Online Data Supplement

Adoptive T cell transfer to treat lymphangioleiomyomatosis.

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Extended Materials and Methods

Cell culture

Tumor cells were isolated from a 21-month-old male *Tsc2*$^{+/-}$ mouse (The Jackson Laboratory, B6;129S4-*Tsc2*^{tm1Djk}/J) by tissue homogenization through a 70 µm cell strainer (ThermoFisher Scientific Inc.). Cells were cultured for over 10 passages and loss of functional Tsc2 expression was confirmed at protein level by immunoblotting (see below). Mouse kidney tumor cells, Human Embryonic Kidney (HEK293) cells (American Type Culture Collection, CRL-1573) and Phoenix-E viral producer cells (American Type Culture Collection, CRL-3214) were maintained in high glucose Dulbecco's Modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and standard penicillin, streptomycin and amphotericin antibiotics/antimycotic solution (all from Gibco Life Technologies). Mouse T cells were maintained in RPMI1640 media supplied with 10% heat-inactivated FBS, 1x non-essential amino acids (Corning Inc.), 50 U/ml penicillin streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM 4-(2-hydroxyl)-1-piperzineethanesulfonic acid (Gibco), 50 µM 2-mercaptoethanol (MilliporeSigma), and 30 IU/ml rhIL-2 (NIH).

Generating LAM-like target cells

Mouse gp100 cDNA was amplified using full-length primers flanked by NheI and PmeI restriction sites 5'-CGGCCGCTAGCATGGGTGTCCAGAGAAGGAGC-3' (forward) and 5'-

CCGGCGTTTAAACTCAGACCTGCTGTCCACTGAG-3'(reverse) from an existing plasmid and inserted into pLKO.AS33W lentiviral plasmid (derived from Addgene biological resource center #10878). The resulting construct was sequence-verified before transfecting HEK293T cells. Stable gp100-overexpressing LAM-like cells were generated by transfecting HEK293T cells with the lentiviral, gp100-encoding plasmid together with the packaging and helper plasmids: pEnvy and pHelper, (a kind gift from Dr. Hui-Kuan Lin, Wake Forest School of Medicine) at a 2:1:1 ratio using Fugene transfection reagent (ThermoFisher Scientific). After 48 and 72 hours, viral supernatant was collected and filtered to exclude producer cells. $Tsc2^-$ kidney tumor cells were spinfected with viral supernatant and 8 μg/ml polybrene (MilliporeSigma). Forty-eight hours later, transgene expressing cells were selected in presence of 2 μg/ml puromycin (ThermoFisher Scientific) for a week. The $gp100^+$ LAM-like cells were maintained at 1 μg/ml puromycin to ensure continued expression of gp100. To enhance tumorigenicity, 2×10^6 viable $mgp100^+$, $Tsc2^-$ kidney tumor cells were suspended in 100 μl 1:4 v/v matrigel/PBS and subcutaneously injected into 4 week-old female SCID mice, allowing tumors to develop for 2 weeks. Single tumor cells were harvested and expanded for tail vein injection.

To generate luciferase-expressing LAM-like cells, Phoenix-E cells were transfected with an MSCV-based, self-made GFP-luciferase expression plasmid (a kind gift from Dr. Hui-Kuan Lin) and the viral supernatant was collected 48 hours and 72 hours after transfection. LAM-like cells were spinfected with collected and filtered supernatant in presence of 8 μg/ml polybrene at 1,000g for 2 hours. After recovering and expanding infected cells, GFP positive cells were isolated through GFP sorting assisted by the Northwestern University Flow Core.

Amino acid deprivation and immunoblotting

LAM-like cells and control HEK293 cells were plated $5x10^5$ cells/well in 6-well tissue culture plate. For amino acid-deprived samples, cells were washed before incubation in Dulbecco's Modified Eagle's

Medium (DMEM) with 10% dialyzed FBS without amino acids (ThermoFisher Scientific) for 2 and 4 hours, while control samples were maintained in replete medium. Cells were harvested on ice and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 1% nonyl phenoxypolyethanol (NP40), 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), and 50 mM NaF). SDS sample buffer was added to the lysate and 10% SDS-polyacryl amide gel electrophoresis (PAGE) was run. Samples were transferred to a polyvinylidene difluoride (PVDF) membrane for immunostaining with D93F12 anti-tuberin antibody, Ser235/236 anti-phospho S6 antibody and 54D2 anti-S6 antibody (all purchased from Cell Signaling Technology).

Generating pmel-1 transduced primary mouse T cells

The mouse pmel-1 TCR α and β chains were amplified from commercially generated DNA and sub-cloned into the pBABE-GFP vector (available via Addgene biological resource Center) between the BamHI and SnaBI restriction enzymes using primers 5'-CGCCGGATCCATGGGCACCAGGCTTCTTG-3' (forward) and 5'-CCGGCTACGTATCATGAATTCTTTCTTTTG-3' (reverse). Phoenix-E cells were transfected with the original pBABE-GFP vector or with pBABE-pmel-1-GFP using FuGene transfection reagent (ThermoFisher). Viral supernatant was collected after 48 and 72 hours. Total T cells and CD8 T cells were isolated from spleens of 8-10 week old C57BL/6 mice using a mouse T cell negative selection kit or CD8a positive selection kit (both Stemcell Technologies) per the manufacturer's protocol. Resulting T cells were activated with Dynabeads Mouse T Activator CD3/CD28 beads (Gibco) per manufacturer's instructions and cultured in T cell medium with 30 IU/ml rhIL-2 for 24 hours before transduction. Cells were transduced by centrifugation at 2,000 x g for 2 hours in presence of viral supernatant in a retronectin-coated 24 well, non-tissue culture plate, then incubated for 4 hrs before replacing the viral supernatant with fresh medium supplemented with 30 IU/ml rhIL-2. A second and third transduction after 24 and 48 hrs, respectively followed the same protocol. Transduced T cells were reactivated with

CD3/CD28 beads at 1:1 beads to cell ratio for 2 days. Transduction efficiency was confirmed by flow cytometry where cells were stained with anti-TCRVβ13-PE (MR12-3; BD Biosciences), anti-CD4-PECy7 (GK1.5; BioLegend), anti-CD8a BV711 (53-6.7; BD Biosciences) and LIVE/DEAD Fixable Near-IR Dead Cell dye (ThermoFisher Scientific).

The mouse pmel-1 TCR was additionally cloned into the MMLV retroviral construct by Northwestern University Feinberg School of Medicine's Gene Editing, Transduction and Nanotechnology Core, Skin Biology & Diseases Resource-Based Center. Phoenix-E cells were transfected with pmel-1-MMLV and additional pECO using GeneJuice transfection reagent (MilliporeSigma). Viral supernatant was collected after 48 and 72 hours. CD3 T cells were isolated from spleens of C57BL/6 mice as described above and T cells were activated overnight with anti-mouse CD3ε (145-2C11; BD Biosciences), anti-mouse CD28 (37.51; BD Biosciences), and 50 IU/mL rhIL-2 (R&D Systems). T cells were then resuspended in viral supernatant, 50 IU/mL rhIL-2, and 1 μg/mL polybrene (MilliporeSigma) and plated into a retronectin coated plate for transduced by centrifugation at 2,000 x g for 90 minutes. T cells were incubated for 4 hours and viral supernatant was subsequently replaced with fresh media containing 50 IU/mL rhIL-2. This transduction protocol was repeated a total of 3 times before confirming efficiency was confirmed by flow cytometry. Untransduced controls were produced in the same way, but received Opti-MEM I media (Gibco) instead of pmel-1-MMLV during Phoenix-E transfection. Cells were stained with anti-TCRVβ13-PE (MR12-3; BD Biosciences) and LIVE/DEAD Fixable Near-IR Dead Cell dye (ThermoFisher Scientific).

All data was collected on a BD FACSymphony flow cytometer equipped with FACS Diva software (Becton Dickinson) and analyzed with FlowJo (FlowJo, LLC). Gating strategies in all instances proceeded in the following order: time gate, SSC-A versus FSC-A, two times doublet gating exclusion on FSC-H versus FSC-W and SSC-H versus SSC-W, live cells versus FSC-A, and ultimately gating on the marker of interest. Gating schemes were first performed on control samples, before adding the same gate to experimental samples for comparison.

In vitro co-culture experiments

Murine T cells were added to co-culture experiments in complete RPMI media (Corning Inc.) containing rhIL-2 to promote survival. T cell viability was confirmed both by counting and in live/dead FACS analysis and was maintained at 90%+. Effector:target ratios were based on viable cell counts.

Transduced mouse T cells (see above) or untransduced isolated T cells from C57/BL6 and pmel-1 mice, were combined with $2x10^4$ $Tsc2^-$ kidney tumor cells with or without gp100 expression at 2:1 and 1:1 target:effector ratios in triplicate wells of a 96-well plate. Target tumor cells were pre-pulsed with 1 μM human $gp100_{25-33}$ peptide, and co-cultures were incubated for 48 hours. Supernatants were saved for a mouse IFN-γ ELISA (Abcam). T cells were aspirated and target cells lifted and counted to measure cytotoxicity as [1- (live target cells incubated with T cells)/(live target cells maintained without effectors)] x100% using a Countess II FL Automated Cell Counter (ThermoFisher Scientific).

In repeat experiments using T cells with higher transduction efficiency Tsc2- kidney tumor cells either transduced with gp100 (or not) were plated at $1x10^5$ and pulsed with 40 ng/ml mIFN-γ (Invitrogen) for 5 hours at 37 degree Celsius. TSC2- kidney tumor cells transduced with gp100 subsequently received 1 μM human $gp100_{25-33}$ peptide for 1 hour at 37 degree Celsius. Transduced or untransduced T cells were then added 1:1 overnight. The following morning Brefeldin A (BioLegend) was added at 1X for 5 hours at 37 degrees Celsius. Supernatant was collected and run in a mouse IL-2 Quantikine ELISA (R&D Systems) per manufacturer's protocol and cells were lifted and stained for FACS analysis. T cells were stained with anti-CD8a-FITC (53-6.7; BD Biosciences) and LIVE/DEAD Fixable Near-IR Dead Cell dye (ThermoFisher Scientific) prior to being fixed and permeabilized (BD Biosciences) and incubated with anti-IFN-γ-BV421 (XMG1.2, BioLegend). Cells were run on a BD FACSymphony flow cytometer equipped with FACS Diva software (Becton Dickinson) and data was analyzed in FlowJo (FlowJo LLC). Images of co-culture wells were taken on a Revolve microscope (Echo) at an E:T ratio of 10:1.

Adoptive T cell transfer and tumor monitoring

Groups (N=4-5) of 6-8 week old SCID/beige immunodeficient mice (Taconic) were incoculated with 10^6 LAM-like cells with or without luciferase expression via tail vein injection. In mice treated by adoptive T cell transfer, one million T cells isolated from either pmel-1 or from C57BL/6 mice (control) were injected 2 and 3 weeks post tumor inoculation. Mice received IL-2 injections ($6x10^4$ U/mouse) 3x per week. In one experiment, hamster anti-mouse PD-1 antibody (200 ug/mouse) or polyclonal American Hamster IgG control antiserum (both Bioxcell, NH, USA) was administered 3x per week for 2 weeks to 3 mice from each group. To evaluate tumors sizes throughout this experiment, mice received a 15 mg/ml D-luciferin (Perkin Elmer, Waltham, MA, USA) intraperitoneal injection 10 minutes before imaging using the Xenogen IVIS Spectrum (Perkin Elmer) under a consistent setting of 1 minute exposure, large bin and factor of 1. Living Image Software 3D Analysis Package (Perkin Elmer) was used to analyze the imaging data and calculate relative tumor sizes. Upon euthanasia, mice were carefully examined and samples of the lungs, spleen and skin (partial) were stored for further analysis.

In situ immunostaining

For immunofluorescent (IF) and immunohistochemical (IHC) staining, subcutaneous tumor or lung tissues were frozen in optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc.) and 8 μm cryosections cut on a CM3050S cryostat (Leica BioSytems). Sections were air-dried and fixed in ice cold acetone for 10 minutes. All sections were blocked with Super Block (ScyTek Laboratories) and sections being stained for CD3ε or F4/80 received an additional incubation with Biotin Block (ScyTek Laboratories). Immunostaining was performed with goat antiserum to PD-1 (R&D Systems) detected by FITC-labeled rabbit anti-goat antibodies (SouthernBiotech); with rabbit antiserum to PD-L1 (E1L3N) XP (Cell Signaling Technology) detected by DyLight 488-labeled donkey anti-rabbit antibodies (BioLegend);

with goat antiserum to PMEL-17/SILV (gp100) (Novus Biologicals) detected by either Alexa Fluor 568-labeled donkey anti-goat antibodies (Invitrogen) or FITC-labeled rabbit F(ab) anti-goat antibodies (SouthernBiotech); with biotin labeled rat antiserum to F4/80 (BM8, BioLegend) detected by PE-labeled streptavidin (SouthernBiotech); or with biotin labeled 145-C211 antibody to CD3ε (Becton Dickinson) detected by PE-labeled streptavidin (SouthernBiotech). For Treg staining, sections were fixed and permeabilized using the True Nuclear Transcription Factor Buffer Set (BioLegend) prior to blocking and incubation with PE labeled hamster antiserum to CD3ε (145-C211, BD Biosciences) and AF488 labeled rat antiserum to FoxP3 (MF-14, BioLegend). All fluorescently labeled slides also received incubation with DAPI (BD Biosciences) prior to imaging. For hematoxylin and eosin staining (H&E) freshly cut cryosections were air-dried and fixed in 10% neutral buffered formalin (ThermoFisher Scientific Inc.) for 10 minutes before being stained in Mayer's hematoxylin (ThermoFisher Scientific Inc.), blued in Scott's Tap Water (MilliporeSigma), and stained in Alcoholic Eosin Y (MilliporeSigma). Images were taken on a Revolve microscope (Echo) and quantifications were performed in Photoshop (Adobe). For PD-L1/gp100 co-staining sections were stratified into groups of no (0%), small (1-33%), intermediate (34-65%), or large (66-100%) tumor based off the percentage of tissue area that the tumor covered in the field of view.

Statistics

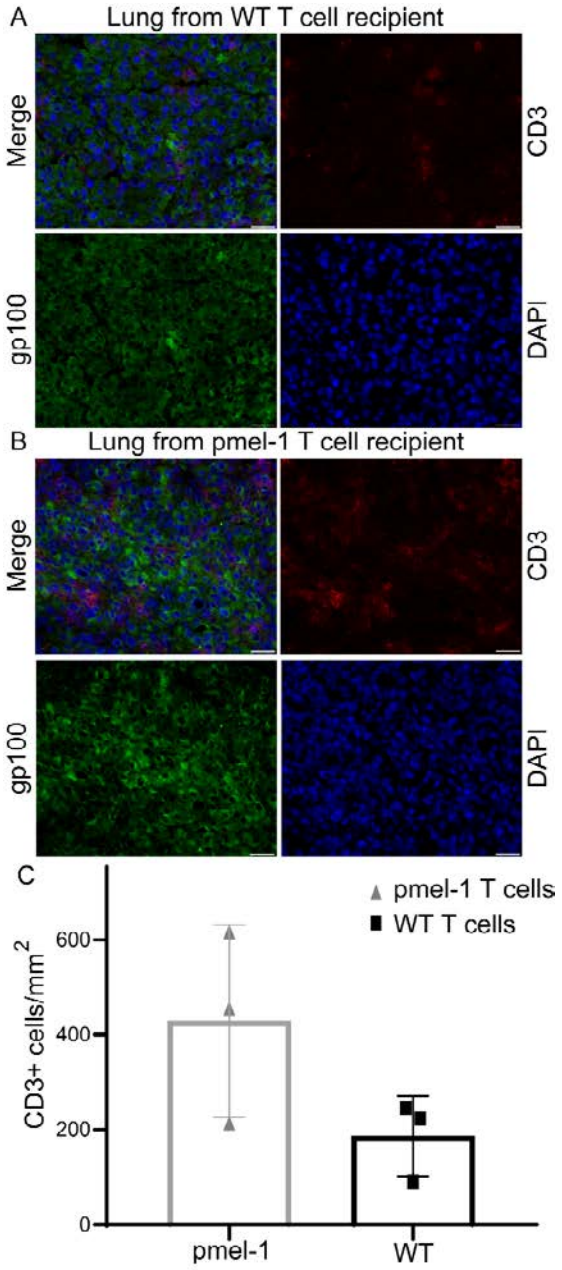
All statistics were performed in Prism software (GraphPad) and parametric or non-parametric tests were assigned after performing applicable normality tests to determine data distribution. All graphs are represented as means ± SD. For the cytotoxicity assay a two-way ANOVA with a Tukey's multiple comparison's test was performed to determine significance across all groups. The significance between numbers of surface lung nodules, as well as the significance between PD-L1/gp100 staining and tumor burden, was determined via a one-way ANOVA (Kruskell-Wallis test) with Dunn's multiple comparisons. A two-tailed, unpaired t-test with Welch's correction was run for PD-1+/PD-1+CD3+

staining in lung tissue. For all tests a value less than 0.05 was considered significant and p values are represented as the following: *$p < 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

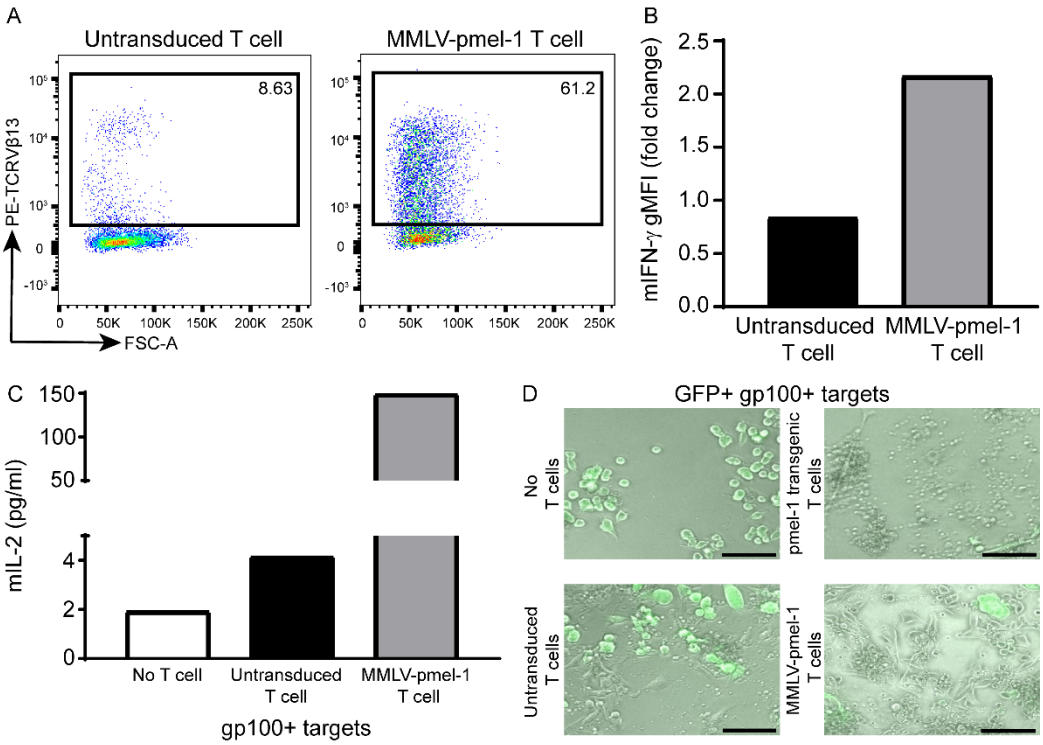
Study Approval

All animal studies were approved by the Institutional Animal Care and Use Committee of Northwestern University and followed the institutional guidelines, as well as experimental set-up as outlined in the approved protocols.

Supplementary data



Supplementary Figure E1. T cell infiltration in pulmonary tissues from adoptively transferred mice. Representative images of CD3+ T cells and gp100+ LAM-like cells in frozen pulmonary tissues from mice treated with (**A**) wild type or (**B**) pmel-1 T cells. Quantification of (**C**) CD3+ cells/mm^2. Scale bar = 50 µm.



Supplementary Figure E2. High efficiency transduction of murine CD3 T cells with pmel-1.

(**A**) Expression of TCRVβ13 analyzed by flow for untransduced T cells and MMLV-pmel-1 transduced murine T cells. (**B**) Expression of IFN-γ represented as gMFI fold change from FACS analysis between gp100+ transduced TSC2- kidney tumor cells and empty vector tumor cells incubated overnight 1:1 with untransduced or MMLV-pmel-1 transduced T cells (N=1). (**C**) Expression of IL-2 (pg/ml) in supernatants from gp100+ TSC2- kidney tumor targets (pre-pulsed with IFN-γ and gp100 peptide) co-culture 1:1 with either no T cells, untransduced T cells, or MMLV-pmel-1- transduced T cells; analyzed by ELISA (N=1) (**D**) Representative images of GFP+ gp100+ transfected TSC2- kidney tumor cells (pre-pulsed with IFN-γ and gp100 peptide) after 48 co-culture with either no T cells, pmel-1 transgenic T cells, untransduced T cells, or MMLV-pmel-1 transduced T cells at an E:T ratio of 10:1. Scale bar = 50 µm.