Supplementary Materials and Methods

Immunohistochemistry

For all except staining performed on frozen samples, samples were prepared with 10% neutral buffered formalin (VWR International, Radnor, PA), embedded in paraffin, and sectioned by the Johns Hopkins Medical Institutions Reference Histology Laboratory. All antibodies are shown in Supplementary Table 1. For CD4 and CD8 stains, pancreata were frozen in Tissue-Tek OCT (Sakura Finetek USA, Inc, Torrance, CA) with liquid nitrogen and cut in 5-μm sections using a cryostat, followed by -20°C acetone fixation. Sections were incubated with primary antibodies to CD4, CD8, or isotype control (R35-95) (BD Pharmingen, San Jose, CA) for 1 hour and with biotin-conjugated secondary antibody (G28-5; BD Pharmingen) for 30 minutes. Antigen retrieval was performed by steam heating in Citra antigen retrieval solution (BioGenex, Fremont, CA) for Ly6G and with proteinase K (Dako North America, Inc, Carpinteria, CA) for F4/80 antibody staining. The corresponding biotinylated secondary antibodies were supplied by Vector Laboratories, Inc (Burlingame, CA). For Foxp3, slides were stained by the Johns Hopkins IHC laboratory using the Envision FLEX, high-pH system (Dako) for automated Dako platforms. For patient samples, sections were stained with anti-CD3 using ultraView Detection (Ventana Medical Systems, Inc, Tucson, AZ) according to standard Ventana protocols in the Johns Hopkins University Immunopathology Laboratory. For RORγt, antigen retrieval was performed with EDTA at 95°C for 20 minutes and slides were stained using Leica auto stainers and Bond Polymer Refine Detection (Leica Microsystems, Inc, Buffalo Grove, IL).

Flow Cytometry

The antibodies used in flow cytometry staining are shown in Supplementary Table 2. The Live/Dead Fixable Dead Cell Stain Kit (Life Technologies) and FcBlock (anti-CD16/CD32) (BD Biosciences) were used routinely before staining with antibodies to cell surface markers and intracellular cytokines.

CD8- and IL17-Depletion Assays

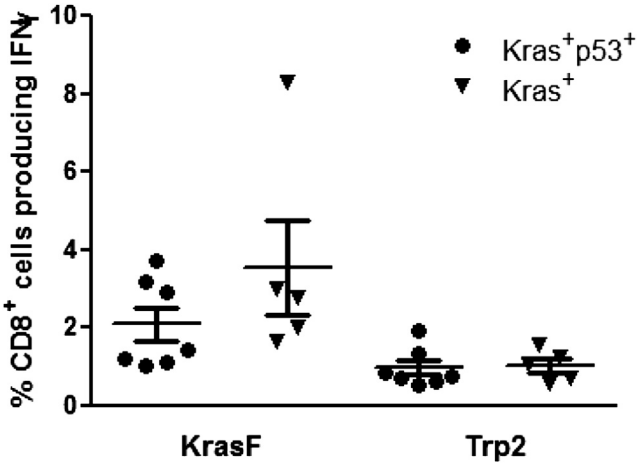
The CD8-depleting antibody 2.43 (500 μg intraperitoneal injection)[1] or control rat IgG was given every other day for 1 week before vaccination, followed by twice-weekly injections throughout the 5-week course of vaccination and Treg depletion. CD8 depletion was verified routinely throughout the study by flow cytometry. Anti-IL17 antibody, a gift from Dr Jerry Niederkorn,[2] or control IgG was given in a 200-μg dose twice weekly for the first week, followed by 100 μg twice-weekly injections for the duration of a 5-week cycle of LM-Kras and Treg depletion.

Histologic Analysis of Pancreatic Lesions

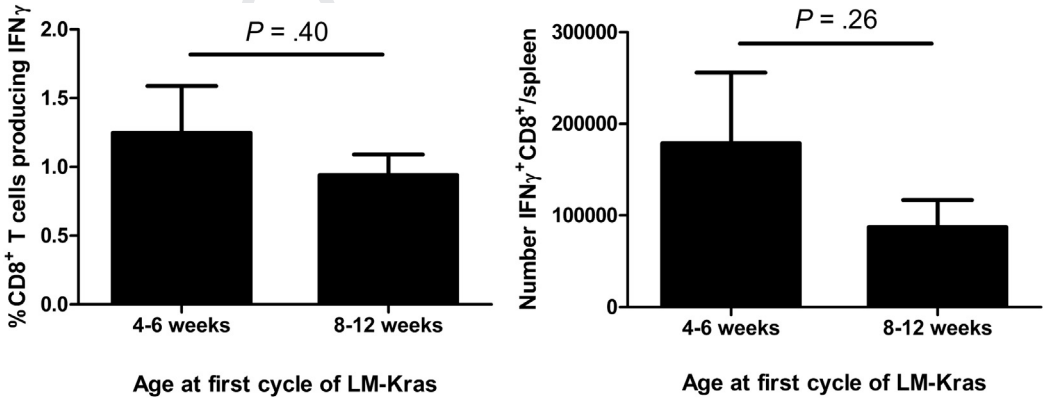
For the CD8-depletion and IL17-depletion studies, the Cavalieri estimator method was used to estimate PanIN or PDA volumes present in the pancreata.[3] Two sections per pancreas were scanned at a magnification of 20× (0.498 μm/pixel) using a ScanScope CS (Aperio, Inc, San Diego, CA). Briefly, a point counting grid with a grid spacing of 75 μm was applied to each whole slide image in custom Java software (freely available at https://bitbucket.org/tcornish/point-counting). The percentage of tissue occupied by each histologic category was calculated based on intersection points counted for each feature.

References

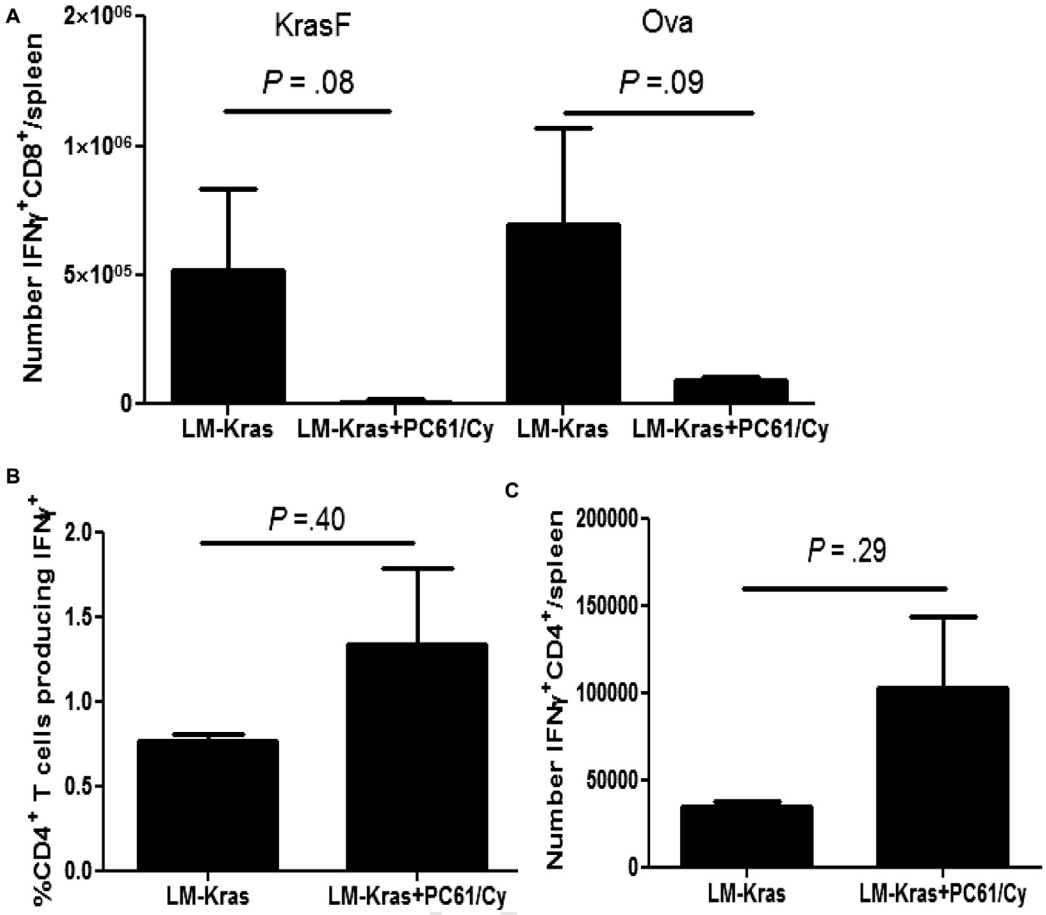
1. Reilly RT, Gottlieb MB, Ercolini AM, et al. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. Cancer Res 2000;60:3569–3576.
2. De Paiva CS, Chotikavanich S, Pangelinan SB, et al. IL-17 disrupts corneal barrier following desiccating stress. Mucosal Immunol 2009;2:243–253.
3. Mouton PR. Principles and practices of unbiased stereology. Baltimore: Johns Hopkins University Press, 2002.



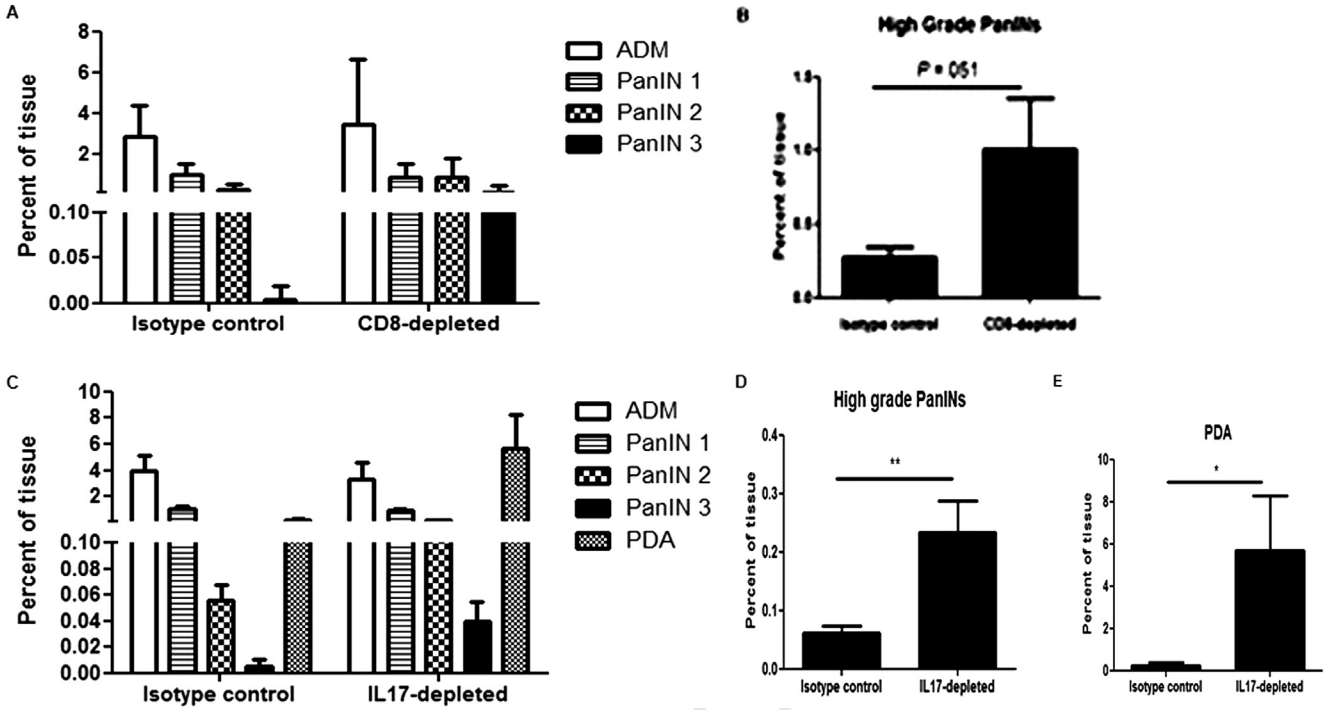
Supplementary Figure 1. $CD8^+$ T-cell IFNγ production in mice comparing the p53 mutation status. Four- to 6-week-old KC (n = 5) and KPC (n = 7) mice were treated with LM-Kras twice, 4 weeks apart, killed 1 week post-vaccine, and splenocytes were harvested. Intracellular cytokine staining was used to assess response to KrasF and Trp2 (negative control) peptides between mice expressing and not expressing the p53 mutant allele. No significant difference in IFNγ production was found in the response to KrasF.



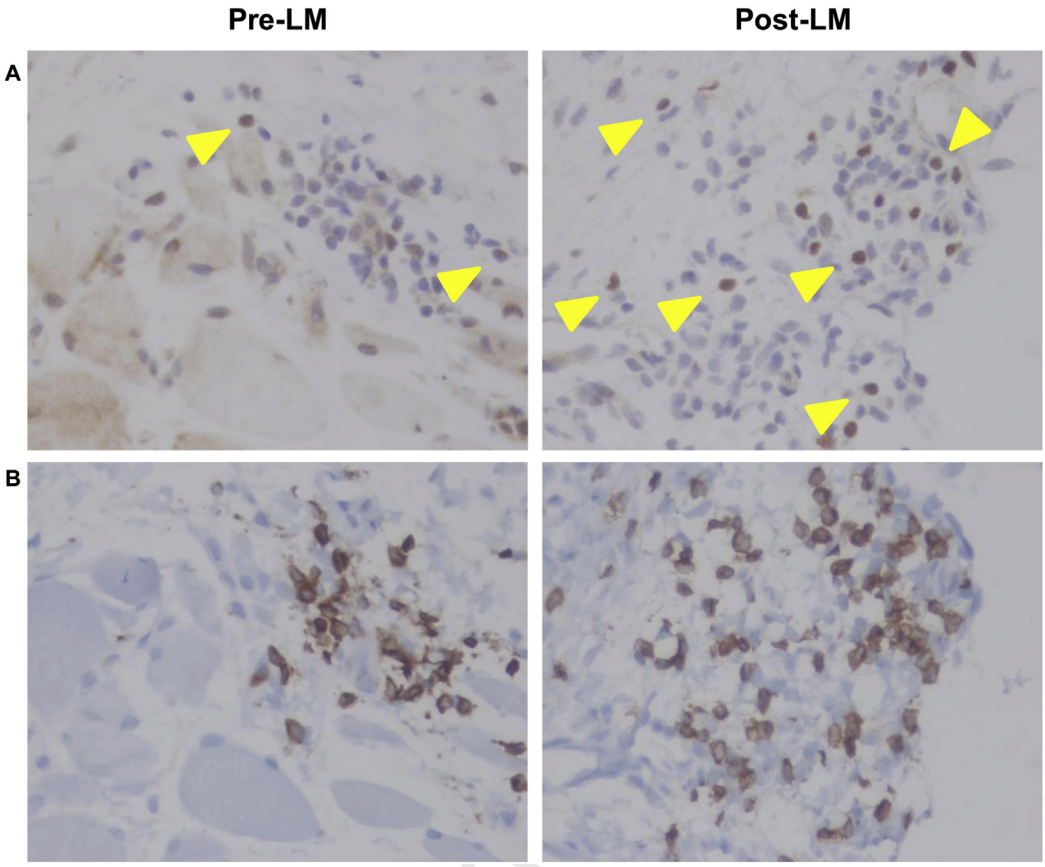
Supplementary Figure 2. Systemic $CD8^+$ T-cell responses in KPC mice with early and late-stage PanINs. Young (4–6 wk, correlating with early stage PanINs) and older (8–12 wk, late-stage PanINs predominate) KPC mice were vaccinated with LM-Kras and splenocytes were harvested 1 week later, stimulated with KrasF and Trp2 peptides, and intracellular cytokine assay and flow cytometry was used to assess response. The $CD8^+$ T-cell response to KrasF was normalized for control peptide (Trp2) response. Total numbers of $CD8^+$ T cells responding to KrasF were calculated based on the percentage of responders and the total number of $CD8^+$ T cells per spleen (n = 5–6 mice per group). Q44



Supplementary Figure 3. $CD8^+$ and $CD4^+$ T-cell responses with LM-Kras and Treg depletion. (*A*) Splenocytes from 4- to 6-week-old treated KPC mice were harvested 1 week after vaccine; stimulated with KrasF, Ova, or Trp2 peptides; and assayed for IFNγ production. Responses to Trp2 were subtracted from each KrasF and Ova response and the total number of $CD8^+$ T-cell responders was calculated using the total numbers of $CD8^+$ T cells per spleen (n = 6–9 per group). (*B*) KPC mice were treated as in panel *A* and splenocytes were stimulated with anti-CD3/CD28 beads overnight, followed by a 5-hour incubation with Golgi-Stop, and assayed for IFNγ production. IFNγ was gated on nonstimulated controls (n = 2–3 per group). Representative of 2 independent experiments. Q45



Supplementary Figure 4. PanIN progression with the depletion of $CD8^+$ cells or IL17. (*A–D*) Mice were treated with LM-Kras and Treg depletion (PC61/Cy) for 2 cycles of therapy together with depleting antibodies for (*A* and *B*) CD8 (n = 12 per group) or (*C* and *D*) IL17 (n = 15–16 per group) or with isotype control before and throughout the course of treatment. (*A* and *C*) After harvest, 2 slides per pancreas were assessed for the percentage of tissue occupied by each PanIN stage, acinar-ductal metaplasia (ADM), and PDA. (*B* and *D*) The percentage of tissue occupied by high-grade PanINs (PanINs 2 and 3) was summed. (*E*) The percentage of tissue occupied by PDA also was included for the anti-IL17 treatment. $^*P < .05$, $^{**}P < .01$.



Supplementary Figure 5. Immune infiltrates in prevaccination and postvaccination mesothelioma biopsy specimens with a *Listeria* vaccine targeting mesothelin. Serial sections from biopsy specimens of malignant pleural mesothelioma in a patient receiving a *Listeria* vaccine targeting mesothelin (CRS-207) before administration (*left panels*) and 2 weeks after the second dose (2 doses were given 2 weeks apart) of CRS-207 (*right panels*) were stained with antibodies to (*A*) RORγt or (*B*) CD3. *Arrowheads* designate cells or areas with cells containing intranuclear staining for RORγt. Magnification, 40×.

Supplementary Table 1. Antibodies Used for Immunohistochemical Staining

Antibody	Clone	Vendor	Concentration/dilution
CD4	H129.19	BD Pharmingen	5 μg/mL
CD8	53-6.7	BD Pharmingen	5 μg/mL
F4/80	C1:A3-1	AbD Serotec (Raleigh, NC)	1:100
Ly6G	1A8	BD Biosciences	1:2000
Foxp3	N/A	Abbiotec, LLC (San Diego, CA)	1:100
CD3	2GV6	Ventana Medical Systems, Inc	Per Ventana protocol
RORγt	6F3.1	EMD Millipore Corporation (Billerica, MA)	1:200

Supplementary Table 2. Flow Cytometry Antibodies

Antibody	Fluorochrome	Clone	Vendor
CD3	Allophycocyanin	145-2C11	BD Pharmingen
CD8	PerCP, Pacific Blue	53-6.7	BD Pharmingen
IFNγ	PE, AF700	XMG1.2	BD Pharmingen
Foxp3	PE	FJK-16s	eBioscience, Inc (San Diego, CA)
CD3	APC-cyanine 7 (Cy7)	145-2C11	BioLegend (San Diego, CA)
CD4	PerCP	GK1.5	BioLegend
CD62L	AF647	MEL-14	BioLegend
CD44	AF700	IM7	BD Pharmingen
TNFα	AF488	MP6-XT22	BD Pharmingen
RORγt	PE	Q31-378	BD Pharmingen
CD45	APC-Cy7	30-F11	BD Pharmingen
Gr-1	AF700	RB6-8C5	BD Pharmingen
IL12	APC	C15.6	BD Pharmingen
IL17	AF647	eBio17B7	eBioscience, Inc
Tbet	PE-Cy7	4B10	eBioscience, Inc
F4/80	PE-Cy7	BM8	eBioscience, Inc
Ly6C	PerCP-Cy5.5	HK1.4	eBioscience, Inc
IL10	V450	JES5-16E3	BD Horizon (San Jose, CA)
Ly6G	V450	1A8	BD Horizon
CD11b	PE-Texas Red	M1/70.15	Life Technologies

AF, Alexa Fluor; APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein complex; TNF, tumor necrosis factor.