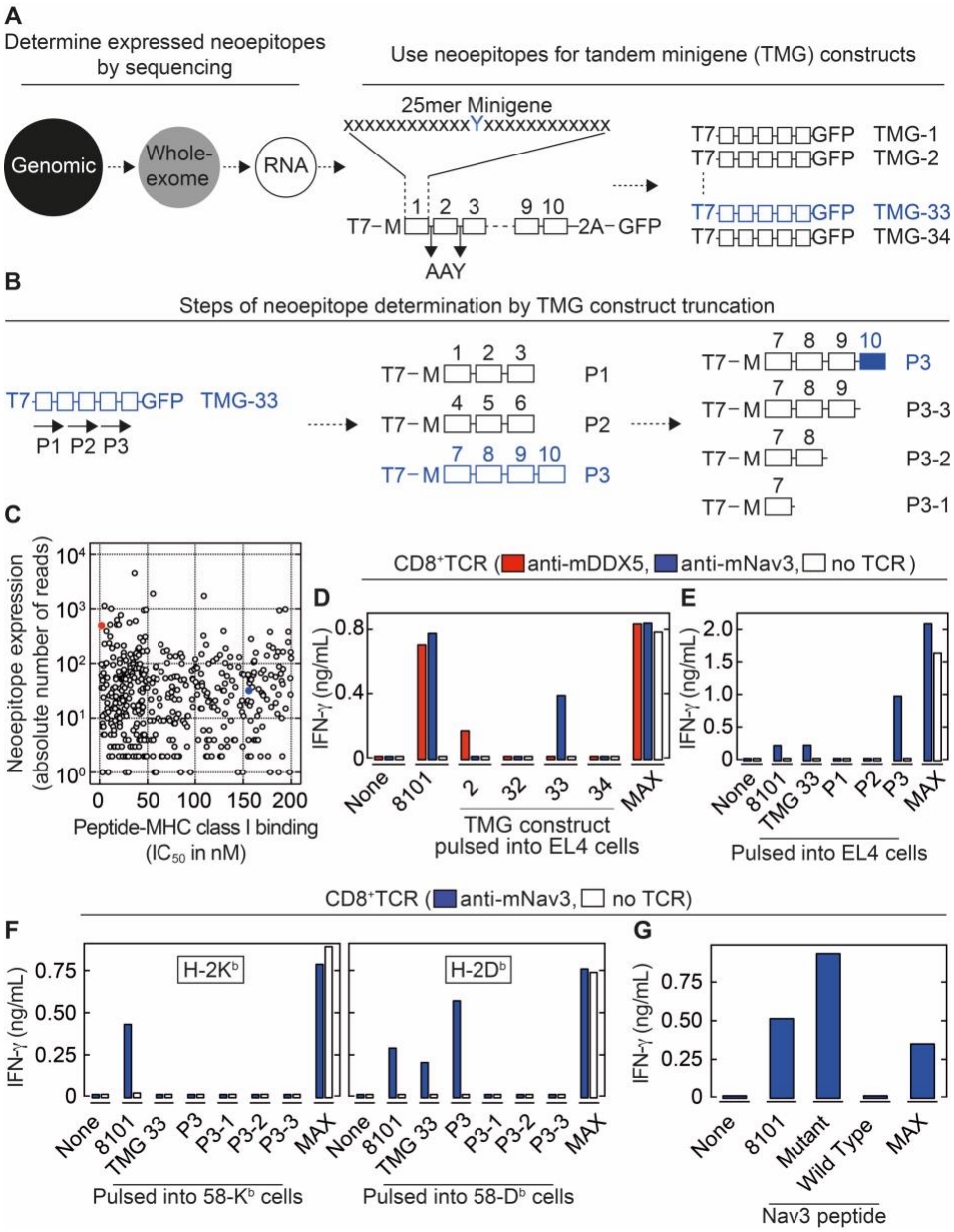
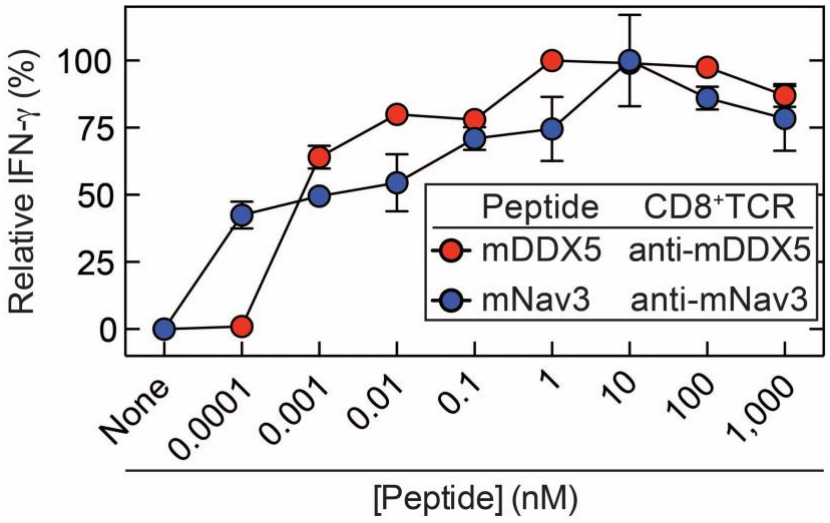
Supplementary Data

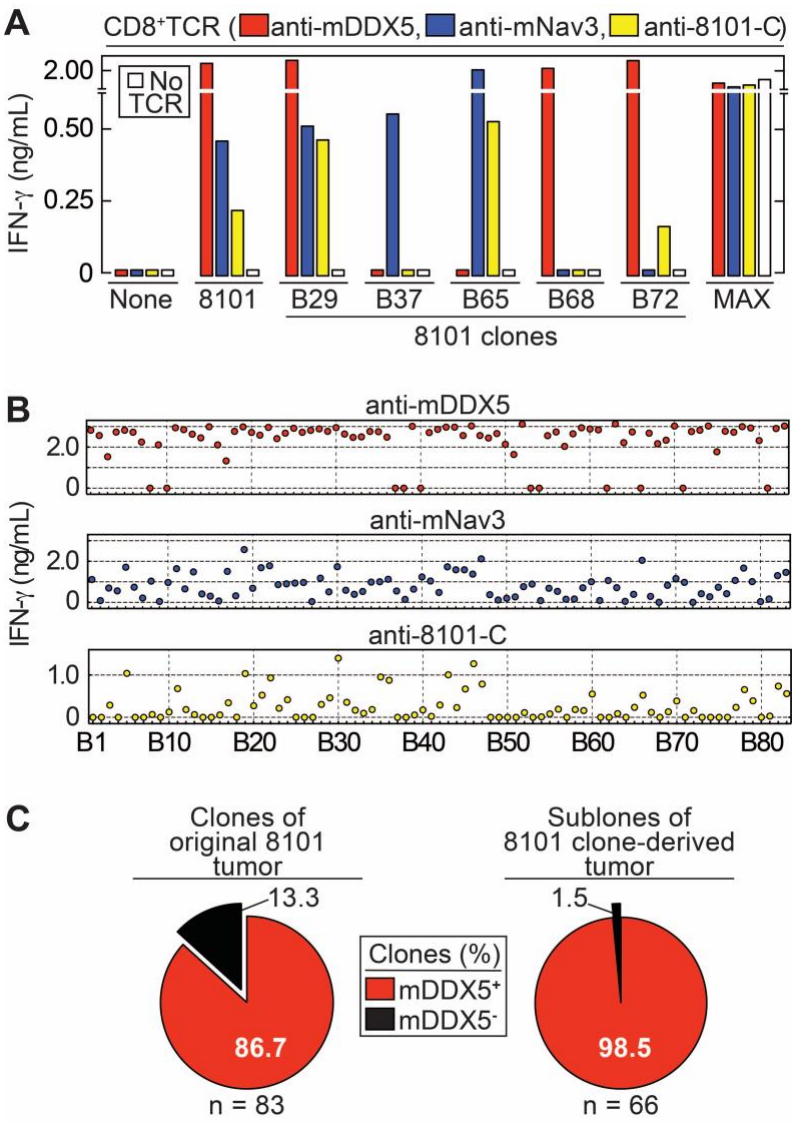


Supplementary Figure 1. Minigene approach to determine the mNav3 specific CD8⁺ T cell response. (A – B) Workflow to determine the genetic origin of an antigen specific T cell response. **(A, left)** Determination of expressed neoepitopes. The 8101 cancer cell line harbors 4,139 genomic mutations, 3,710 were detected in the exome and 1,207 can be detected on mRNA level and are potential 8101-specific neoepitope targets. Tandem minigene (TMG) construct design **(right)**. Tandem decamers of 25mer neoepitope sequences with the mutated amino acid at the center position, concatenated with an AAY proteasomal cleavage site, were linked via 2A with

GFP. Each TMG contained 10 different neoepitopes. **(B**) Neoepitope determination. TMG reactive construct is divided into sub-regions containing 3 to 4 minigenes. Smaller subregions are again tested for T cell stimulation and further truncated to determine the genetic origin. **(C**) 444 neoepitopes with a binding affinity of ≤ 200 nM to either H-2K^{b} or H-2D^{b} were chosen for T cell analysis and 45 TMG-constructs were generated. The mDDX5 (red) and mNav3 (blue) neoepitopes are highlighted. **(D – G**) Spleen cells from C57BL/6 mice were used as CD8^{+} T cell source and were TCR-engineered with indicated CD8^{+}TCRs. Co-cultures were performed for 24 h and supernatants were analyzed for IFN-γ concentrations by ELISA. For TCR-independent T cell stimulation ionomycin and phorbol myristate acetate (PMA) were used (MAX). Data are means of duplicates ± SD and show one representative out of 2 independent experiments. **(D)** EL4 cells were pulsed with multiple different TMGs and used for T cell stimulation. Indicated TMG-2 construct contained mDDX5 as positive control. **(E**) TMG-33 was truncated and the P3 subregion gave specific T cell stimulation. **(F)** P3 subregion was further reduced and only stimulated T cells when minigene position 10 was preserved and presented by H-2D^{b} expressing 58$^{-/-}$ cells. **(G**) Mutant but not wild type Neuron Navigator 3 (Nav3) peptide loaded onto spleen cells from C57BL/6 mice resulted in a specific T cell response. *In vitro* cultured 8101 was used as control.

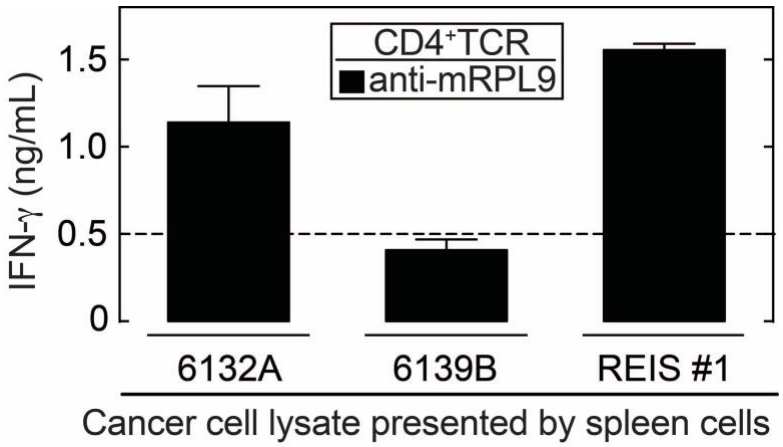


Supplementary Figure 2. The CD8$^+$TCRs specific for either mDDX5 or mNav3 have high avidity. TCR-engineered CD8$^+$ T cells expressing indicated TCRs were incubated with H-2K^b and H-2D^b positive EL4 cells together with graded amounts of either mDDX5 or mNav3 peptide. Supernatants were analyzed by IFN-γ ELISA. Data are means of duplicates ± SD. Shown is one out of two independent experiments.

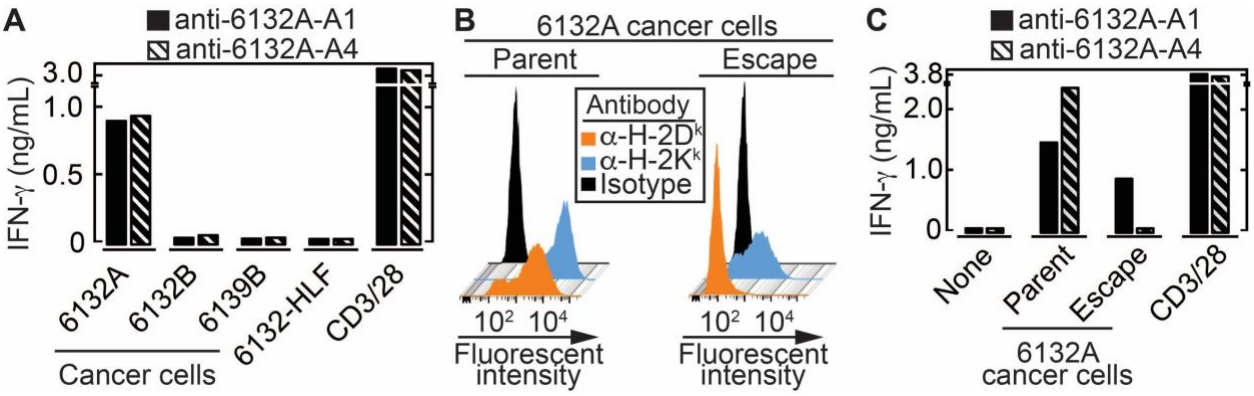


Supplementary Figure 3. 8101 specific CD8$^+$TCRs recognize independent cancer-specific antigens shown by distinct recognition patterns of 8101 cancer cell clones. (A – B) Co-cultures were performed for 24 h and supernatants were analyzed for IFN-γ concentrations by ELISA. Spleen cells from C57BL/6 mice were used as CD8$^+$ T cell source and were engineered with indicated CD8$^+$TCRs. 8101 cancer cell clones from the "B series" (see Material and Methods) were used for T cell stimulation. **(A)** Represented is the recognition pattern of CD8$^+$TCR-engineered T cells against exemplary chosen 8101 clones and the original 8101 cancer cell line used in **Figure 1**. For TCR-independent T cell stimulation ionomycin and PMA were used (MAX). Data are means of duplicates and shown is one representative out of 2 independent experiments. **(B)** Heterogeneity of all 83 cancer cell clones used for T cell stimulation. Shown is one

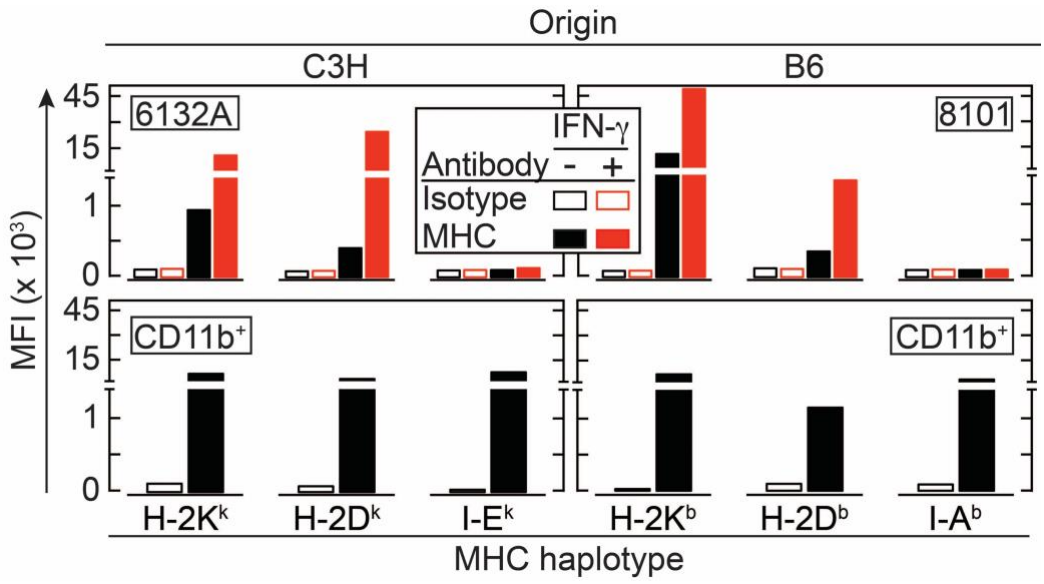
representative out of two independent experiments. For comparison, recognition of the clones by anti-mDDX5 as previously published (22) were used to visualize recognition and stimulation differences. **(C)** Clone A12 was grown in B6 $Rag^{-/-}$ mice for 25 days before reisolated. After adaption in culture new subclones were made. Shown is the percentage of mDDX5 positive clones generated either from the 8101 “B series” (n = 83 clones) or from the reisolated A12-tumor derived cancer cell line #1 (n = 66 clones) which was determined by recognition of anti-mDDX5 T cells.



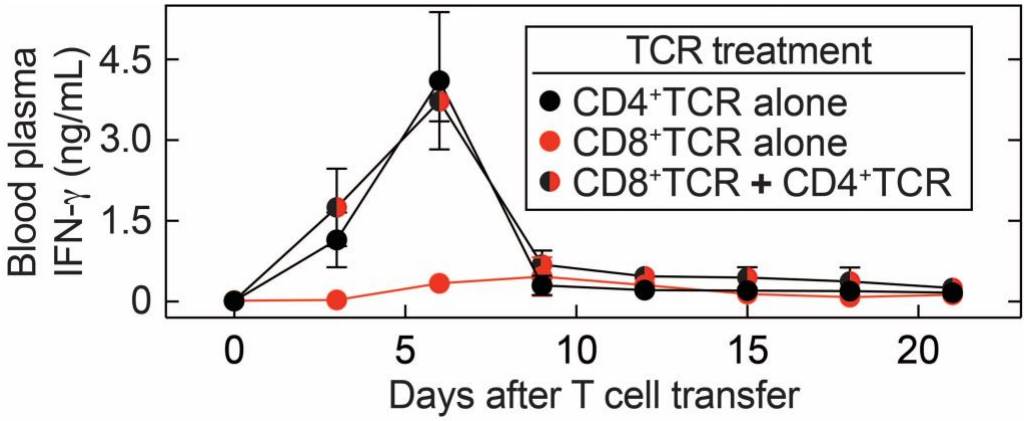
Supplementary Figure 4. Reisolated 6132A cancer cells retain the mRPL9 antigen after relapse. Spleen cells from C3H $CD8^{-/-}$ were used as $CD4^+$ T cell source for TCR-engineering. Anti-mRPL9 TCR-engineered $CD4^+$ T cells were co-cultured for 24 h with C3H/HeN spleen cells and indicated cancer cell lysates. Supernatants were analyzed for IFN-γ concentrations by ELISA. Data are means of duplicates ± SD. Examined was a 6132A tumor that was readapted *in vitro* after relapse of anti-mRPL9 $CD4^+$TCR-therapy.



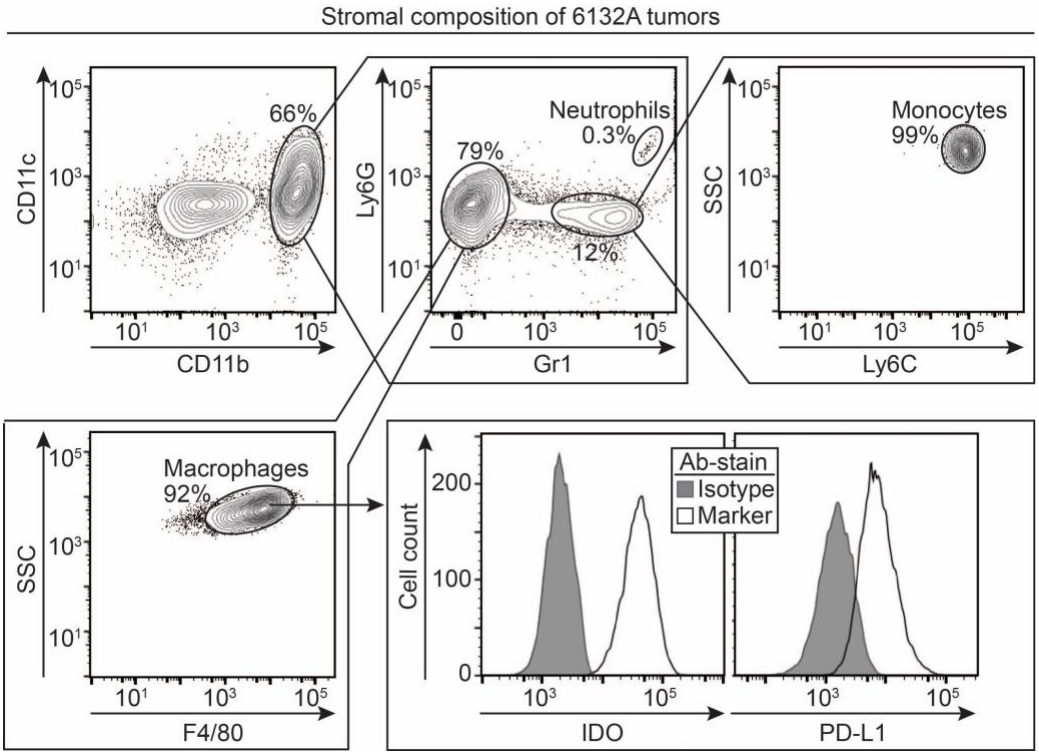
Supplementary Figure 5. 6132A variant that escaped from anti-6132A-A4 CD8$^+$TCR has lost H-2D^k expression. **(A – C)** Co-cultures were performed for 24 h and supernatants were analyzed for IFN-γ concentrations by ELISA. Spleen cells from C3H CD4$^{-/-}$ mice were used as CD8$^+$ T cell source and were TCR-engineered with indicated CD8$^+$TCRs. For TCR-independent T cell stimulation CD3- and CD28-specific antibodies were used. Data are means of duplicates and shown is one representative out of 3 independent experiments. **(A)** CD8$^+$TCR-T cells were used for co-cultures with indicated *in vitro*-cultured cancer cell lines or HLF. **(B)** MHC class I stain of the parental 6132A cancer cell line was compared to the anti-6132A-A4 CD8$^+$TCR escape variant which lacks H-2D^k expression. **(C)** *In vitro*-cultured parental 6132A and the escape cancer cell lines were used for CD8$^+$ T cell stimulation.



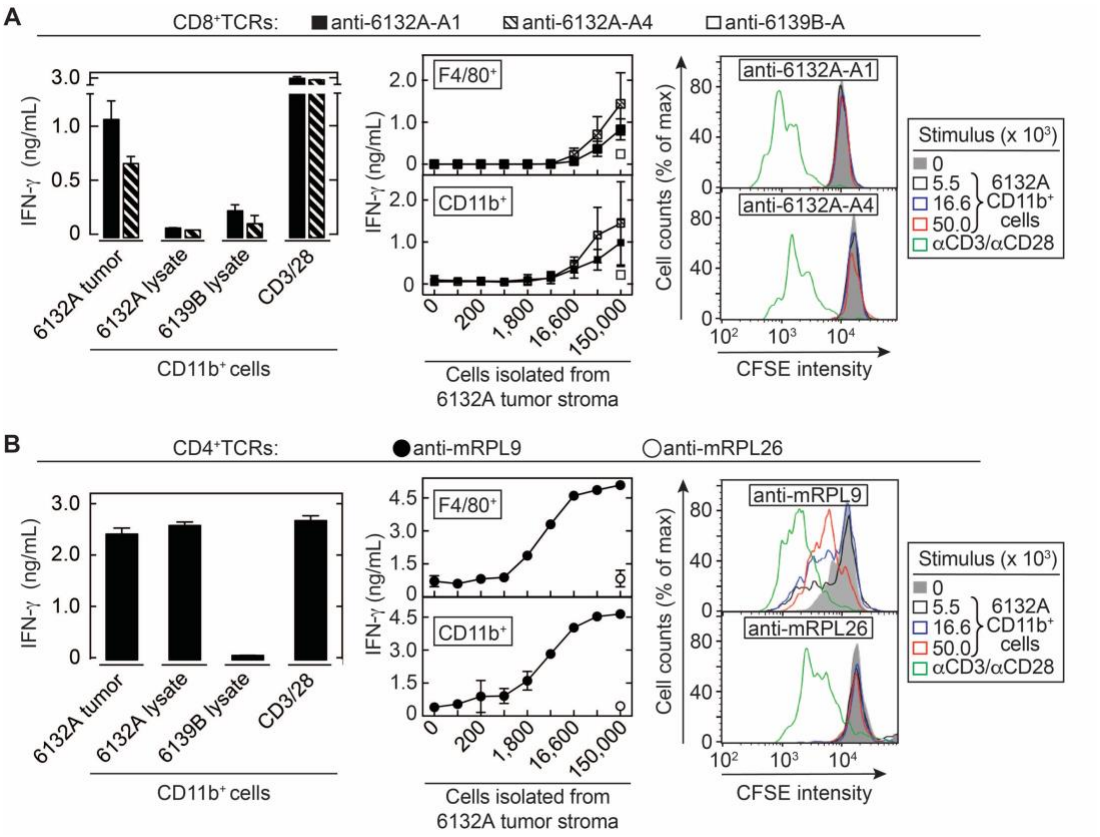
Supplementary Figure 6. Upregulation of MHC class I but not class II after exposure to IFN-γ *in vitro* by 6132A and 8101 cancer cells. 6132A and 8101 cancer cells were cultured for 48 h with or without IFN-γ before MHC class I and II surface expression was analyzed by flow cytometry. $CD11b^+$ cells from peripheral blood from C3H $Rag^{-/-}$ and B6 $Rag^{-/-}$ mice were also analyzed for MHC class I and II molecules. Isotype antibodies were used as controls. Shown is one representative out of two independent experiments.



Supplementary Figure 7. CD4$^+$TCR-engineered T cells are the main IFN-γ producer *in vivo*. IFN-γ concentrations in blood plasma samples collected every 3 days from 6132A tumor-bearing C3H Rag$^{-/-}$ mice after start of T cell transfer were analyzed by flow cytometry. Evaluated were mice which received either CD8$^+$TCR only (n = 3), CD4$^+$TCR only (n = 4) or a combination of both (n = 3). C3H CD4$^{-/-}$ and C3H CD8$^{-/-}$ mice were used as CD8$^+$ and CD4$^+$ T cell source and engineered with either the anti-6132A-A1 or anti-6132A-A4 CD8$^+$TCR or with the anti-mRPL9 CD4$^+$TCR. Data are means ± SD and are summarized from two independent experiments.



Supplementary Figure 8. 6132A-tumor stroma consists mainly of immunosuppressive macrophages. For an unbiased representation, 6132A-tumors grown in C3H Rag$^{-/-}$ mice were minced and digested. Tumor cell suspensions were analyzed by flow cytometry. The majority of cancer cells were excluded by SSC/FSC gating and the remaining whole myeloid population was analyzed for life populations of CD11c, CD11b, F4/80, Gr1, Ly6G and Ly6C. Tumor-associated macrophages were identified as CD11b^{+} and F4/80^{+} and were characterized by stainings of IDO and PD-L1. Shown is one representative out of 3 independently analyzed tumors.



Supplementary Figure 9. CD8$^+$TCR-transduced T cells recognize cross-presented antigen only on tumor-derived APCs. (A – B) Spleen cells from C3H CD4$^{-/-}$ or C3H CD8$^{-/-}$ mice were used as CD8$^+$ or CD4$^+$ T cell source for TCR-engineering. TCR-engineered CD8$^+$ or CD4$^+$ T cells were co-cultured for 24 h. Supernatants were analyzed for IFN-γ concentrations by ELISA. Data are means of duplicates ± SD and shown is one representative out of at least 2 independent experiments. **(left)** T cells were used for co-cultures with CD11b$^+$ cells isolated from 6132A tumors grown in C3H Rag$^{-/-}$ mice or isolated from the spleen of C3H/HeN wild type mice loaded with cancer cell lysates. For TCR-independent T cell stimulation CD3- and CD28-specific antibodies were used. **(middle)** Serial dilution of either tumor-associated macrophages (F4/80$^+$) or CD11b$^+$ cells were used for stimulation. **(right)** Indicated CFSE-labeled T cells were cocultured for 72 h with CD11b$^+$ cells isolated from 6132A tumors grown in C3H Rag$^{-/-}$ mice. Shown are one out of two independent experiments**. (A)** anti-6132A CD8$^+$TCR-T cells were used. Populations positive for CD3$^+$, CD8$^+$ and Vβ6$^+$ (anti-6132A-A1) or Vβ8.3$^+$ (anti-6132A-A4) were analyzed by flow cytometry for CFSE signal. **(B)** anti-mRPL9 CD4$^+$TCR-T cells were used. Populations positive for CD3$^+$, CD4$^+$ and Vβ6$^+$ (anti-mRPL9) or Vβ2$^+$ (control) were analyzed by flow cytometry for CFSE signal. Shown is one out of two independent experiments.

Supplementary Video S1. 6132A cancer cells disappear when incubated with 6132A-specific CD4$^+$ and CD8$^+$ T cells. *In vitro* time-lapse imaging of the four cell types 6132A cancer cells (cerulean), CD11b$^+$ cells (DiD), anti-mRPL9 CD4$^+$ T cells (GFP), anti-6132A-A1 CD8$^+$ T cells (mCherry)). Positions were imaged every 90 seconds for 5.5 h. All four 2 x 2 positions are shown in sequence from 0 h to 23 h. One out of two independent experiments is shown. See Material and Methods for details.

Supplementary Video S2. 6132A cancer cells proliferate when incubated with 6132A-specific CD8$^+$ T cells and non-specific CD4$^+$ T cells. *In vitro* time-lapse imaging of the four cell types (6132A (cerulean), CD11b$^+$ cells (DiD), anti-mRPL26 CD4$^+$ T cells (GFP), anti-6132A-A1 CD8$^+$ T cells (mCherry)). Positions were imaged every 90 seconds for 5.5 h. All four 2 x 2 positions are shown in sequence from 0 h to 23 h. One out of two independent experiments is shown. See Material and Methods for details.

Supplementary Table S1. Comparison of the mean counts of cell-to-cell interactions of 6132A cancer cells with CD8$^+$ T cells, CD4$^+$ T cells and CD11b$^+$ cells in the antigen-specific and the antigen-non-specific settings.

Cancer cells in cell-to-cell interactions with	Mean count of specific (SD)[a]	Mean count of non-specific (SD)[a]	Difference of means (SD)	*t*-Statistic[b]	*p*-Value	Significance
CD8$^+$	15.634 (7.17)	16.296 (8.35)	-0.662 (8.32)	0.275	0.794	ns
CD8$^+$ and CD11b$^+$	9.382 (5.64)	6.751 (2.91)	2.632 (3.99)	2.228	0.0764	ns
CD8$^+$, CD4$^+$ and CD11b$^+$	17.056 (8.66)	5.517 (4.82)	11.539 (3.55)	5.861	0.00205	**

[a] Summarized from two independent experiments

[b] All *t*-tests conducted with 3 degrees of freedom

Supplementary Table S2. TCR CDR3 sequences generated from 8101 and 6132A cancer cell line specific $CD8^+$ T cell clones.

$CD8^+$TCR	α-Chain	β-Chain
anti-mNav3	TRAV5D - CAASGTGGYKVVF - TRAJ12	TRBV13 - CASGAGQGPEQFF - TRBJ2
anti-8101-C	TRAV21 - CILRVAQGTGSKLSF - TRAJ58	TRBV2 - CASSQDRGFSNERLFF - TRBJ1
anti-6132A-A1	TRAV3D - CAVSNDSGYNKLTF - TRAJ11	TRBV19 - CASTPTGIQDTQYF - TRBJ2
anti-6132A-A4	TRAV9D - CALSAINTGNYKYVF - TRAJ40	TRBV13 - CASSPDWGGFAEQFF - TRBJ2
anti-6139B-A	TRAV3 - CAVSNTDKVVF - TRAJ34	TRBV13 - CASMLGGRFEQYF - TRBJ2

Supplementary Table S3. TCR CDR3 sequences isolated from 6132A and 6139B cancer cell line specific $CD4^+$ T cell hybridomas.

$CD4^+$TCR	α-Chain	β-Chain
anti-mRPL9	TRAV4 - CAAGYGGSGNKLIF - TRAJ32	TRBV19 - CASSIGTGGNERLFF - TRBJ1
anti-mRPL26	TRAV13 - CAMVTGANTGKLTF - TRAJ52	TRBV1 - CTCSAHNNQAPLF - TRBJ5