Supplementary Text and Figures

Cell culture

The human embryonic kidney 293T and Phoenix-Amp cell lines, both used to package retroviruses carrying RNA encoding TCR$\alpha\beta$, were grown in DMEM with 10% Fetal Bovine Serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), 200nM L-glutamine, 1% MEM non-essential amino acids and antibiotics. The mouse melanoma B16BL6 (B16WT) clone (selected for high TRP2 expression) and cell line, B16:A2-YLEP clone and cell-line, and other B16 cell lines expressing gp100 antigen variants, as well as tumor cells isolated from *in vivo* tumors were cultured using the same medium. Mouse splenocytes as well as TILs (the latter starting from single tumor cell suspensions) were cultured in mouse T cell medium consisting of RPMI 1640 medium supplemented with 25 mM HEPES, 200 nM L-glutamine, 10% FBS, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, antibiotics and 50 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands).

Mice

The HLA-A2 transgenic (tg) mice (kindly provided by prof. François Lemonnier, Paris, France), are β2m$^{-/-}$ and H-2D$^{b-/-}$ and express a chimeric HLA-A*0201 transgene (HHD, referred to as HLA-A2) composed of human β2m, the α1 and α2 domains of HLA-A*0201 and the α3 domain of H-2D^{b} (1). Inbred C57BL6 (B6) mice were purchased from Harlan Laboratories (Hillcrest, UK). Experiments were approved by the Experimental Animal Committee of the Erasmus MC Cancer Institute and carried out in accordance with institutional and national guidelines.

TCR and antigen constructs

Making of constructs

TCRα and β genes specific for human glycoprotein 100 (gp100$_{280\text{-}288}$) peptide (YLEPGPVTA) presented by HLA-A2 (gp100/A2) were derived from CTL-296 clone as described earlier (2).

Gp100/A2-specific TCRα and β genes were murinized as described previously (3), codon optimized (GeneArt, Regensburg, Germany) and cloned separately in pBullet retroviral vector (4) (vectors are abbreviated as pB:TCRα+β and pB:optTCRα+β). In addition, the codon optimized gp100/A2 TCR genes were cloned into the pMP71 vector (kindly provided by prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany) with TCRα and β genes separated by an optimized T2A ribosome skipping sequence (abbreviated as pMP71:opt TCR β-T2A-α). TCRα and β genes specific for mouse tyrosinase-related protein-2 ($TRP2_{180-188}$) peptide (SVYDFFVWL) presented by H2-K^b (TRP2/H2-K^b) were derived from CTL-LP9 clone (5) as described earlier (6). In short, our laboratory obtained TCR-V DNAs by 5′ RACE and identified the full length TCRα and β genes according to www.imgt.org: TRAV12N-3/J31/C and TRBV26/D1/J2-7/C2. TCR sequences were cysteine modified (according to (7)**)** codon optimized and cloned into the pMX vector with TCRα and β genes separated by a P2A sequence (abbreviated as pMX:opt TCRβcys-P2A-TCRαcys, and kindly provided by prof. Ton Schumacher, NKI-AvL, Amsterdam, the Netherlands). The A2-YLEP antigen gene was a fusion between HHD (kindly provided by prof. Francois Lemonnier) and the gp100 peptide (YLEPGPVTA). This antigen gene was obtained by inserting DNA that covered the leader sequence of HLA-A2, the gp100 peptide sequence and a $(G_4S)_3$ linker and was flanked by *Eco*RI sites (synthesized by GeneArt) into a pLXSN vector that already contained HHD (pLXSN:A2). HHD cDNA and human gp100 cDNA (kindly provided by prof. Gosse Adema, Nijmegen, The Netherlands) were subcloned as *Xho*I fragments into the retroviral vectors pLXSN and pLXSH, respectively (Clontech Laboratories Inc., Mountain View, CA) (pLXSN:A2 and pLXSH:gp100). We obtained a pLXSH:gp100AV variant containing an altered gp100 peptide ligand with improved HLA-A2 binding (8) by replacing the anchor alanine residue by a valine by site-directed mutagenesis (QuikChange II kit, Stratagene, Cedar Creek, TX). All TCR and antigen inserts, once cloned into retroviral vectors, were sequence verified (Service XS, Leiden, The Netherlands)**.**

Retroviral transduction

Total mouse splenocytes were isolated, activated with Concanavalin A and rhIL-2 in mouse T cell medium, and transduced with the retroviral supernatant as described by Pouw and colleagues (3). T cells were transduced with TCR genes or empty retroviral vector (TCR and Mock T cells, respectively). B16 cells were retrovirally transduced using a similar transduction protocol with the exception that GALV-pseudotyped viruses together with polybrene (4 μg/ml) were used and cells were incubated with the retroviral supernatant for 24h, followed by a second incubation for 8h. B16:A2-YLEP cells (transgenes: pLXSN:A2-YLEP+pLXSH:gp100) were cultured with neomycin (1 mg/ml) (G418, Calbiochem, La Jolla, CA) and hygromicin (0.5 mg/ml, Clontech Laboratories). In addition to our standardly used B16:A2-YLEP cells, we have transduced B16 cells with pLXSN:A2-YLEP but not pLXSH:gp100 (B16A2-YLEP w/o gp100). B16:A2/gp100 (transgenes: pLXSN:A2+pLXSH:gp100) and B16:A2/gp100AV cells (transgenes: pLXSN:A2+pLXSH:gp100AV) were cultured in the presence of both neomycin and hygromycin.

Validation of TCR and tumor antigen

Expression of TCR genes and antigens was validated by standard flow cytometry using a FACSCalibur and CellQuest software (BD). Surface expression of TCR transgenes was detected with the following antibodies and reagents: FITC-labeled anti-TCR-Vβ27 mAb (gp100/A2 TCR, clone CAS1.1.3, Beckman Coulter, Marseille, France); gp100/A2K^b PE-labeled tetramers (gp100/A2 pMHC generated according to (9)); FITC-labeled anti-TCR-Vα12N-3 mAb (TRP2/H2-K^b TCR, clone B21.14; eBioscience, Hatfield, UK); and PE–labeled anti-TCR-Vβ26 mAb (TRP2/H2-K^b TCR, clone KJ25; BD, Oxford, UK). Stainings for TCRs were performed in combination with PerCP-labeled anti-CD3ε and APC-labeled anti-CD8α mAbs. Expression of surface MHC class I molecules and intracellular antigens were detected with the following antibodies: FITC-labeled anti-HLA-A2; anti-human gp100 followed by 2nd step PE-labeled anti-mouse IgG; PE-labeled anti-H2-K^b; and anti-mouse TRP2 followed by 2nd step PE-labeled anti-rabbit IgG (see Materials and Methods). Stainings for TCRs and antigens are exemplified in Figures S1A, S1B (gp100/A2) and S12A (TRP2/H2-K^b). For most

in vivo experiments, B16:A2-YLEP cells were single-cell sorted for high HLA-A2 expressing cells on a FACSAria cell sorter using the anti-HLA-A2 antibody, and B16WT cells were single-cell sorted for high mouse TRP2 expressing cells. TCR T cells were functionally validated by IFNγ production after 20h co-cultivation with B16 tumor cells using ELISA measurements in supernatants (see Materials and Methods) (Figures S1C and S12A). We observed that among the various gp100/A2 TCR and antigen constructs the pMP71:opt TCR β-T2A-α transduced T cells and B16:A2-YLEP cells were most optimal according to surface expression of transgene, *in vitro* T cell function and *in vivo* growth kinetics of antigen-positive B16 tumor cells (see Figure S1), and were used for *in vivo* adoptive T cell transfer experiments.

Adoptive T cell therapy

Conditioning with Busulphan and Cyclophosphamide

HLA-A2 tg mice were conditioned at day -3 prior to tumor transplant as described for the curative model in Materials and Methods. At day 4 after start of treatment with chemotherapeutic drugs, blood was drawn from conditioned and unconditioned control mice. Following lysis of erythrocytes with NH_4Cl absolute cell numbers of lymphocyte subsets were determined using Flow-Count Fluorospheres (Beckman Coulter). Cells were stained with the following mAbs: CD3ε-PerCP (clone 145-2C11), CD8-APC (clone 53-6.7), CD4-FITC (clone L3T4), CD19-PE (clone 1D3), NK1.1-PE (clone PK136) and CD11c-APC (clone HL3) (all BD Pharmingen, San Diego, CA) and analyzed by flow cytometry (see Figure S2).

Tumor prevention model

HLA-A2 tg mice were conditioned as mentioned above, and at day 0 mice were injected s.c. with $0.5x10^6$ B16:A2-YLEP cells, followed at day 1 with injection of 2.5, 7.5 or $20x10^6$ TCR or $20x10^6$ Mock T cells i.v. in a volume of 100μl. Numbers of TCR T cells reflect T cells that specifically bind gp100/A2 pMHC, which covered 70-80 % of total T cells injected. T cell injection was followed by injections of IL-2 ($1x10^5$ IU per dose) s.c. on 4 consecutive days (days 1-4) (Figure S3).

Tumor curative model

See Materials and Methods for a description of the tumor curative model.

Tumor growth

Tumor growth was measured by caliper 3 times a week and tumor volumes were estimated with the formula 0.4 x (A x B^2) where A represents the largest diameter and B the diameter perpendicular to A (10). Animals were sacrificed once the tumor was ulcerated, the largest diameter reached 20 mm or there was > 20% loss in body weight.

Analyses of T cells following T cell therapy

T cell persistence and memory

Peripheral blood was drawn at day 4 after T cell transfer and at weekly intervals thereafter. Absolute T cell counts were determined using Flow-Count Fluorospheres. TCR T cells were monitored as described above (see *Validation of TCR*). To detect low frequencies of memory TCR T cells, mice that remained tumor-free for > 90 days after the first tumor challenge (in prevention model) were re-challenged with $0.5x10^6$ B16:A2-YLEP cells. Spleens from mice that again remained tumor-free for > 90 days were isolated and amplified for 5 days in the presence of gp100 peptide (0.005 μg/ml) starting at $2.5x10^6$ cells/ml in mouse T cell medium supplemented with 20 IU/ml IL-2. Spleens from mice that remained tumor-free after tumor regression (i.e. no macroscopic tumor) for > 75 days (in curative model) underwent similar procedures. T cells were harvested and stained with gp100/A2 pMHC, anti-CD3ε, anti-CD8α, APC-labeled anti-CD44 (clone IM7) and FITC-labeled anti-CD62L mAbs (clone MEL-14) (all mAb from BD). In addition, T cells were stimulated O/N with B16WT and B16:A2-YLEP tumor cells in the presence of Golgi Plug (according to manufacturer instructions, BD), stained with anti-CD8α mAb, permeabilized and stained with PE-labeled anti-IFNγ mAb (clone XMG1.2, BD). All samples were analyzed on a FACSCalibur using CellQuest software.

T cell responses against antigens other than gp100/A2

Mice that were treated with gp100/A2 TCR T cells and cured were tested for endogenous T cell responses against epitopes other than gp100/A2. To this end, splenocytes ($2x10^6$) were co-cultured with irradiated (160Gy) B16:A2-YLEP tumor cells ($1x10^6$) in a total volume of 1 ml mouse T cell medium supplemented with 20 IU/ml IL-2 per well in a 24 well plate. After 5-7 days of T cell amplification, cells were analyzed for pMHC binding and IFNγ production in response to B16:A2-YLEP and B16WT by flow cytometry (see also legend to Figure S7).

Analyses of tumors following T cell therapy

Antigen mRNA and genomic DNA

Tumor RNA and DNA were purified with use of silica-based membranes (Mini kits, Qiagen, Valencia, CA). Quantitative PCRs were performed to assess levels of antigen mRNA and genomic antigen DNA. Levels were calculated using the formula $2^{-\Delta CT}$, in which C_T is defined as the cycle number at which the amplified fluorescence signal crosses a pre-set threshold and ΔC_T is defined as the difference between the C_T value of the A2-YLEP gene and that of an endogenous reference gene, namely mouse TRP2. Sequences of primers and probes used to quantify levels of A2-YLEP and A2 DNAs are provided in Figure S9. Sequences of primers and probe to quantify TRP2 DNA were: forward primer, 5'-TAATTGTGGAGGCTGCAAGTTC-3'; reverse primer, 5'-AGGATGGCCGGCTTCTTC-3'; and probe, FAM-5'-CTGGACCGGCCCCGACTGTAATC-3' (Applied Biosystems, Foster City, CA). Detection was monitored with an MX3000P real-time PCR System using MX Pro data analysis (Stratagene, La Jolla, CA). For further analyses, retrovirally introduced antigen transgenes were amplified from tumor DNA (100 ng) using: the pLXSN forward primer, 5'-GCCTCCGCCTCCTCTTCCTCCATC-3'; the pLXSN reverse primer, 5'-GCTCAGAAGAACTCGTCAAGAAGG-3'; and Phusion High-Fidelity DNA polymerase (Thermo Scientific Finnzymes, Vantaa, Finland). PCR products were analyzed by gel electrophoresis and sequencing, which allowed the design of primers and probes and performance of quantitative PCRs to distinguish between antigen transgenes with or without the gp100 peptide sequence (see legend to Figure S10 for details).

Methylation of antigen promoter

To assess the methylation status of antigen promoter, DNA was treated with sodium bisulphite (EZ DNA Methylation, Zymo research; Irvine, CA) according to the manufacturer's protocol. Methylation-specific PCR was performed on treated DNA using primers specific for either unmethylated (U) or methylated (M) DNA sequences of the 5' LTR promoter of the pLXSN provirus, upstream of the antigen gene. Primer sequences were: U forward primer, 5'-TGTGTTTTATTTGAATTAATTAATTAGTTT-3'; U reverse primer, 5'-CACAATCTATCAAAAAACTAACACC-3'; M forward primer, 5'-TTTTATTTGAATTAATTAATTAGTTCGT-3'; and M reverse primer, 5'-GCAATCTATCGAAAAACTAACGC-3'.

Ex vivo treatment of tumor cells to up-regulate antigen expression

Cultures of isolated tumor cells were either non-treated or subjected to one of the following *ex vivo* treatments prior to measurements of antigen expression by flow cytometry. Azacitidine (AZA) was added to the culture media at day 4 for 3 consecutive days (final concentration 2-10μM) (Sigma-Aldrich); mouse IFNγ was added at day 5 (100 Units/ml) (Sanquin) and tumor cells were left untreated for another 48h; and tumor cells were cultured for extended periods of 14-21 days.

Functional antigen expression

Cultures of isolated tumor cells were tested for their ability to induce IFNγ production by TCR T cells. In addition, target cell lysis was measured with two types of assays. The WST-1 (Water Soluble Tetrazolium salts) assay was adapted from (11). In brief, tumor cells were seeded in flat-bottom 96-wells plates ($1x10^4$ per well in 200 µl B16 culture medium) and incubated overnight at 37ºC and 5% CO_2. Next, 100 µl supernatant was removed from the wells and TCR or Mock T cells were added ($8x10^4$ T cells per well in 50 µl mouse T cell medium). Cells were left to incubate for 20h at 37ºC and 5% CO_2. In a last step, 50 µl of 5x diluted WST-1 reagent (Roche, Basel, Switzerland) was added to each well and incubation was continued for 3h at 37ºC and 5%CO_2, after which absorbance was measured (450 nm).

Tumor cells only and a 1% triton solution were used to set 0 and 100% target cell killing, respectively. Absorbance of WST-1 reagent in medium (no cells) was used to determine background activity and subtracted from all data. Data was presented as % target cell killing, which was calculated as follows:

$$\% \text{ killing} = \frac{Abs^{\text{tumor cells only}} - Abs^{\text{Sample}}}{Abs^{\text{tumor cells only}} - Abs^{\text{Triton}}}$$

A standard ^{51}Cr-release assay was performed as described previously (3).

Genome-wide expression analysis

RNA from collected tumors was purified with use of silica-based membranes (Mini kit, Qiagen) and subjected to genome-wide expression analysis using Illumina expression bead chips (ServiceXS, Leiden, The Netherlands). In short, RNA concentration was measured with a Nanodrop ND1000 spectrophotometer, and RNA quality and integrity were checked with an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Two hundred ng total RNA was amplified and labeled with biotin-16-UTP (TotalPrep-96 RNA amplification kit). Biotinylated cRNA samples were hybridized onto Illumina MouseWG-6 v2 beads, after which beads were washed, stained and scanned with an iScan array scanner. Initial gene expression analysis and subsequent data processing and normalization were performed using Genomestudio v. 2011.1 and 'lumi' (12) packages, respectively. Normalized sample data were averaged and used to calculate signal differences between relapsed, regressed or progressed tumors, which, followed by log2 transformations, represented fold changes in gene expressions between the different tumor types.

References

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Figure S1

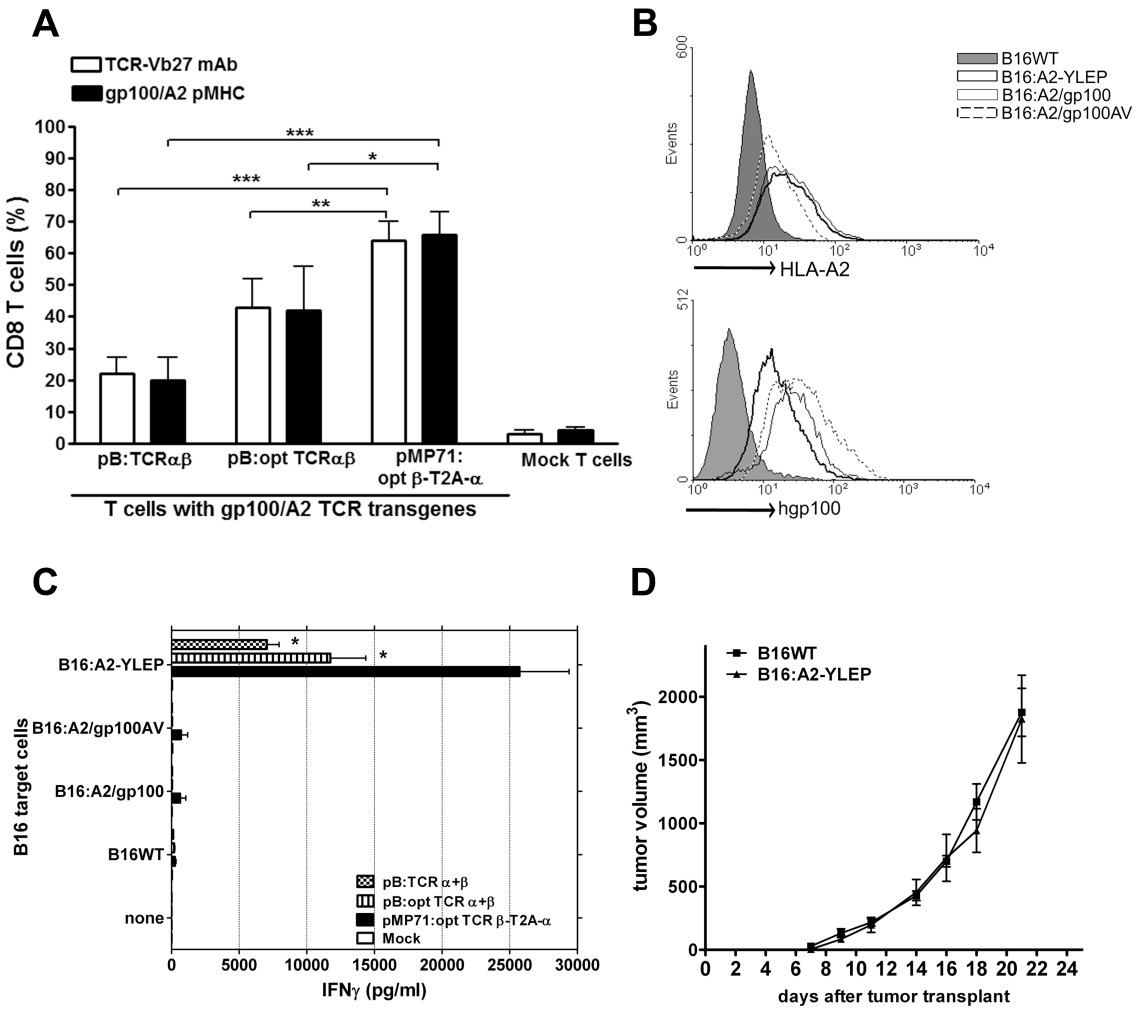

Figure S1. Validation of TCR and antigen gene constructs

(**A**) Murine splenocytes were transduced with the following gp100/A2 TCR constructs: pB:TCRα+β (two separate pBullet vectors with wild type sequences for TCRα and TCRβ); pB:optTCRα+β (two separate pBullet vectors with codon optimized sequences for TCRα and TCRβ); and pMP71:optTCRβ-T2A-α (single pMP71 vector with codon optimized TCRαβ in a T2A cassette). See *Supplementary text* for details on the construction of TCR genes. At day 5 following T cell activation CD8 T cells were analyzed for TCR-Vβ27 expression (white bars) and gp100/A2 pMHC binding (black bars) by flow cytometry. Data represent mean % ± SEM, n=5 separate transductions. (**B**) B16 cells were transduced with the following gp100/A2 antigen constructs: pLXSN:A2+pLXSH:gp100 (B16:A2/gp100 cells, dashed histogram); pLXSN:A2+pLXSH:gp100AV (B16:A2/gp100AV cells, black histogram) and pLXSN:A2-

YLEP+pLXSH:gp100 (B16:A2-YLEP cells, bold black histogram). See *Supplementary text* for details on the construction of antigen genes. Tumor cells were stained with a FITC-labeled anti-HLA-A2 mAb, or anti-human gp100 mAb followed by a PE-labeled anti-mouse IgG and analyzed by flow cytometry. Non-transduced B16WT cells were used as a negative control (grey filled histogram). (**C**) TCR-transduced splenocytes (from S1A) were incubated with different populations of B16 target cells (from S1B) and supernatants were harvested after 20h and analyzed for murine IFNγ. Data are presented as mean pg/ml IFNγ + SEM, n=5. (**D**) B16:A2-YLEP or B16WT cells ($0.5x10^6$) were injected s.c. into HLA-A2 tg mice. Tumor volumes were measured 3 times a week with a caliper and data are presented as mean mm^3 ± SEM, n=5. Statistical differences were calculated with Student's *t* tests: *p<0.05, **p<0.01, *** p<0.0001.

Figure S2

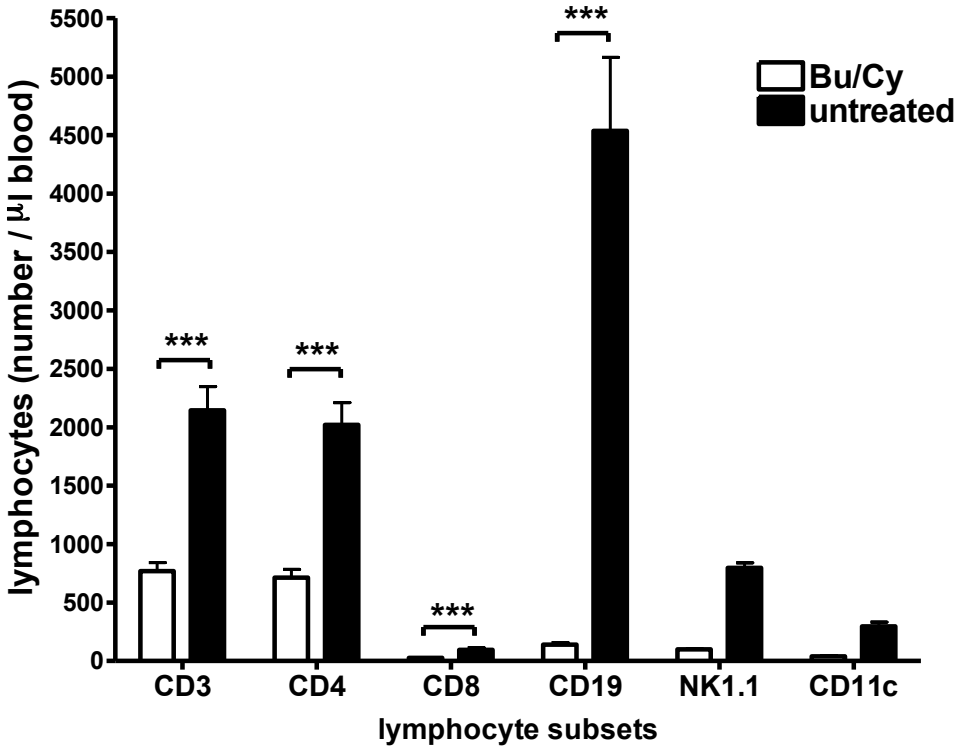

Figure S2. Conditioning with Busulphan and Cyclophosphamide results in effective lymphodepletion *in vivo*

HLA-A2 tg mice were conditioned with Bu/Cy as described in the *Supplementary text* (n=24 mice, tumor-bearing, no T cell treatment). As a control, HLA-A2 tg mice were left untreated (n=8). Peripheral blood was collected one day after conditioning and analyzed for different lymphocyte subsets by flow cytometry (for NK1.1 and CD11c, n=2). Data are displayed as mean numbers ± SEM. Statistical differences were calculated with Student's *t* tests. ***p<0.0001.

Figure S3

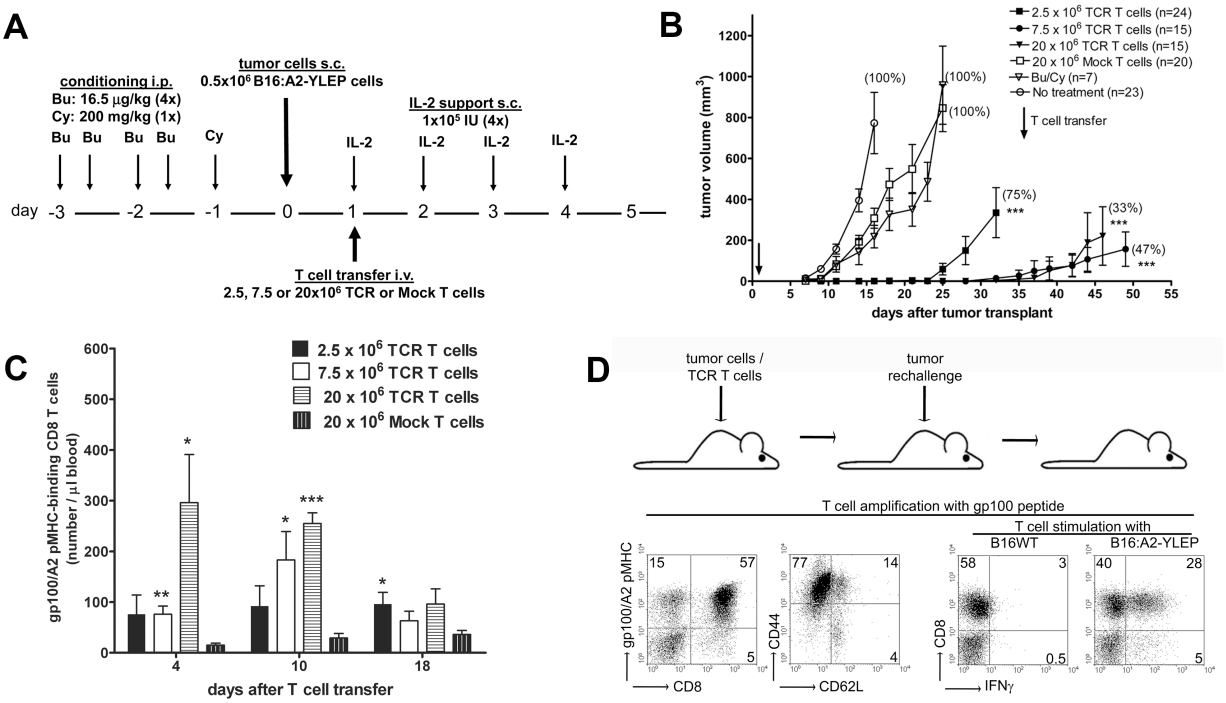

Figure S3. Treatment with gp100/A2 TCR T cells prevents or delays tumor growth and results in the generation of antigen-specific memory T cells

(**A**) Schedule of tumor prevention model: HLA-A2 tg mice were conditioned with a total of 4 Bu injections i.p., twice daily on 2 consecutive days, followed by a single Cy injection i.p. prior to transplantation of $0.5x10^6$ B16:A2-YLEP tumor cells s.c. at day 0. At day 1 mice were either injected i.v. with gp100/A2 TCR T cells (2.5, 7.5 or $20x10^6$ T cells that bind gp100/A2 pMHC), Mock T cells (number of Mock T cells equal to number of T cells in $20x10^6$ TCR T cell group) or left untreated. T cell injection was followed by injections of IL-2 ($1x10^5$ IU per dose) s.c. on 4 consecutive days. (**B**) Tumor sizes were measured 3 times a week with a caliper. Data are presented as mean mm^3 ± SEM. The percentages of mice that presented with a tumor are indicated in parentheses. (**C**) Peripheral blood was collected from mice at the indicated time points and absolute numbers of gp100/A2 pMHC-binding CD8 T cells were determined by flow cytometry. Data are presented as mean numbers ± SEM (n=8). (**D**) Mice that remained tumor-free for > 90 days after tumor challenge were re-challenged with B16:A2-YLEP cells (n=13). Tumor growth was delayed in all re-challenged mice when

compared to primary challenged mice and 30% of mice remained again tumor-free until the end of the experiment (> 90 days). Splenocytes of tumor-free mice were amplified *in vitro* in the presence of gp100 peptide and assessed for gp100/A2 pMHC-binding and expression of CD44 and CD62L. In addition, peptide- amplified cells were stimulated O/N in the presence of B16:A2-YLEP or B16WT cells and monitored for intracellular IFNγ expression by CD8 T cells. Representative dot plots from 1 of 4 mice are shown and the % of cells in each quadrant is indicated. Statistical significances were calculated with Student's t-tests: * $p<0.05$, ** $p<0.005$, *** $p<0.0001$.

Figure S4

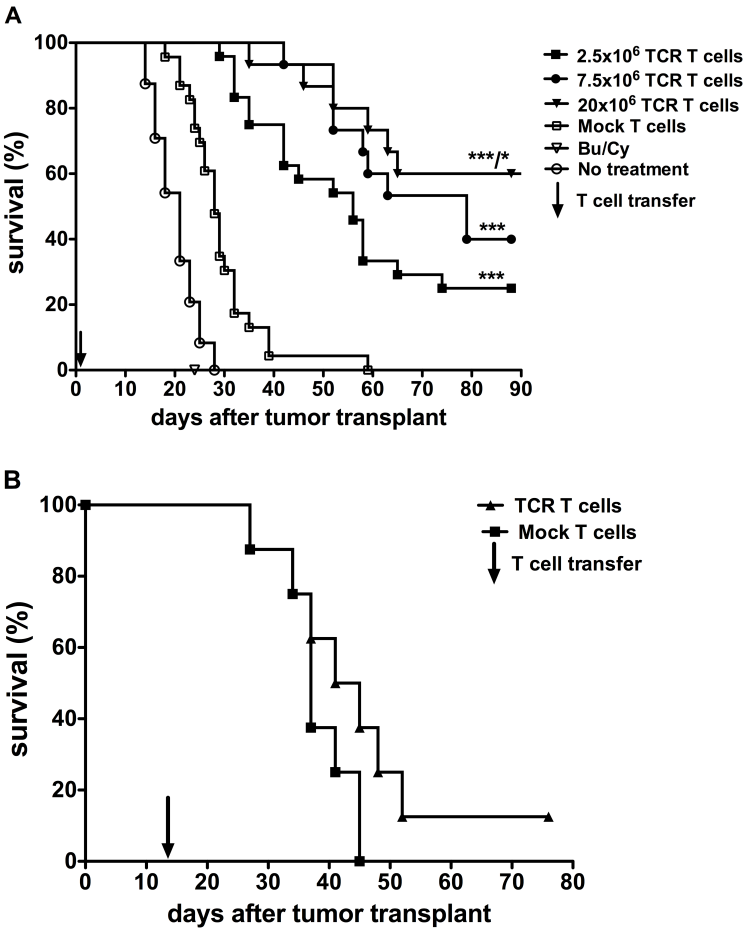

Figure S4. TCR T cells provide a survival advantage in preventive but not curative model

(**A**) HLA-A2 tg mice were treated as described in the legend to Figure S3A. (**B**) Mice were treated with 20x10^6 TCR or Mock T cells as described in the legend to Figure 1A. Statistical significances between TCR and Mock T cells and among different doses of TCR T cells were calculated with the Mantel-Cox test: *** $p<0.0001$ (preventive model, TCR vs Mock T cells); * $p<0.05$ (preventive model, 20x10^6 vs 2.5x10^6 TCR T cells); and not significant (curative model, TCR vs Mock T cells; and preventive model, 20x10^6 vs 7.5x10^6 TCR T cells).

Figure S5

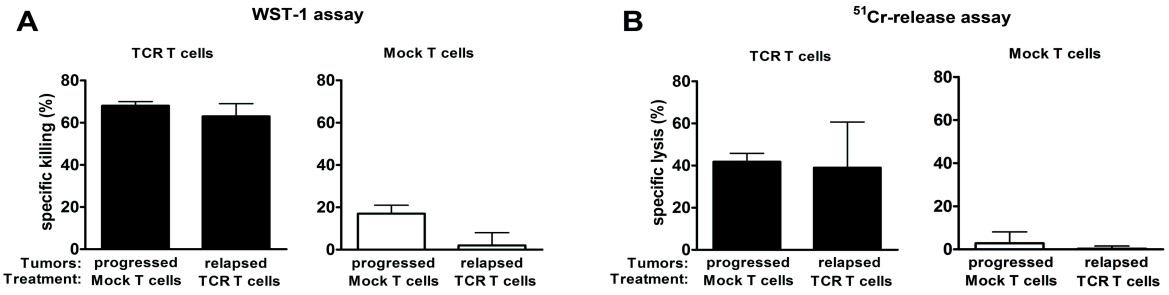

Figure S5. Cells isolated from relapsed tumors are killed by TCR T cells *ex vivo*

HLA-A2 tg mice bearing established tumors from B16:A2-YLEP clone were conditioned and treated with TCR or Mock T cells as described in legend to Figure 1A. Following T cell treatment, mice with progressing (treated with Mock T cells) and relapsing (treated with TCR T cells) tumors were sacrificed when tumor sizes were maximal (the largest diameter reached 20 mm). Tumors were isolated, single cell suspensions were prepared and short-term cultures (4-7 days) were set up. Tumor cells were exposed to TCR or Mock T cells and target cell lysis was measured by (**A**) a WST-1 assay (effector-to-target (E:T) ratio of 8:1) and (**B**) a ^{51}Cr-release assay (E:T ratio of 40:1). Both assay were performed in triplicate and data are presented as mean % specific killing + SEM (n=8 individual tumors in A; and n=2 individual tumors in B).

Figure S6

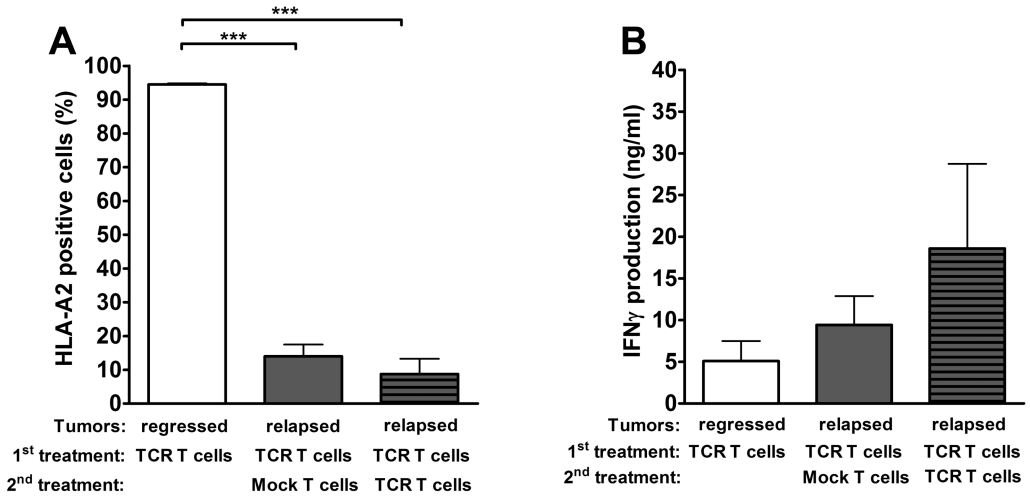

Figure S6. Relapsed tumors that progress following a second T cell treatment have not lost expression of antigen

HLA-A2 tg mice bearing relapsed B16:A2-YLEP tumors received a second treatment with either TCR or Mock T cells at day 34 (after tumor transplant). Mice bearing a B16:A2-YLEP tumor and receiving a first treatment with TCR T cells (day 14 after tumor transplant) served as controls. Mice were sacrificed, tumors were isolated, single cell suspensions were prepared and short-term cultures (4-7 days) were set up (n=3-5 mice per group). (**A**) Tumor cell HLA-A2 surface expression was measured by flow cytometry and data are presented as mean % positive cells in viable gate + SEM. (**B**) IFNγ production by TCR T cells upon a 20h exposure to tumor cells *ex vivo* was analyzed by ELISA. Data are presented as mean ng/ml + SEM. Statistical significances were calculated with Student's t-tests: *** $p < 0.0001$. See legends to Figures 3B and C for details.

Figure S7

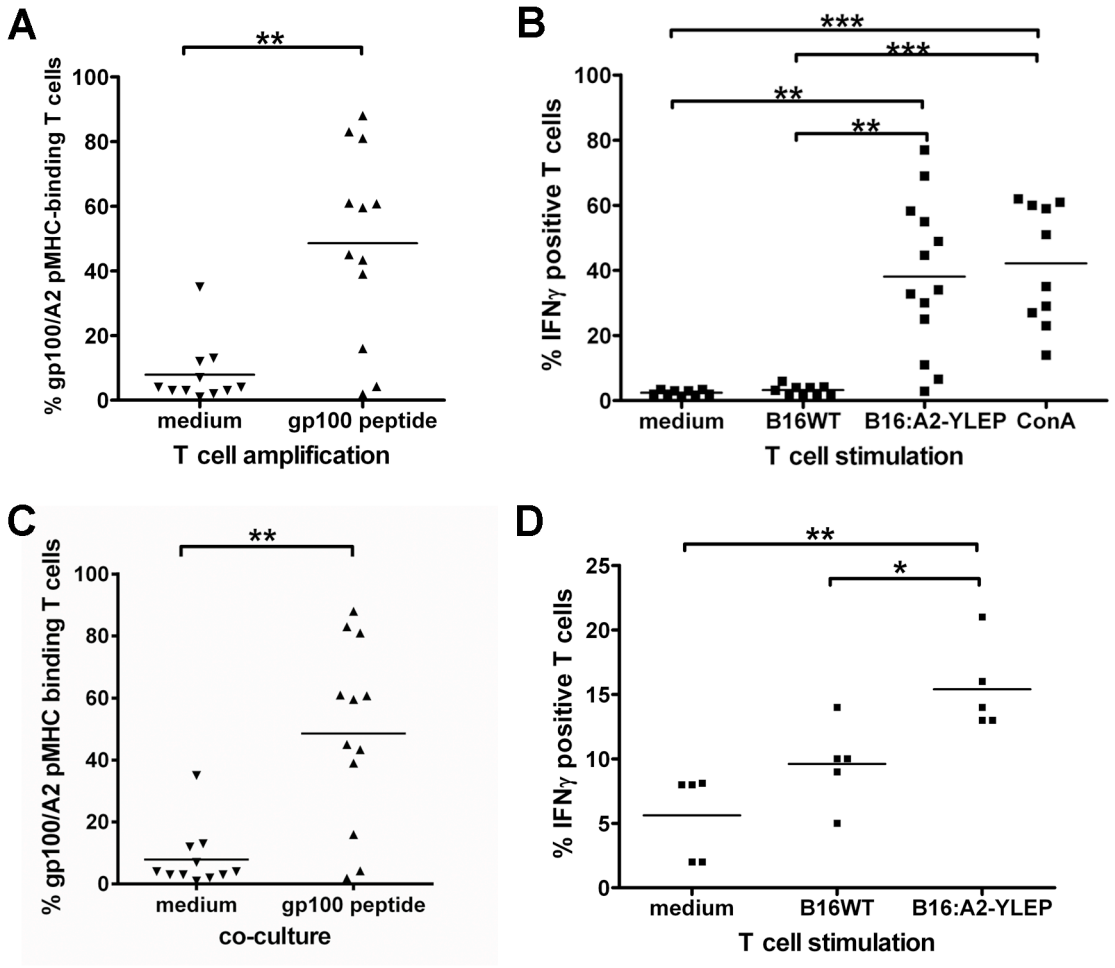

Figure S7. T cell responses in cured mice are not directed against tumor antigens other than the target antigen

(**A-D**) B16:A2-YLEP tumor bearing mice were treated as described in the legend to Figure 1A. (**A**) Splenocytes from cured mice were cultured in the presence or absence of gp100 peptide for 5-7 days and analyzed for the % of gp100/A2 pMHC-binding T cells by flow cytometry. (**B**) Splenocytes amplified in the presence of gp100 peptide were stimulated *in vitro* with B16WT or B16:A2-YLEP cells for 20h and analyzed for IFNγ production by flow cytometry. Stimulations with medium and Concanavalin A were used as negative and positive controls, respectively. (**C**) Splenocytes from cured mice were also amplified in the presence of B16:A2-YLEP cells for 7 days and analyzed for the % of gp100/A2 pMHC-binding T cells by flow cytometry. (**D**) Splenocytes amplified in the presence of B16:A2-YLEP cells were also stimulated *in vitro* with B16WT or B16-A2-YLEP cells for 20h and analyzed

for IFNγ production by flow cytometry. IFNγ responses were defined positive in case T cells demonstrated at least 10% binding of gp100/A2 pMHC and a % that stained positive for IFNγ that was at least 2.5 fold higher compared to medium-stimulated T cells. Concanavalin A responses ranged between 35 and 60% IFNγ–positive T cells (not shown). Each dot represents an individual mouse with lines indicating mean % pMHC binding or IFNγ staining. Statistical significances were calculated with Student's t-tests: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

Figure S8

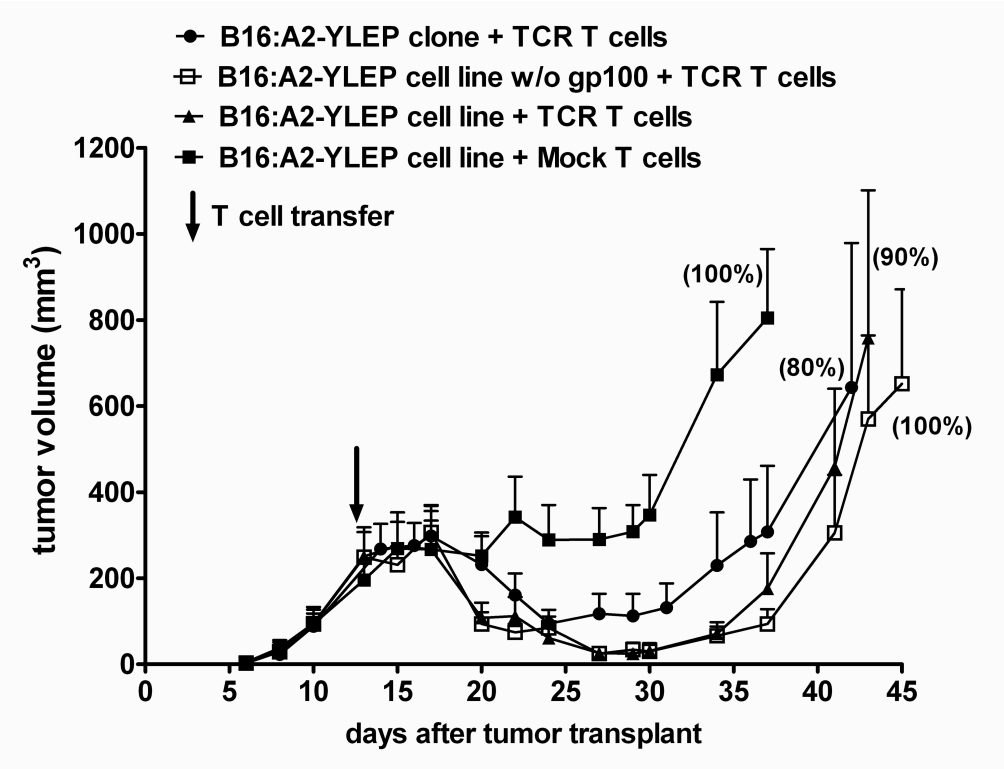

Figure S8. Frequency and kinetics of tumor relapse are neither affected by presence of antigen-negative tumor cells nor cross-presentation of gp100 antigen

HLA-A2 tg mice were transplanted either with a B16:A2-YLEP cell line that expresses wild type gp100 protein (B16:A2-YLEP) or a B16:A2-YLEP cell line or clone that does not express wild type gp100 protein (B16:A2-YLEP w/o gp100 or B16:A2-YLEP clone, respectively). Once tumors were established, mice were conditioned and received $20x10^6$ TCR or Mock T cells (see legend to Figure 1A). Tumor sizes were measured 3 times a week with a caliper. Data are expressed as mean mm^3 ± SEM, n=8, and % of mice with tumor relapse are indicated in parentheses.

Figure S9

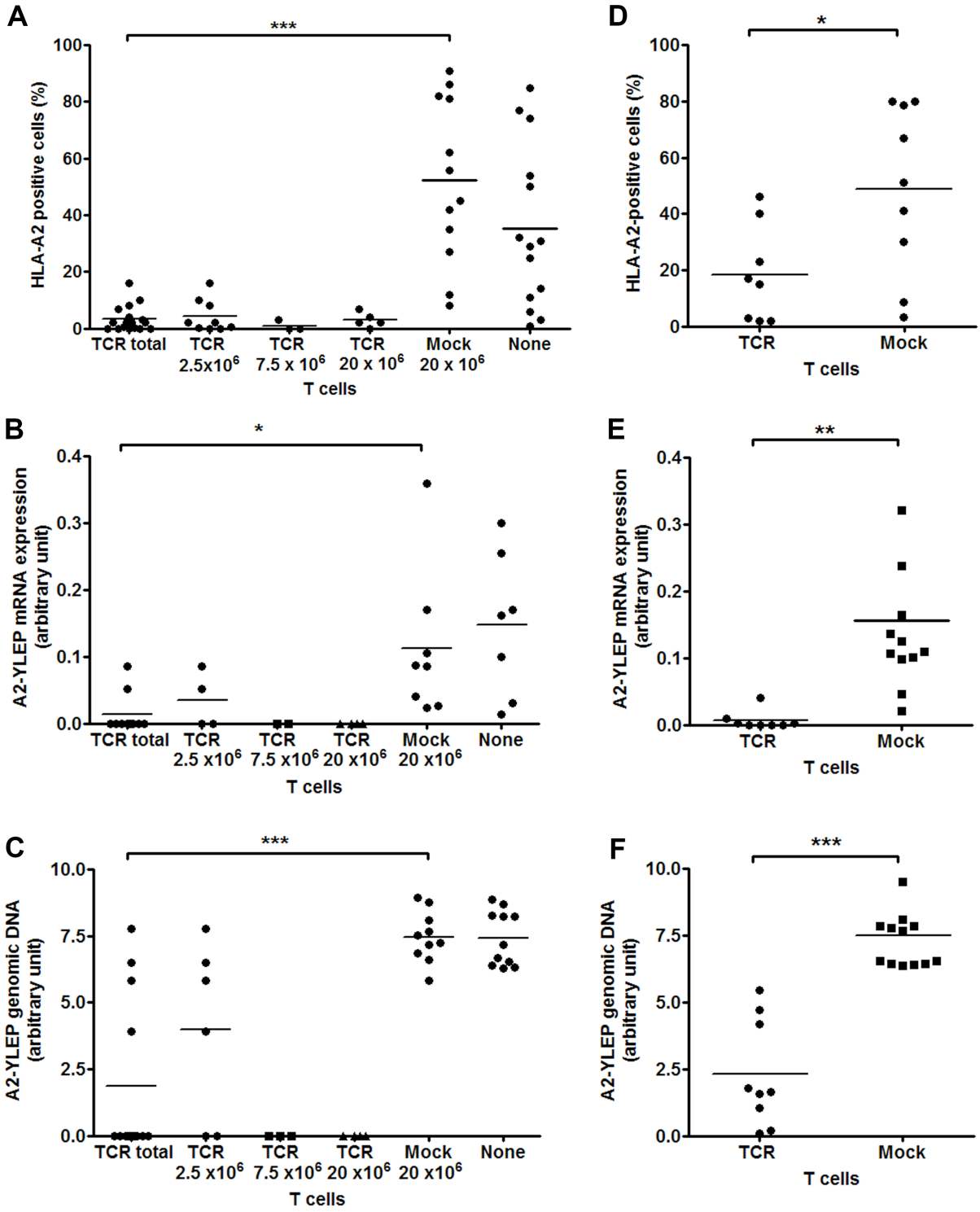
A
***
HLA-A2 positive cells (%)
100
80
60
40
20
0
TCR total
TCR 2.5x10^6
TCR 7.5 x 10^6
TCR 20 x 10^6
Mock 20 x 10^6
None
T cells
D
*
HLA-A2-positive cells (%)
TCR
Mock
T cells
B
*
A2-YLEP mRNA expression (arbitrary unit)
0.4
0.3
0.2
0.1
0.0
TCR total
TCR 2.5 x10^6
TCR 7.5 x10^6
TCR 20 x10^6
Mock 20 x10^6
None
T cells
E
**
A2-YLEP mRNA expression (arbitrary unit)
TCR
Mock
T cells
C
***
A2-YLEP genomic DNA (arbitrary unit)
10.0
7.5
5.0
2.5
0.0
TCR total
TCR 2.5 x10^6
TCR 7.5 x10^6
TCR 20 x10^6
Mock 20 x10^6
None
T cells
F
***
A2-YLEP genomic DNA (arbitrary unit)
TCR
Mock
T cells

Figure S9. TCR T cells select for tumors that lack antigen DNA

HLA-A2 tg mice were conditioned, transplanted with tumor cells and treated with T cells as described in the legend to Figure S3A (preventive model: **A-C**) or Figure 1A (curative model: **D-F**). Tumors were either treated with TCR T cells (analyzed as a total group or per individual dosis), Mock T cells or not treated. Under conditions where tumor diameters ≥ 20 mm, mice were sacrificed, tumors were isolated and single cell suspensions were prepared. (**A** and **D**) HLA-A2 surface expression was measured by flow cytometry and data are presented as % positive cells in viable gate. Horizontal lines represent mean % in each treatment group. Levels of (**B** and **E**) mRNA and (**C** and **F**) genomic DNA were measured by quantitative PCR and data are presented relative to the expression level of the endogenous reference gene TRP2, with lines representing mean levels of mRNA or DNA in each treatment group. Statistical significances were calculated with Student's t-tests: *$p<0.05$, **$p<0.005$, and ***$p<0.0001$. Note that individual points do not necessarily reflect identical mice and TCR and Mock T cell-treated mice should be analyzed as a group rather than individually.

Figure S10

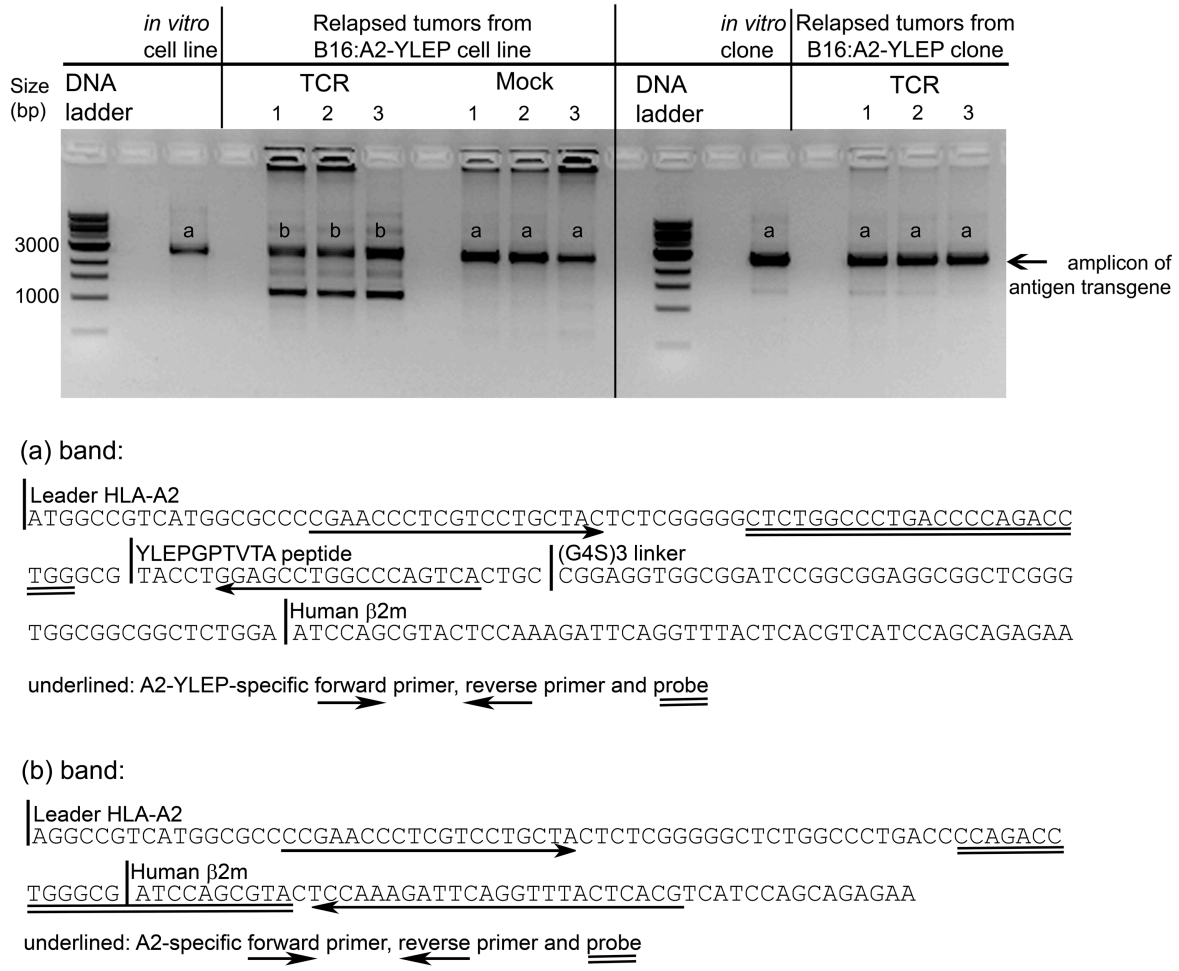

Figure S10. Antigen-negative cells in relapsed tumors originate from antigen-negative cells already present in pre-treatment tumors

Relapsed tumors were derived from either B16:A2-YLEP cell lines or clones and used to assess antigen gene levels. PCR was performed to amplify retrovirally introduced A2-YLEP DNA using primers specific for pLXSN flanking sequences (see *Supplementary text*). Upper part: products were subjected to gel electrophoresis and dominant bands (termed: (a) and (b) bands, indicated by an arrow) were sequenced. The (b) band, present in relapsed tumors derived from B16:A2-YLEP cell line contained HLA-A2 (A2) without the YLEPGPVTA peptide and $(G_4S)_3$ linker DNA (i.e., 1448 bp A2 +1329 bp retroviral sequence = 2777 bp), whereas the (a) band, present in relapsed tumors derived from B16:A2-YLEP clone contained A2-YLEP DNA (i.e., 1523 bp A2-YLEP + 1329 bp retroviral sequence = 2852 bp). Tumors

treated with Mock T cells or *in vitro* cultures of B16:A2-YLEP cell line or clone, all contained the (a) band with A2-YLEP. Note that the lower bands, only present in relapsed tumors derived from B16:A2-YLEP cell line, were non-specific and did not contain A2 sequences. Shown are 3 representative samples of cell line-derived tumors treated with TCR (n=9) and Mock T cells (n=12), and clone-derived tumors treated with TCR T cells (n=3). Lower part: complete sequences of (a) and (b) bands indicating the position of leader, peptide, linker and β2m domains, and primers/probes used to distinguish between A2-YLEP and A2 DNAs.

Figure S11

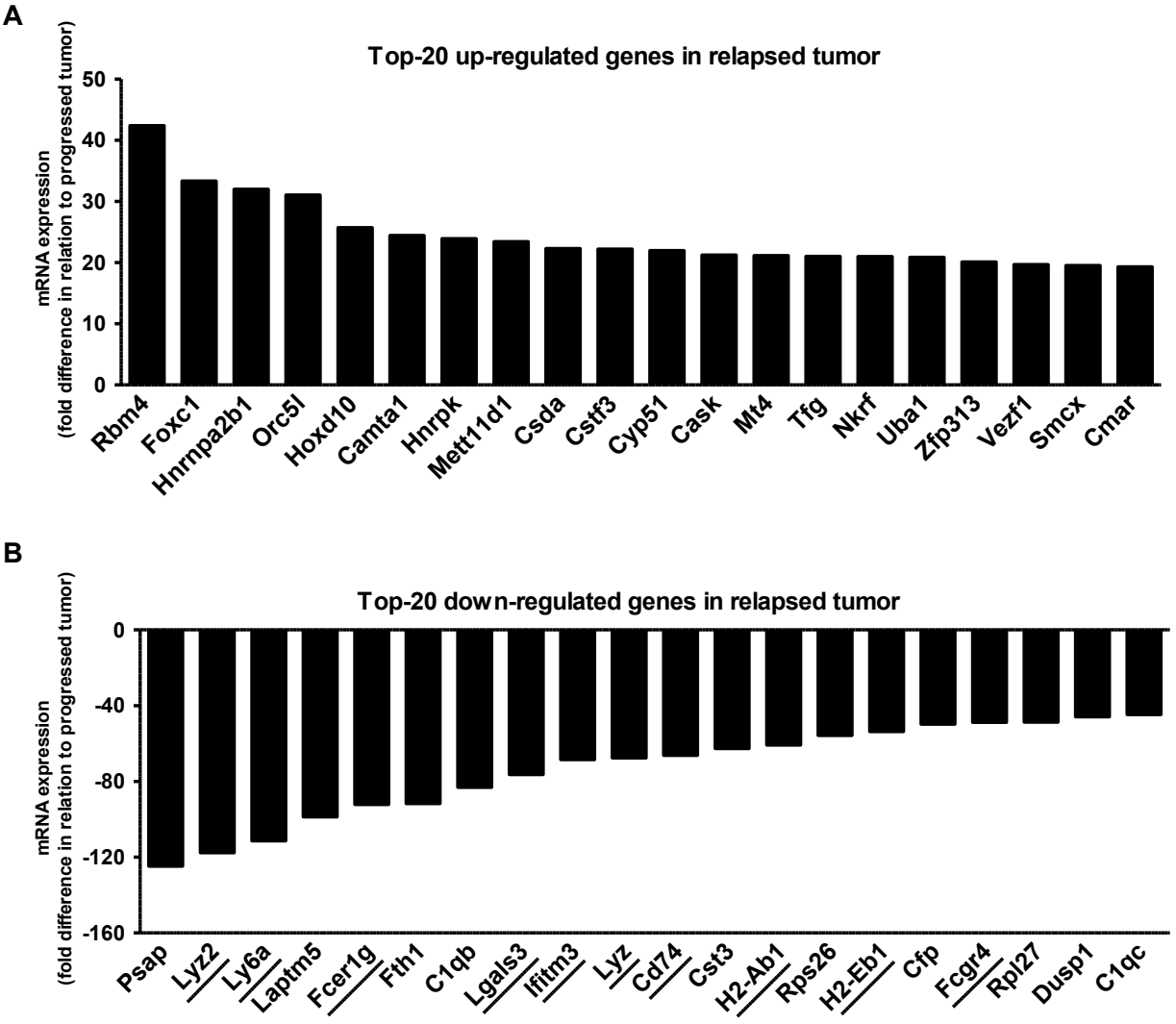

Figure S11. Ranking of genes that are most differentially expressed in relapsed tumors

Tumors were collected and used for RNA isolation, after which RNA was processed and analyzed for genome-wide expression analysis (see legend to Figure 5). A total set of 24,000 genes was analyzed for differential expression in relapsed tumors (see *Supplementary text* for details). Figures (**A**) and (**B**) present twenty genes that are ranked according the highest fold increase (up-regulated genes) or decrease (down-regulated genes) in expression in relapsed versus regressed tumors. Calculations were based on mean values, n=3, per tumor type (see legend to Figure 3A for a description of regressed and relapsed tumors). Genes are annotated according to www.genecards.org. Genes that are related to immunity according to gene ontogeny analyses are underlined. Statistical significances were calculated with Sign t-tests: * $p < 0.05$.

Figure S12

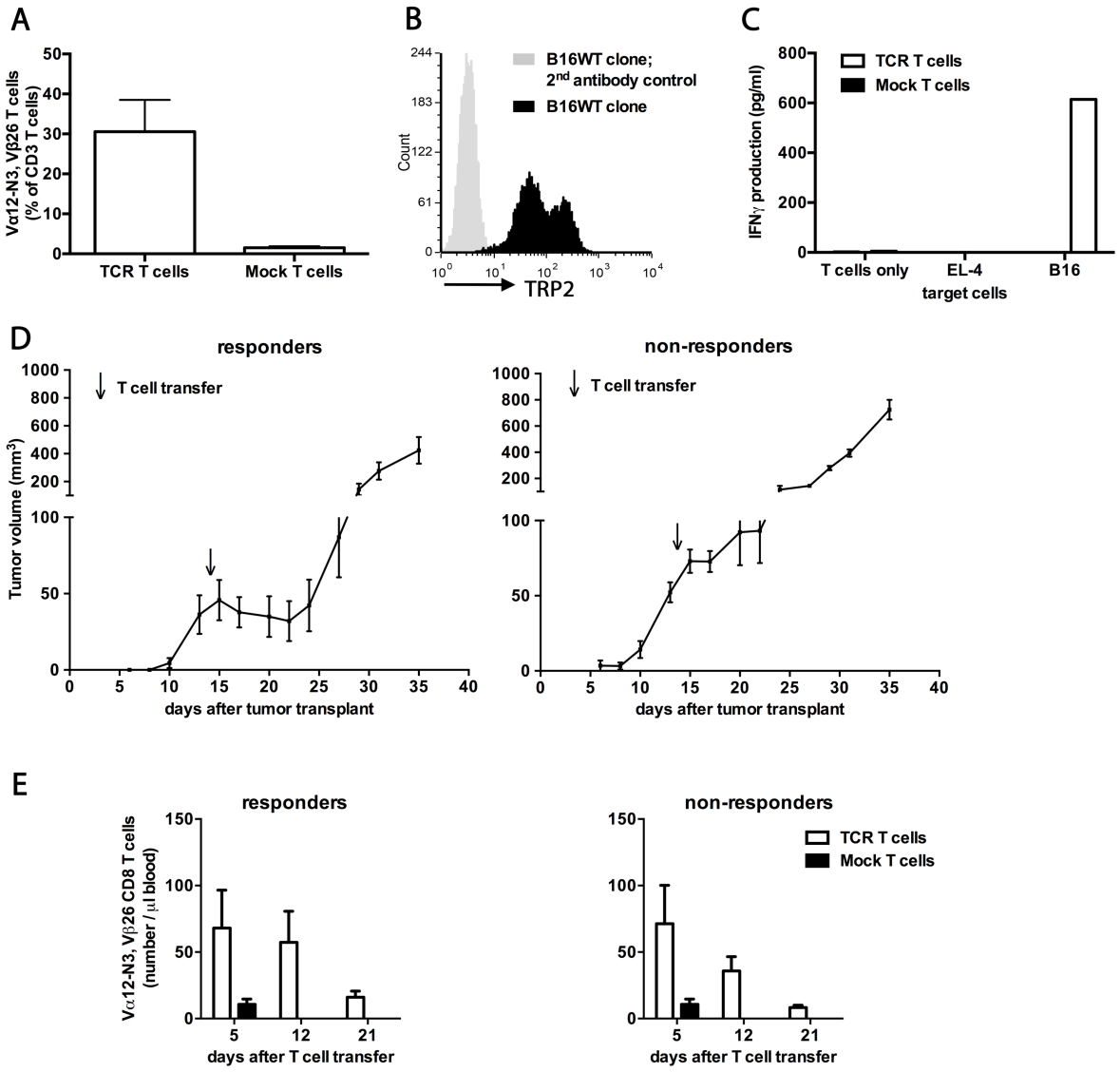

Figure S12. Validation of TRP2 TCR in vitro and in vivo

(**A**) Murine splenocytes from B6 WT mice were transduced with a TRP2/H2-K^b TCR. See *Supplementary text* for details on the construction of TCR vectors. At day 5 following T cell activation CD8 T cells were analyzed for TCR-Vα12N-3 and TCR-Vβ26 expression by flow cytometry. Data represent mean % ± SEM, n=3 separate transductions. (**B**) B16WT clone was stained with anti-TRP2 mAb followed by a PE-labeled anti-goat Ig (H+L) and analyzed by flow cytometry (histogram with solid line). B16WT cells stained with the secondary Ig only were used as a negative control (grey histogram). (**C**) TRP2/H2-K^b TCR-transduced splenocytes (from S12A) were incubated with B16WT and the TRP2 negative mouse tumor cell line EL4. Supernatants were harvested after 20h and analyzed for murine IFNγ. A

representative experiment out of 3 is shown and data are presented as pg/ml IFNγ (**D**) B6 mice bearing established B16WT tumors were treated as described in legend to Figure 7. Treatment with TCR T cells resulted in a decrease in tumor size of ≥ 30% relative to the day of T cell treatment in 70% of mice (responders, n=9), and less or no decrease in tumor size in 30% of mice (non-responders, n=4). Tumor sizes were measured and presented as described in legend to Figure 1A. (**E**) Peripheral blood was collected from mice in Figure S12D at the indicated time points and absolute numbers of TCR-Vα12N-3, TCR-Vβ26 CD8 T cells were determined by flow cytometry. Data are presented as mean numbers ± SEM.