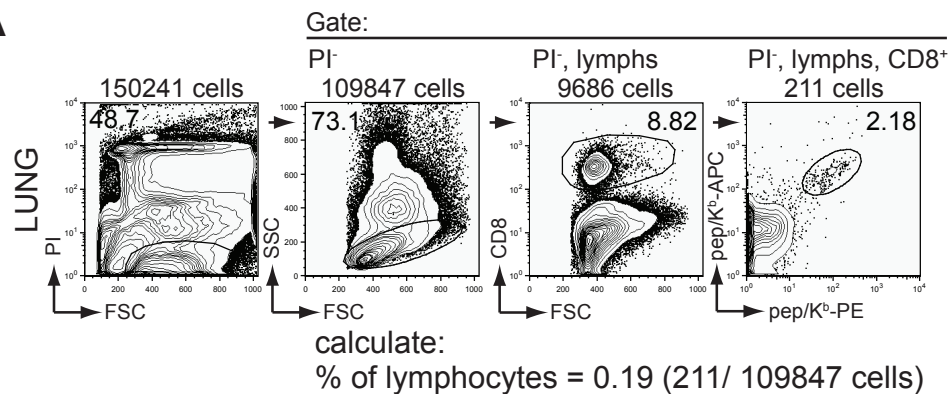
**Pathological analysis of H&E stained tumor sections did not reveal the presence of any other types of tumor-infiltrating immune cells such as neutrophils, basophils, or eosinophils.

(C) Lung tumors induced with Lenti-x or Lenti-LucOS in *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} mice stained with anti-Foxp3 eight weeks after tumor initiation. Scale = 50µm (inset 10µm).

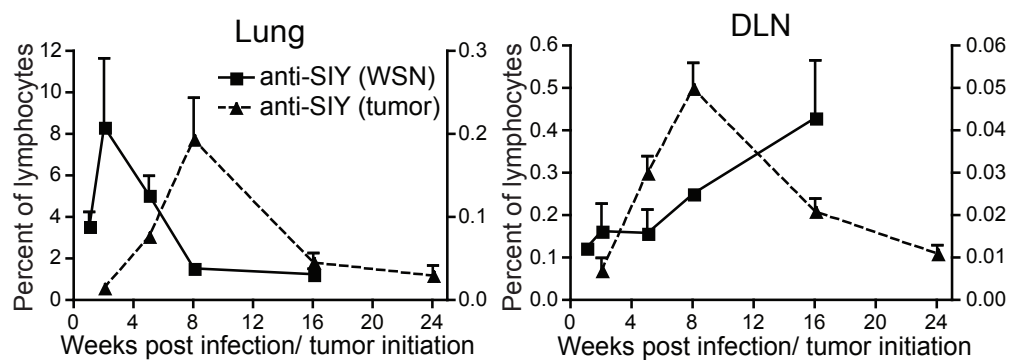
(D) Quantification of the T regulatory cell infiltrates by IHC 8 and 16 weeks after tumor initiation (n= 2-5 mice, 19-101 tumors, per group). Mean ± SEM.

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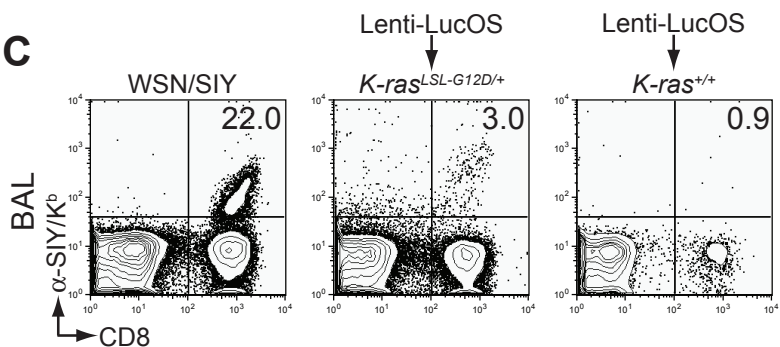
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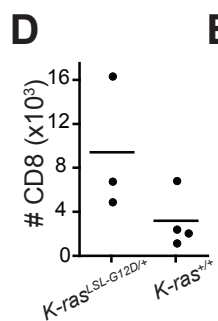
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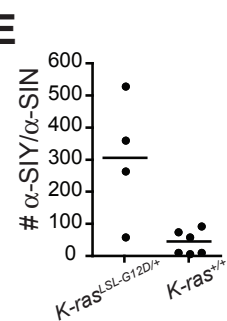
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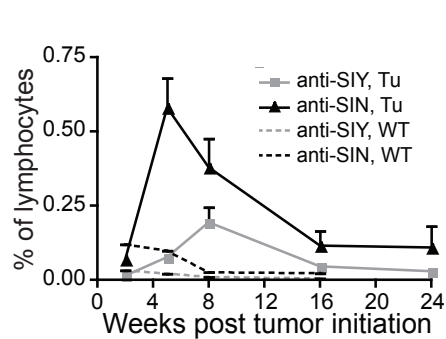
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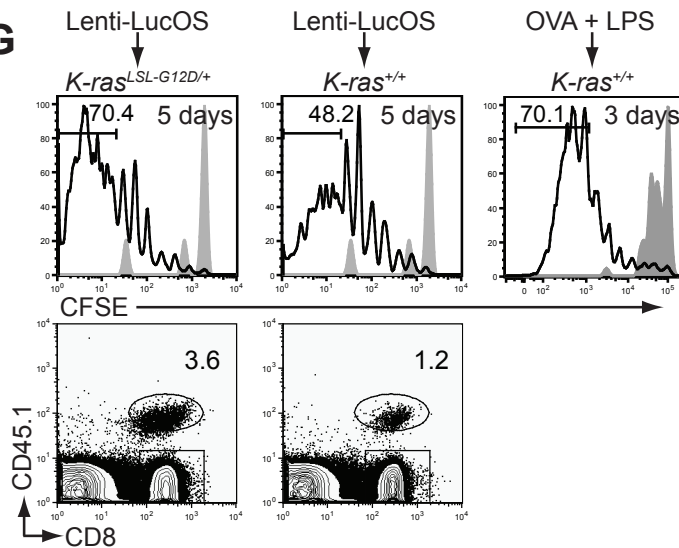
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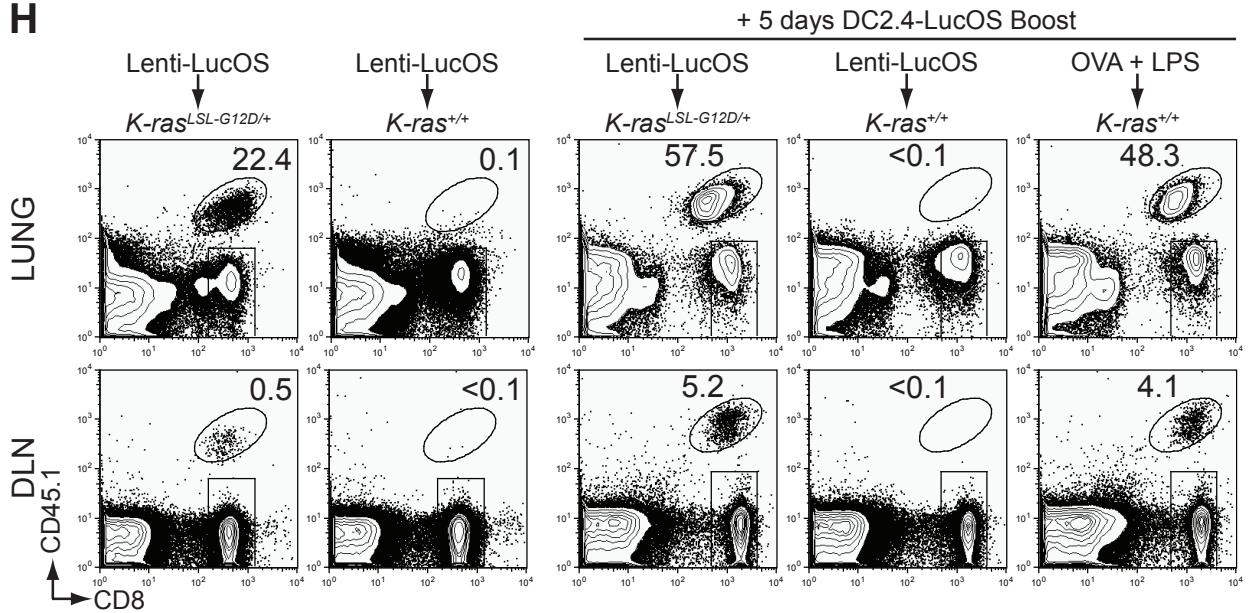


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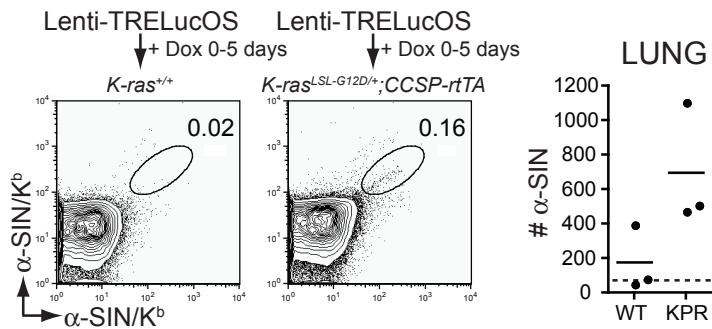


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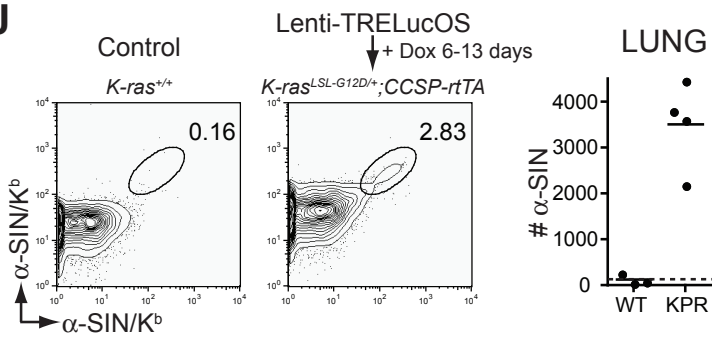
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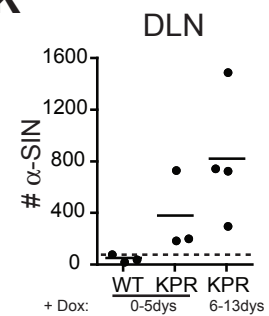
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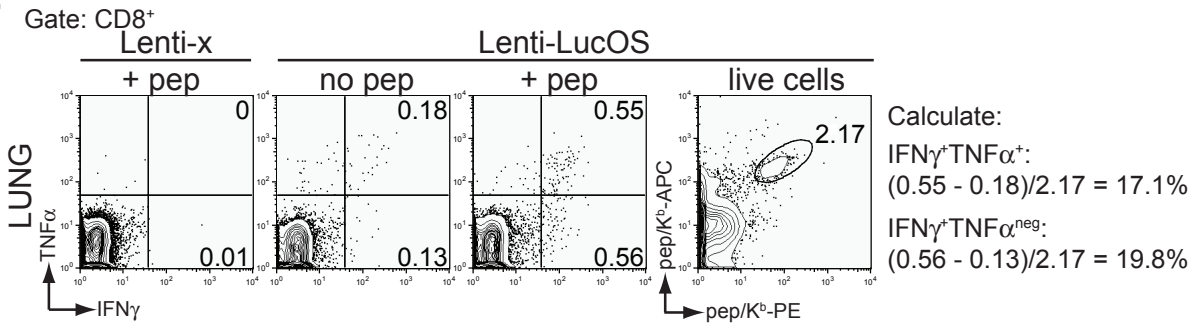


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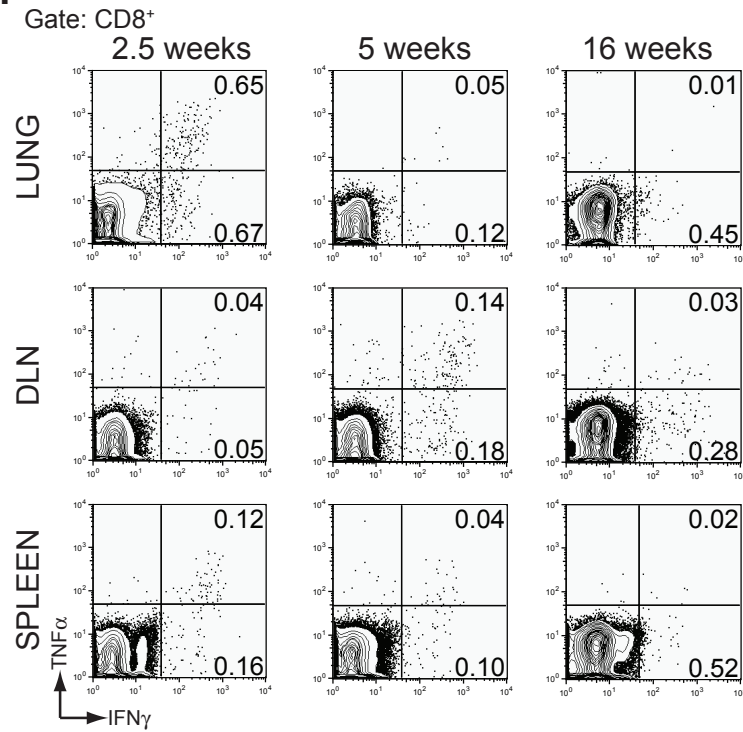


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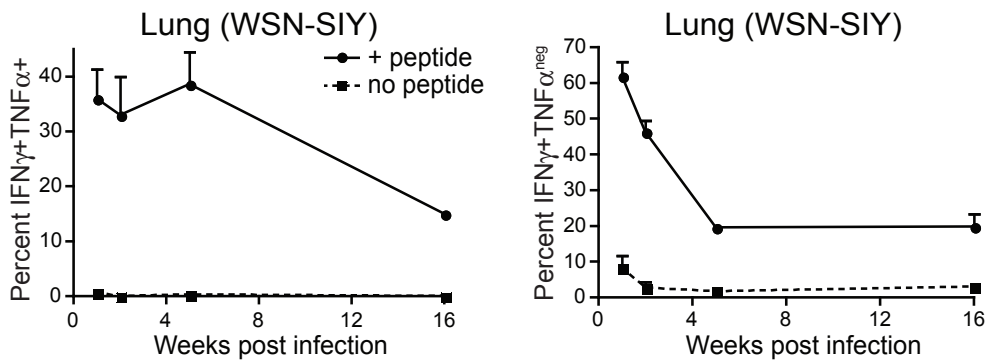


Figure S2 (related to Figure 2). Monitoring endogenous T cell responses after Lenti-LucOS or WSN-SIY infection

(A) Representative FACS plots from a single cell suspension of lung cells from a Lenti-LucOS tumor-bearing mouse demonstrates the gating procedure for calculating the percent of total lymphocytes specific for SIY or SIN. Cells were gated in order from left to right to give the total number of PI-negative, lymphocyte⁺ (based on FSC and SSC), CD8⁺, pep/K^b-PE⁺, pep/K^b-APC⁺ cells. The total number of cells gated in each plot is shown above the plots (percent gated shown within plots). We calculated the percent of lymphocytes by dividing the number of pep-specific cells by the number of PI-negative lymphocytes (shown below plots).

(B) T cell responses to WSN-SIY were measured by the percent of lymphocytes specific for SIY in the lung and the mediastinal draining lymph node (DLN) at defined times after WSN-SIY infection (solid lines, left y-axis, n= 2-3 mice per time-point). For reference, tumor-specific anti-SIY T cells are shown from Figure 2 (dotted lines, right y-axis).

(C) FACS analysis for the presence of anti-SIY T cells from bronchoalveolar lavage (BAL) 7 days after infection of *K-ras*^{LSL-G12D/+} or *K-ras*^{+/+} mice with WSN/SIY or Lenti-LucOS (gated on PI negative cells). Percent of CD8⁺ T cells specific for SIY is shown. Data is representative of infections from at least three different lentiviral preparations.

(D) Quantification of the number of CD8⁺ T cells in the BAL of *K-ras*^{LSL-G12D/+} or *K-ras*^{+/+} mice infected with Lenti-LucOS or Lenti-LucS. Data is representative of infections from at least three different lentiviral preparations.

(E) Quantification of the number of anti-SIY or anti-SIN T cells in the BAL of *K-ras*^{LSL-G12D/+} or *K-ras*^{+/+} mice infected with Lenti-LucOS or Lenti-LucS. Data is representative of infections from at least three different lentiviral preparations.

(F) Percent of lymphocytes specific for SIN or SIY in the lungs of mice after infection with Lenti-LucOS. Solid lines show the percent of lymphocytes in *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} mice that form tumors (Tu) while dashed lines show the percent of lymphocytes in *K-ras*^{+/+}; *p53*^{fl/fl} mice that do not form tumors (WT). Percent of lymphocytes is calculated as shown above in Figure S2A. n= 2-5 mice per time-point.

(G) FACS analysis comparing CFSE dilution (top) in naïve 2C or OT-I cells (gated: CD8⁺CD45.1⁺) from the mediastinal DLN five days after transfer of 2x10⁵ CFSE-labeled naïve 2C cells into *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} or *K-ras*^{+/+}; *p53*^{fl/fl} mice followed by intratracheal administration of Lenti-LucOS lentivirus or three days after transfer of 2x10⁵ CFSE-labeled naïve OT-I T cells followed by intratracheal administration of 10⁻⁵ µmol Ovalbumin + 1µg LPS (as a positive control). The percent of cells fully diluted of CFSE is shown. Grey histograms represent DLN cells transferred into uninfected control mice. FACS analysis comparing the percentage of CD8⁺CD45.1⁺ transferred 2C cells of the total CD8⁺ in the DLN of *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} or *K-ras*^{+/+}; *p53*^{fl/fl} mice five days after Lenti-LucOS infection (bottom). FACS plots are representative of results from 2-3 mice per group.

(H) FACS analysis for the persistence of transferred naïve OT-I T cells after infection with Lenti-LucOS in *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl} or *K-ras*^{+/+}; *p53*^{fl/fl} mice. 5x10⁵ naïve OT-I T cells were transferred into mice prior to infection with Lenti-LucOS and the presence of OT-I T cells (gate: CD8⁺CD45.1⁺) was analyzed after 6 weeks (n= 2 mice per group) or 5 days after i.p. injection of 5x10⁵ DC2.4-LucOS cells (8 weeks after infection, n= 2 mice per group). As a positive control, mice were inoculated intratracheally with 10⁻⁵ µmol Ovalbumin + 1µg LPS.

The experiments in panels C-H demonstrate that Lenti-LucOS infection itself can initiate a small and transient T cell response against the OVA and SIY antigens.

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(I) FACS analysis for the presence of anti-SIN T cells from the lungs of wildtype or *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl}; *CCSP-rtTA*⁺ (KPR) mice five days after infection with TRELucOS (see Supplemental Experimental Procedures) and Dox feeding. Percent of PI-negative, CD8⁺ cells is shown in the FACS plots and the total number of anti-SIN cells per lung from 3 mice in each group is shown (the approximate limit of detection is indicated by the dashed line). TRELucOS allowed for restricted expression of LucOS in lung epithelial cells.

(J) FACS analysis for the presence of anti-SIN T cells from the lungs of *K-ras*^{LSL-G12D/+}; *p53*^{fl/fl}; *CCSP-rtTA*⁺ (KPR) mice infected with TRELucOS and then fed Dox food for 7 days beginning 5 days after infection (days 6-13). Percent of PI-negative, CD8⁺ cells is shown in the FACS plots and the total number of anti-SIN cells per lung from 3 mice is shown (the approximate limit of detection is indicated by the dashed line). Three uninfected wildtype mice served as controls in this experiment. In this experiment, TRELucOS allowed for delayed expression of LucOS in lung epithelial cells beginning 6 days after lentiviral infection.

(K) The total number of anti-SIN cells per DLN from the experiments described in panels I and J (the approximate limit of detection is indicated by the dashed line).

(L) Procedure used to determine IFN- γ and TNF- α cytokine production in SIY and SIN-reactive T cells from the lungs of Lenti-LucOS tumor-bearing mice. Lenti-x tumor-bearing mice served as a negative control and were completely negative in the IFN- γ ⁺TNF- α ⁺ or IFN- γ ⁺TNF- α ^{neg} quadrants after SIY and SIN peptide stimulation. The percentage of SIY and SIN-specific T cells from the lungs that were IFN- γ ⁺TNF- α ⁺ or IFN- γ ⁺TNF- α ^{neg} was determined by analysis of cytokine production from CD8⁺ cells in the absence of stimulation (no pep) or in the presence of SIY or SIN peptides (+ pep). The percent of CD8⁺ cytokine producing cells was then divided by the percent of SIY or SIN-specific CD8⁺ cells from duplicate samples stained with DimerX (as shown to right).

(M) Representative IFN- γ and TNF- α cytokine production by CD8⁺ cells from the lung, DLN, and spleen of Lenti-LucOS tumor-bearing mice stimulated with SIY and SIN peptides at several time points during tumor development (n= 2-7 mice per time-point). Gated on CD8⁺ cells.

(N) Functional T cell responses to WSN-SIY were measured by the percentage of SIY-specific CD8⁺ cells that are IFN- γ ⁺TNF- α ⁺ or IFN- γ ⁺TNF- α ^{neg} +/- peptide at several times after WSN-SIY infection as in Figure S2L (n= 2-3 mice per time-point).

Data are mean \pm SEM.

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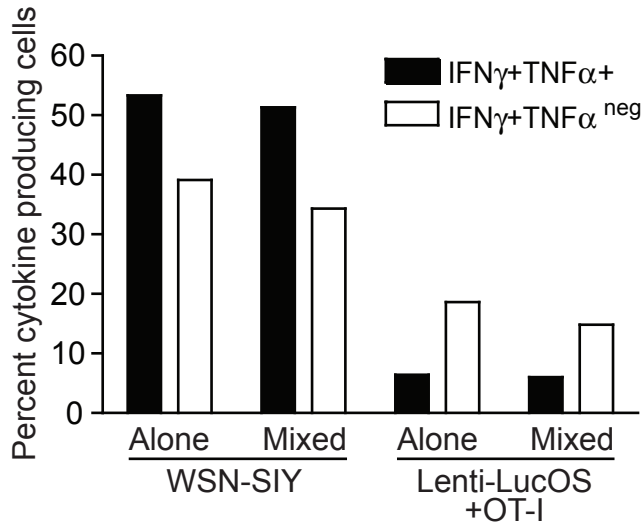


Figure S3 (related to Figure 3). Cytokine production by T cells is unaltered by the conditions during *in vitro* stimulation

To confirm that the differences in the capacity to produce cytokines during the *in vitro* stimulation were not the result of either additional stimulatory factors in the lungs of influenza infected mice or additional inhibitory factors in the lungs of tumor-bearing mice, we stimulated cell populations from WSN-SIY infected mice and Lenti-LucOS tumor-bearing mice alone or mixed together and monitored cytokine production. Cell suspensions from the lungs of mice infected with WSN-SIY (+ 4 weeks) or Lenti-LucOS tumor-bearing mice receiving naïve OT-I T cells (14 days prior) were stimulated with peptides alone (Alone) or in combination (Mixed) and analyzed for cytokine production. To determine the percent of cytokine producing cells reactive to WSN-SIY, we monitored the endogenous T cells specific to SIY (WSN-SIY), whereas for tumor-specific T cells, we monitored the transferred CD45.1⁺ OT-I T cells specific to SIN (Lenti-LucOS + OT-I). Cytokine production by influenza-reactive and tumor-reactive T cells was not altered when the cell suspensions were stimulated in combination (Mixed), indicating that the differences in T cell function in response to influenza or autochthonous tumors is due to inherent or acquired differences in the functional states of these T cells and not the priming conditions *in vitro*.

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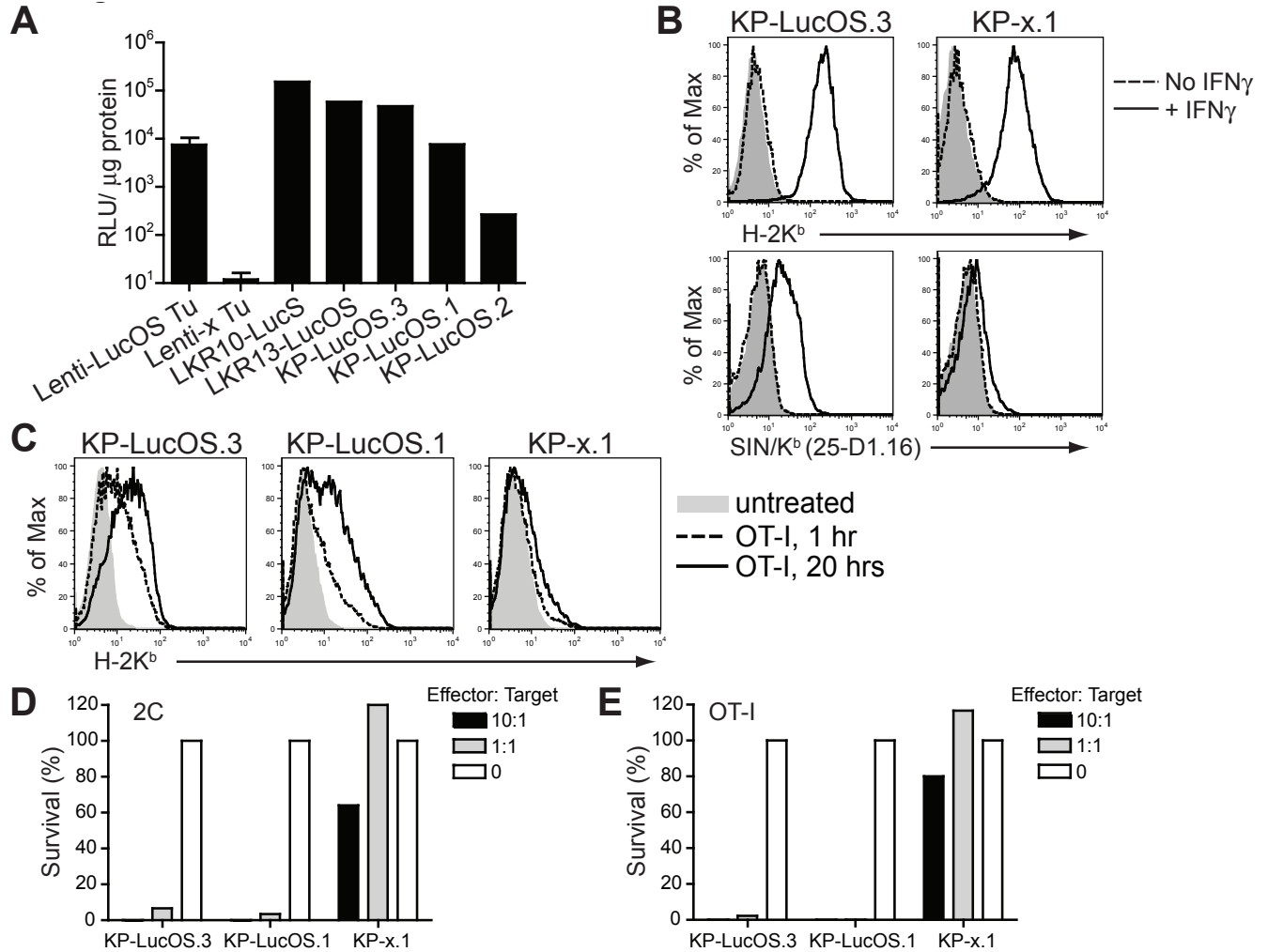


Figure S4 (related to Figure 4). MHC class I presentation of antigens is induced by IFN γ and T cell recognition

(A) Comparison of antigen expression by assaying luciferase activity (RLU/ μ g protein) in freshly explanted Lenti-LucOS or Lenti-x tumors, or cell lines derived from Lenti-LucOS tumors (KP-LucOS.1, KP-LucOS.2, KP-LucOS.3) or cell lines in which LucS or LucOS were introduced by lentiviral infection (LKR10-LucS and LKR13-LucOS). Mean \pm SEM. (B) H-2K^b and SIN/K^b (25D1.16 antibody) expression in a representative Lenti-LucOS cell-line (KP-LucOS.3) and Lenti-x cell-line (KP-x.1) after treatment with 100 U/mL IFN γ for 48 hours (solid line) or no treatment (dotted line). Note: similar results with 10 U/ml IFN γ . Filled histograms are negative control antibody stains for CD8. (C) H-2K^b expression on KP-LucOS.3, KP-LucOS.1, and KP-x.1 after co-culture with *in vitro* activated OT-I T cells. Tumor cell lines were plated and the following day OT-I T cells were added at a low density (\sim 2 tumor cells to 1 T cell). After 1, 2, 3, 4 or 20 hours, OT-I cells were washed away from adherent tumor cells. H-2K^b expression was analyzed on all tumor cell line samples by FACS 20 hours after initial T cell addition (T cells were excluded using CD45.1). As little as 1 hour co-culture of OT-I T cells with tumor cells was sufficient to upregulate MHC I and only occurred on cell lines that expressed SIN. MHC I expression increased with increasing culture time (only 1 and 20 hours shown).

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(D and E) Modified kill assays were performed by plating 10, 1, or 0 *in vitro* activated 2C (D) or OT-I T cells (E) to tumor cells (Effector: Target) on Day 1, removing the T cells with three PBS washes on all samples on Day 2, and then counting the total number of tumor cells on Day 4. Survival percentages were determined by dividing the number of cells after co-culture with T cells by the number of cells without co-culture. Specific killing only occurred in KP-LucOS cell lines and not a KP-x control cell line. Tumor cells were not treated with IFN- γ prior to co-culture, indicating T cells were capable of specifically recognizing KP-LucOS cell lines despite low levels of MHCI surface expression.

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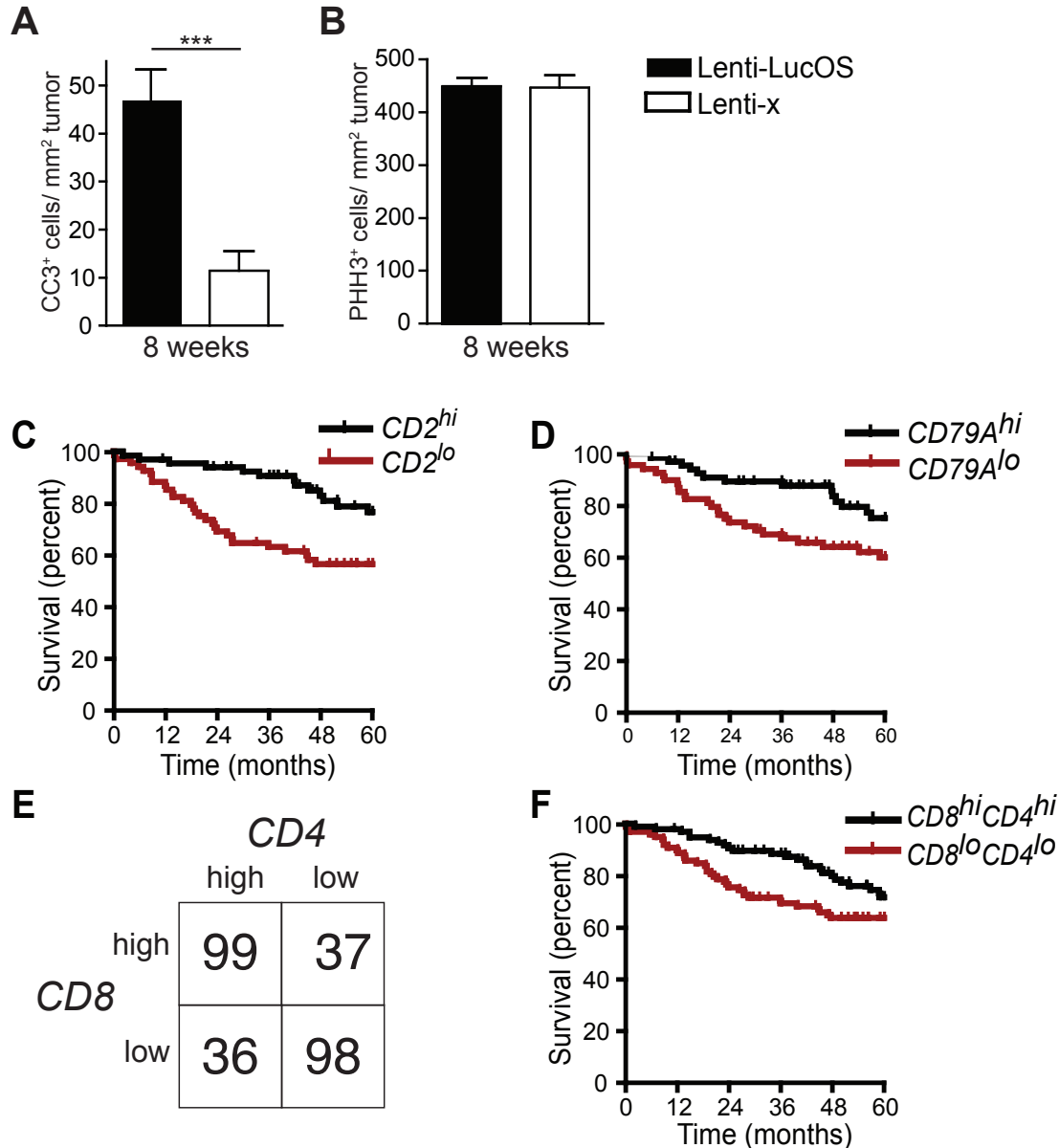


Figure S5 (related to Figure 5). Increased apoptosis in Lenti-LucOS tumors compared to Lenti-x tumors and high expression of canonical lymphocyte genes in patient lung adenocarcinomas correlates with better prognoses

(A and B) Eight weeks after tumor initiation, lung tumors induced with Lenti-LucOS or Lenti-x were stained with anti-Cleaved caspase-3 (CC3) for apoptosis (A) or anti-Phospho-Histone H3 (PHH3) for proliferation (B) and the number of positive cells per mm² of tumor area was quantified. For (A), $p \sim 0.0007$, $n = 2-5$ mice, 38-94 tumors, per group. For (B), $p \sim 0.46$, $n = 4-5$ mice, 16-48 tumors, per group. Mean \pm SEM.

(C and D) Kaplan-Meier survival curves for Stage I/II (lymph node-metastases free N0) lung adenocarcinoma patients comparing the top versus bottom quartile for the indicated genes. Top and bottom quartiles contain 88 patients each. Patient survival based on CD2 expression or CD79A expression, $p \sim 0.003$ and $p < 0.03$, respectively. CD2 (LFA-2) is an adhesion molecule involved in T cell activation. CD79A encodes the Ig α signalling chain of the B cell receptor.

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(E) Correlation between patient tumor samples with high CD4 and high CD8 expression. Contingency table of CD4 and CD8 expression (the average of CD8 α and CD8 β) with samples split into top half or bottom half for each gene, $p < 10^{-14}$.

(F) Patient survival based on the expression of CD4 and CD8 (from E), $p < 0.07$.

Data in panels C-F is from samples collected and processed at four institutes; University of Michigan Cancer Center (UM), Moffitt Cancer Center (HLM), Memorial Sloan-Kettering Cancer Center (MSK), and the Dana-Farber Cancer Institute (CAN/DF) (Shedden et al., 2008).

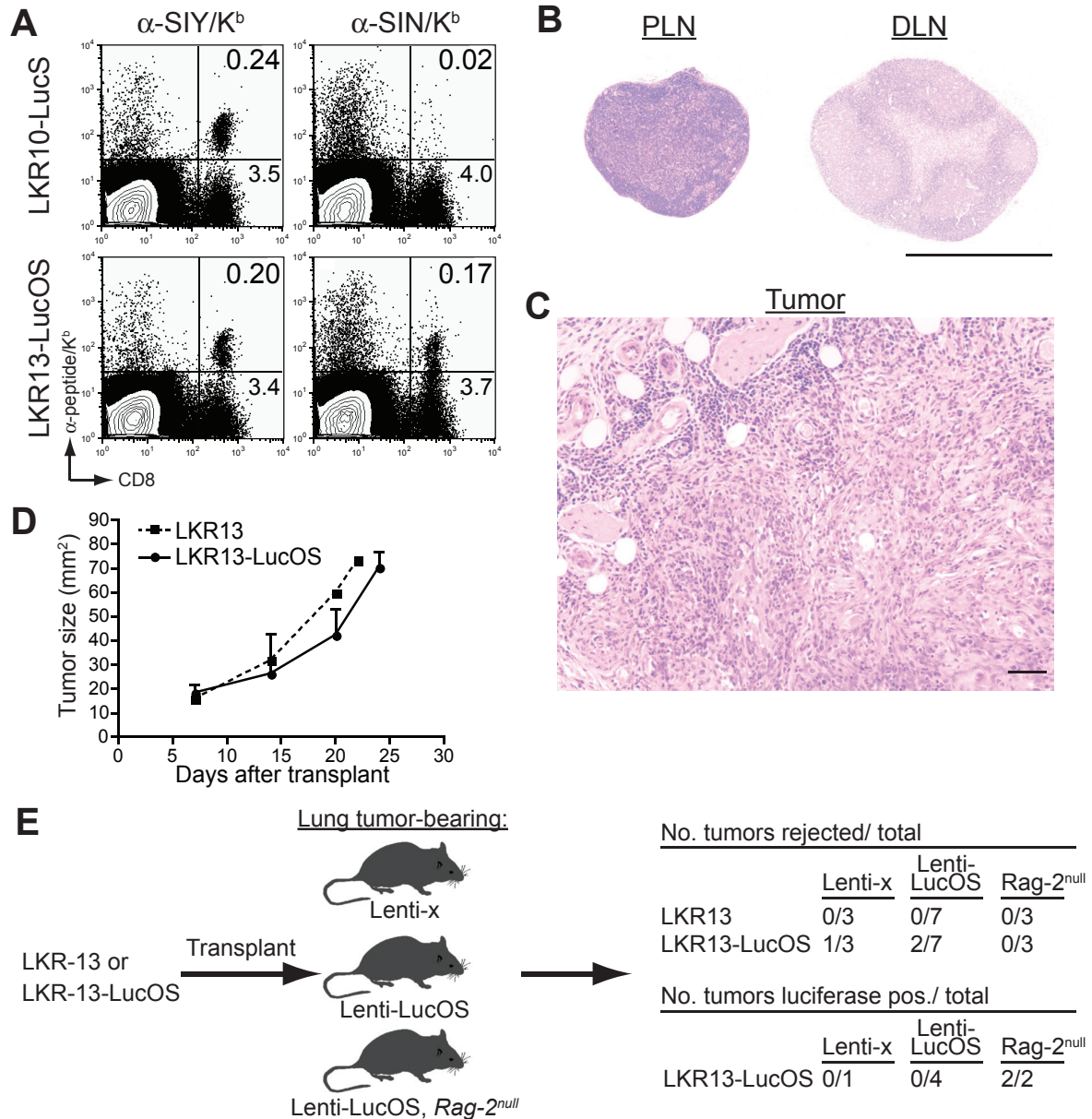


Figure S6 (related to Figure 6). T cell responses to LKR10/LKR13 transplantable models of lung cancer

(A) Splenocytes from mice harboring subcutaneous LKR10-LucS or LKR13-LucOS tumors were stained for CD8 (x-axis) and SIY or SIN loaded MHCII/K^b DimerX (y-axis). (B) H&E stained inguinal lymph nodes show highly reactive (multiple germinal centers) ipsilateral lymph nodes draining the subcutaneously transplanted tumors (DLN) compared to the contralateral lymph nodes (PLN) (scale = 1mm). (C) H&E stained LKR13-LucOS tumor with intratumoral lymphocytes (scale = 50µm). (D) LKR13 and LKR13-LucOS grow at comparable rates in *Rag-2^{-/-}* mice. Mean ± SEM. (E) Endogenous lung tumor-bearing mice do not generate systemic tolerance toward transplanted LKR-13-LucOS tumors. Number of tumors that were rejected or remained luciferase positive of the total analyzed after transplantation into immune-competent or *Rag-2^{null}* mice bearing Lenti-x or Lenti-LucOS-induced lung tumors.

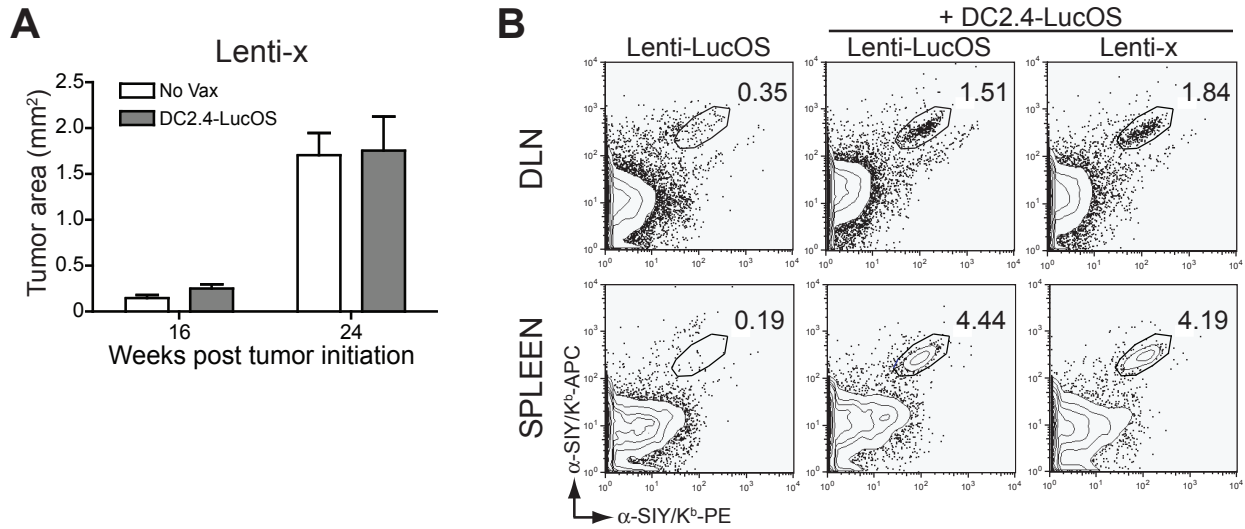


Figure S7 (related to Figure 7). DC2.4-LucOS vaccination leads to a sustained anti-tumor T cell response

(A) Mean tumor area of Lenti-x lung tumors at 16 and 24 weeks after tumor initiation with or without DC2.4-LucOS vaccination (p-values are ~0.06 and ~0.90, respectively, from n= 2-5 mice, 66-97 tumors, per group). Mean ± SEM.

(B) FACS analysis with two peptide-loaded DimerX reagents labeled with PE (x-axis) or APC (y-axis) to detect SIY-specific CD8⁺ T cells in the DLN and spleen of Lenti-LucOS or Lenti-x tumor-bearing mice with or without DC2.4-LucOS vaccination prior to tumor initiation (analyzed 19 weeks after tumor initiation). FACS plots are gated on CD8⁺ cells and the percent of CD8 cells is shown.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and tumor induction

Trp53^{fl} mice were provided by A. Berns (Jonkers et al., 2001).

CCSP-rtTA mice (used in Figure S2I-K) specifically express the reverse tetracycline transactivator in lung epithelial cells using the clara cell secretory protein promoter described in (Meylan et al., 2009).

Lentiviral production and TRELucOS construct

Lentiviruses were produced as described in (Tiscornia et al., 2006).

TRELucOS (used in Figure S2I-K) is similar to Lenti-LucOS but uses a doxycyclin-regulated promoter element (TRE) to control LucOS expression rather than the constitutive UbC promoter (Meylan et al., 2009).

Tumor grading

Grading was performed by R.B. as described previously (DuPage et al., 2009). Briefly, Grade 1 lesions represent hyperplasias and small adenomas, Grade 2 lesions are large adenomas, Grade 3 lesions are adenocarcinomas, Grade 4 lesions are invasive adenocarcinomas.

Flow cytometry

The 1B2 antibody recognizing the 2C TCR is reported here (Kranz et al., 1984).

The 25-D1.16 antibody is reported here (Porgador et al., 1997).

Staining cells with the same ligand labeled with PE or APC for improved sensitivity has been reported (Stetson et al., 2002; Townsend et al., 2001).

Tumor cell-lines and transplantation

Information about LKR10 and LKR13 are cell lines derived from K-ras^{G12D}-driven mouse lung tumors from 129S₄/SvJae mice can be found here (Johnson et al., 2001; Kumar et al., 2007).

DC2.4-LucOS vaccination

DC2.4-LucOS cells were generated from the dendritic cell-line (DC2.4) (Shen et al., 1997) and were previously reported from our lab (Cheung et al., 2008).

Influenza

The WSN-SIY influenza strain was recently reported for tumor studies (Bai et al., 2008; Cheung et al., 2008).

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