



Supplementary Materials for

Cancer Immunotherapy Based on Mutation-Specific CD4⁺ T Cells in a Patient with Epithelial Cancer

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Materials and Methods

Whole-exomic sequencing

Whole-exomic sequencing of cryopreserved tumor tissue (embedded in OCT) and normal peripheral blood cells was performed by Personal Genome Diagnostics (PGDx, Baltimore, MD) as previously described (27). The average number of distinct high quality sequence reads at each base was 155 and 160 for tumor and normal (PBMC) DNA, respectively.

Patient treatment and generation of TIL for adoptive cell therapy

Patient 3737 was enrolled in the institutional-review board (IRB)-approved protocol: "A Phase II Study Using Short-Term Cultured, Autologous Tumor-Infiltrating Lymphocytes Following a Lymphocyte Depleting Regimen in Metastatic Digestive Tract Cancers" (ClinicalTrials.gov number: NCT01174121), which was designed to evaluate the safety and effectiveness of the adoptive transfer of autologous, ex vivo expanded tumor-infiltrating lymphocytes (TIL) in patients with gastrointestinal cancers.

TIL used for patient's first treatment was generated as previously described (28). Briefly, resected tumors were minced into approximately 1-2 mm fragments and individual fragments were placed in wells of a 24-well plate containing 2 ml of complete media (CM) containing high dose IL-2 (6000 IU/ml, Chiron). CM consisted of RPMI supplemented with 10% in-house human serum, 2 mM L-glutamine, 25 mM HEPES and 10 µg/ml gentamicin. Additionally, a mixed tumor digest was also cultured in CM with high dose IL-2. After the initial outgrowth of T cells (between 2-3 weeks), 5e6 T cells from select cultures were rapidly expanded in gas-permeable G-Rex100 flasks using irradiated allogeneic PBMC at a ratio of 1 to 100 in 400 ml of 50/50 medium, supplemented with 5% human AB serum, 3000 IU/ml of IL-2, and 30 ng/ml of OKT3 antibody (Miltenyi Biotec). 50/50 media consisted of a 1 to 1 mixture of CM with AIM-V media. All cells were cultured at 37°C with 5% CO₂. The cells were rapidly expanded for two weeks prior to infusion. Patient 3737 underwent a non-myeloablative lymphodepleting regimen consisting of cyclophosphamide and fludarabine prior to receiving 42.4 billion total T cells in conjunction with four doses of high dose IL-2 (see Fig. S4 for treatment scheme and details).

TIL used for the patient's second treatment was generated in a similar manner as the first treatment with the following changes. The first treatment product (3737-TIL) was composed of a combination of 5 individual TIL cultures. These 5 cultures were individually assessed for expression of CD4 and Vβ22, and reactivity against mutated ERBB2IP, and one culture was found to be highly enriched in Vβ22+ ERBB2IP-mutation-reactive CD4+ T cells (data not shown). This one TIL culture (after the initial outgrowth with high dose IL-2) was then rapidly expanded as described above. The patient underwent an identical non-myeloablative lymphodepleting regimen as the first treatment prior to receiving 126 billion total T cells in conjunction with four doses of high dose IL-2.

Generation of tandem mini-gene (TMG) constructs

The detailed methodology will be published elsewhere, but briefly, for each non-synonymous substitution mutation identified by whole exome sequencing, a "mini-gene" construct encoding the corresponding amino acid change flanked by 12 amino acids of the wild-type protein sequence was made. Multiple mini-genes were genetically fused together to generate a tandem mini-gene (TMG) construct. These mini-gene constructs were codon optimized and synthesized as DNA String constructs (Life Technologies). TMGs were then cloned into the pcDNA3.1/V5-His TOPO vector using In-Fusion technology (Clontech). Site-directed mutagenesis was used to generate the nine "wild-type reversion" TMG-1 constructs (Gene Oracle). The nucleotide sequence of all TMGs was verified by standard Sanger sequencing (Macrogen and Gene Oracle).

Generation of autologous antigen presenting cells (APCs)

Monocyte-derived, immature dendritic cells were generated using the plastic adherence method. Briefly, autologous pheresis samples were thawed, washed, set to 5-10e6 cells/ml with neat AIM-V media (Life Technologies) and then incubated at approximately 1e6 cells/cm² in an appropriate sized tissue culture flask and incubated at 37°C, 5% CO₂. After 90 min, non-adherent cells were collected, and the flasks were vigorously washed with AIM-V media, and then incubated with AIM-V media for another 60 min. The flasks were then vigorously washed again with AIM-V media and then the adherent cells were incubated with DC media. DC media comprised of RPMI containing 5% human serum (collected and processed in-house), 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 800 IU/ml GM-CSF and 800 U/ml IL-4 (media supplements were from Life Technologies and cytokines were from Peprotech). On day 3, fresh DC media was added to the cultures. Fresh or freeze/thawed DCs were used in experiments on day 5-7 after initial stimulation. In all experiments, flow cytometry was used to phenotype the cells for expression of CD11c, CD14, CD80, CD86, and HLA-DR (all from BD Bioscience) to ensure that the cells were predominantly immature DCs (CD11c⁺, CD14⁻, CD80^{low}, CD86⁺, and HLA-DR⁺; data not shown). Antigen presenting B cells were generated using the CD40L and IL-4 stimulation method. Briefly, human CD19-microbeads (Miltenyi Biotec) were used to positively select B cells from autologous pheresis samples. CD19⁺ cells were then cultured with irradiated (6000 rad) 3T3 cells stably expressing CD40L (3T3-CD40L) at approximately a 1:1 ratio in B-cell media. B-cell media comprised of IMDM media (Life Technologies) supplemented with 7.5-10% human serum (in-house), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies), 10 µg/ml gentamicin (CellGro), 2 mM L-glutamine (Life Technologies), and 200 U/ml IL-4 (Peprotech). Fresh B-cell media was added starting on day 3, and media added or replaced every 2-3 days thereafter. Additional irradiated 3T3-CD40L feeder cells were also added as required. Antigen presenting B cells were typically used in experiments 2-3 weeks after initial stimulation.

Generation of in vitro transcribed RNA (IVT) RNA

Plasmids encoding the tandem mini-genes were linearized with the restriction enzyme Sac II. A control pcDNA3.1/V5-His-TOPO vector encoding GFP was linearized with Not I. Restriction digests were terminated with EDTA, sodium acetate and ethanol precipitation. Complete plasmid digestion was verified by standard agarose gel

electrophoresis. Approximately 1 µg of linearized plasmid was used for the generation of IVT RNA using the mmessage mmachine T7 Ultra kit (Life Technologies) as directed by the manufacturer. RNA was precipitated using the LiCl₂ method, and RNA purity and concentrations were assessed using a NanoDrop spectrophotometer. RNA was then aliquoted into microtubes and stored at -80°C until use.

RNA transfections

APCs (DCs or B cells) were harvested, washed 1x with PBS, and then resuspended in Opti-MEM (Life Technologies) at 10-30e6 cells/ml. IVT RNA (4 µg or 8 µg) was aliquoted to the bottom of a 2 mm gap electroporation cuvette, and 50 µl or 100 µl of APCs were added directly to the cuvette. The final RNA concentration used in electroporations was thus 80 µg/ml. Electroporations were carried out using a BTX-830 square wave electroporator. DCs were electroporated with 150 V, 10 ms, and 1 pulse, and B cells were electroporated with 150 V, 20 ms, and 1 pulse. Transfection efficiencies using these settings were routinely between 70-90% as assessed with GFP RNA (data not shown). All steps were carried out at room temperature. Following electroporation, cells were immediately transferred to polypropylene tubes containing DC- or B-cell media supplemented with the appropriate cytokines. Transfected cells were incubated overnight (12-14 h) at 37°C, 5% CO₂. Cells were washed 1x with PBS prior to use in co-culture assays.

Peptide pulsing

Autologous B cells were harvested, washed, and then resuspended at 1e6 cells/ml in B-cell media supplemented with IL-4, and then incubated with 1 µg/ml of a 25-mer peptide overnight (12-14 h) at 37°C, 5% CO₂. After overnight pulsing, B cells were then washed 2x with PBS, and then resuspended in T-cell media and immediately used in co-culture assays. The peptides used were: mutated ERBB2IP (TSFLSINSKEETGHLNGNKYPNLE); wild-type ERBB2IP (TSFLSINSKEETGHLNGNKYPNLE); and, as a negative control, mutated ALK (RVLKGGSVRKLRHAKQLVLELGEEA). The mutated ERBB2IP peptide was purchased from three different sources (GenScript, Peptide 2.0, and SelleckChem) with all yielding the same in vitro results, while the wild-type ERBB2IP and mutated ALK peptides were purchased from Peptide 2.0. For culturing allogeneic EBV-B cells, RPMI media containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies), 10 µg/ml gentamicin (CellGro), and 2 mM L-glutamine was used instead of B-cell media.

T-cell sorting, expansion, and cloning

The BD FACS Aria IIu and BD FACS Jazz were used in all experiments requiring cell sorting. In indicated experiments, sorted T cells were expanded using excess irradiated (4000 rad) allogeneic feeder cells (pool of three different donor leukapheresis samples) in 50/50 media containing 30 ng/ml anti-CD3 antibody (OKT3) and 3000 IU/ml IL-2. Limiting dilution cloning was carried out in 96-well round bottom plates using the above stimulation conditions with 5e4 feeder cells per well and 1-2 T cells per well. Media was exchanged starting at approximately 1 week post stimulation and then every other day or as required. Cells were typically used in assays, or further expanded, at approximately 2-

3 weeks after the initial stimulation.

Co-culture assays: IFN- γ ELISPOT and ELISA, flow cytometry for cell surface activation markers, and intracellular cytokine staining (ICS)

When DCs were used as APCs, approximately 3.5×10^4 to 7×10^4 DCs were used per well of a 96-well flat or round-bottom plate. When B cells were used as APCs, approximately 2×10^5 cells were used per well of a 96-well round-bottom plate. In ELISPOT assays, 1×10^3 to 1×10^4 effector T cells were used per well, and in flow cytometry assays, 1×10^5 effector T cells were used per well. T cells were typically thawed and rested in IL-2 containing 50/50 media (3000 IU/ml IL-2) for two days and then washed with PBS (3x) prior to co-culture assays. All co-cultures were performed in the absence of exogenously added cytokines. For all assays, plate-bound OKT3 (0.1 μ g/ml or 1 μ g/ml) was used as a positive control. In experiments involving HLA blocking antibodies, the following antibodies were used: pan-class-II (clone: IVA12), pan-class-I (clone: W6/32), HLA-DR (clone: HB55), HLA-DP (clone: B7/21), and HLA-DQ (clone: SPV-L3). Cells were blocked with 20-50 μ g/ml of the indicated antibody for 1-2 h at 37°C, 5% CO₂ prior to co-culture with T cells. T4 are T cells that have been transduced with an HLA-DR4-restricted TCR that is reactive against an epitope in tyrosinase. DMF5 is an HLA-A2-restricted T-cell line reactive against MART-1. 624-CIITA is a HLA-A2 and HLA-DR4-positive melanoma cell line that stably expresses MHC-II due to ectopic expression of CIITA (class II, major histocompatibility complex, transactivator), and is positive for MART-1 and tyrosinase expression.

For IFN- γ ELISPOT assays, briefly, ELIIP plates (Millipore, MAIPSWU) were pre-treated with 50 μ l of 70% ethanol per well for 2 min, washed 3x with PBS, and then coated with 50 μ l of 10 μ g/ml IFN- γ capture antibody (Mabtech, clone: 1-D1K) and incubated overnight in the fridge. For OKT3 controls, wells were coated with a mixture of IFN- γ capture antibody (10 μ g/ml) and OKT3 (1 μ g/ml). Prior to co-culture, the plates were washed 3x with PBS, followed by blocking with 50/50 media for at least 1 h at room temperature (RT). After 20-24 h of co-culture, cells were flicked out of the plate, washed 6x with PBS + 0.05% Tween-20 (PBS-T), and then incubated for 2 h at RT with 100 μ l/well of a 0.22 μ m filtered 1 μ g/ml biotinylated anti-human IFN- γ detection antibody solution (Mabtech, clone: 7-B6-1). The plate was then washed 3x with PBS-T, followed by a 1 h incubation with 100 μ l/well of streptavidin-ALP (Mabtech, diluted 1:3000). The plate was then washed 6x with PBS followed by development with 100 μ l/well of 0.45 μ m filtered BCIP/NBT substrate solution (KPL, Inc.). The reaction was stopped by rinsing thoroughly with cold tap water. ELISPOT plates were scanned and counted using an ImmunoSpot plate reader and associated software (Cellular Technologies, Ltd).

Expression of the T-cell activation markers OX40 and 4-1BB was assessed by flow cytometry at approximately t=22-26h post-stimulation. Briefly, cells were pelleted, washed with FACS buffer (1X PBS supplemented with 1% FBS and 2 mM EDTA), and then stained with the appropriate antibodies for approximately 30 min, at 4°C in the dark. Cells were washed at least once with FACS buffer prior to acquisition on a BD FACSCanto II flow cytometer. All data were gated on live (PI negative), single cells.

Cytokine production was assessed using intracellular cytokine staining (ICS) and flow cytometry. Briefly, after target and effector cells were combined in the wells of a 96-well plate, both GolgiStop and GolgiPlug were added to the culture (BD Biosciences). GolgiStop and GolgiPlug were used at 1/2 of the concentration recommended by the manufacturer. At t=6h post stimulation, cells were processed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were pelleted, washed with FACS buffer, and then stained for cell surface markers (described above). Cells were then washed 2x with FACS buffer prior to fixation and permeabilization. Cells were then washed with Perm/Wash buffer and stained with antibodies against cytokines for 30 min, at 4°C in the dark. Cells were washed 2x with Perm/Wash buffer and resuspended in FACS buffer prior to acquisition on a FACSCantoII flow cytometer. All flow cytometry data were analyzed using FlowJo software (TreeStar Inc).

IFN- γ in serum samples was detected using a human IFN- γ ELISA kit as directed by the manufacturer (Thermo Scientific).

Flow cytometry antibodies

The following titrated anti-human antibodies were used for cell surface staining: CCR7-FITC (clone: 150503), CD45RO-PE-Cy7 (clone: UCHL1), CD62L-APC (clone: DREG-56), CD27-APC-H7 (clone: M-T271), CD4-eFluor 605NC (clone: OKT4), CD57-FITC (clone: NK-1), CD28-PE-Cy7 (clone: CD28.2), CD127-APC (clone: eBioRDR5), CD3-AF700 (clone: UCHT1), CD4-FITC, PE-Cy7, APC-H7 (clone: SK3), CD8-PE-Cy7 (clone: SK1), V β 22-PE (clone: IMMU 546), V β 5.2-PE (clone: 36213), OX40-PE-Cy7 or FITC (clone: Ber-ACT35), 4-1BB-APC (clone: 4B4-1), and CD107a-APC-H7 (clone: H4A3). All antibodies were from BD Biosciences, except CD4-eFluor605NC (eBioscience), V β 22-PE and V β 5.2-PE (Beckman Coulter), and 4-1BB-APC and OX40-PE-Cy7 (BioLegend). The following optimally titrated anti-human antibodies were used for intracellular cytokine staining: IFN- γ -FITC (clone: 4S.B3), IL-2-APC (clone: MQ1-17H12), TNF-PerCP-Cy5.5 or APC (clone: MAb11), IL-17-PE (clone: eBio64DEC17), and IL-4-PE-Cy7 (clone: 8D4-8). All ICS antibodies were from eBioscience except IL-4-PE-Cy7 (BD Bioscience). The IO Mark Beta Mark TCR V kit was used to assess the TCR-V β repertoire (Beckman Coulter).

Sequencing of the ERBB2IP mutation

Sanger sequencing was used to validate the *ERBB2IP* mutation found by whole-exomic sequencing. Total RNA was extracted from snap frozen T cells or tumor tissues (OCT block) using the RNeasy Mini kit (Qiagen). Total RNA was then reverse transcribed to cDNA using ThermoScript reverse transcriptase with oligo-dT primers (Life Technologies). Normal and tumor cDNA were then used as templates in a PCR with the following ERBB2IP primers flanking the mutation: ERBB2IP Seq Forward: 5'—TGT TGA CTC AAC AGC CAC AG—3'; and ERBB2IP Seq Reverse: 5'—CTG GAC CAC TTT TCT GAG GG—3'. Phusion DNA polymerase (Thermo Scientific) was used with the recommended 3-step protocol with a 58°C annealing temperature (15 sec) and a 72°C extension (30 sec). PCR products were isolated by standard agarose gel electrophoresis

and gel extraction (Clontech). Products were directly sequenced using the same PCR primers (Macrogen).

Quantitative PCR

Total RNA was extracted from snap frozen T cells or tumor tissues (OCT block) using the RNeasy Mini kit (Qiagen). Total RNA was then reverse transcribed to cDNA using qScript cDNA Supermix (Quanta Biosciences). Gene-specific Taqman primer and probe sets for human β -actin (catalogue #: 401846) and ERBB2IP (catalogue #: 4331182) were purchased from Life Technologies. Quantitative PCR was carried out with TaqMan Fast Advanced Master Mix using the 7500 Fast Real Time PCR machine (both from Applied Biosystems). Specificity of amplified products was verified by standard agarose gel electrophoresis. All calculated threshold cycles (Ct) were 30 or below.

TCR-V β deep sequencing

TCR-V β deep sequencing was performed by immunoSEQ, Adaptive Biotechnologies (Seattle, WA) on genomic DNA isolated from peripheral blood, T cells, and frozen tumor tissue using the DNeasy blood and tissue kit (Qiagen). The number of total productive TCR reads per sample ranged from 279, 482 to 934,672. Only productive TCR rearrangements were used in the calculations of TCR frequencies.

TCR sequencing and construction of the ERBB2IP-mutation reactive TCR

T cells were pelleted and total RNA isolated (RNeasy Mini kit, Qiagen). Total RNA then underwent 5'RACE as directed by manufacturer (SMARTer RACE cDNA amplification kit, Clontech) using TCR-alpha and -beta chain constant primers. Program 1 of the kit was used for the PCR, with a modification to the extension time (2 min instead of 3 min). The sequences of the alpha and beta chain constant primers are: TCR-alpha, 5'—GCC ACA GCA CTG TGC TCT TGA AGT CC—3'; TCR-beta, 5'—CAG GCA GTA TCT GGA GTC ATT GAG—3'. TCR PCR products were then isolated by standard agarose gel electrophoresis and gel extraction (Clontech). Products were then either directly sequenced or TOPO-TA cloned followed by sequencing of individual colonies (Macrogen). For sequencing of known V β 22+ T-cell clones, cDNA was generated from RNA using qScript cDNA Supermix (Quanta Biosciences). These cDNAs then were used as templates in a PCR using the TCR-beta constant primer (above) and the V β 22-specific primer: 5'—CAC CAT GGA TAC CTG GCT CGT ATG C—3'. PCR products were isolated by standard agarose gel electrophoresis and gel extraction (Clontech). Products were directly sequenced (Macrogen) using the nested TCR-beta chain constant primer: 5'—ATT CAC CCA CCA GCT CAG—3'.

Construction of the V β 22+ ERBB2IP-mutation TCR was done by fusing the V β 22+ TCR-alpha V-D-J regions to the mouse TCR-alpha constant chain, and the V β 22+ TCR-beta-V-D-J regions to the mouse TCR-beta constant chains. The alpha and beta chains were separated by a furin SGSG P2A linker. Use of mouse TCR constant regions promotes pairing of the introduced TCR and also facilitates identification of positively transduced T cells by flow cytometry using an antibody specific for the mouse TCR- β chain (eBioscience). The TCR construct was synthesized and cloned into the MSGV1 retroviral vector (Gene Oracle).

TCR transduction of peripheral blood T cells

Autologous pheresis samples were thawed and set to 2×10^6 cells/ml in T-cell media, which consists of a 50/50 mixture of RPMI and AIM-V media supplemented with 5% in-house human serum, 10 $\mu\text{g/ml}$ gentamicin (CellGro), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin, 1.25 $\mu\text{g/ml}$ amphotericin B (Fungizone) and 2 mM L-glutamine (all from Life Technologies). 2×10^6 cells (1 ml) were stimulated in a 24-well plate with 50 ng/ml soluble OKT3 (Miltenyi Biotec) and 300 IU/ml rhu IL-2 (Chiron) for 2 days prior to retroviral transduction. To generate transient retroviral supernatants, the retroviral vector MSGV1 encoding the V β 22-positive, ERBB2IP-mutation-specific TCR (1.5 $\mu\text{g/well}$) and the envelope encoding plasmid RD114 (0.75 $\mu\text{g/well}$) were co-transfected into the retroviral packaging cell line 293GP (1×10^6 cells per well of a 6-well poly-D-lysine-coated plates, plated the day prior to transfection) using Lipofectamine 2000 (Life Technologies). Retroviral supernatants were collected at 42-48 h after transfection, diluted 1:1 with DMEM media, and then centrifuged onto Retronectin-coated (10 $\mu\text{g/ml}$, Takara), non-tissue culture-treated 6-well plates at 2,000 g for 2 h at 32°C. Activated T cells (2×10^6 per well, at 0.5×10^6 cells/ml in IL-2 containing T-cell media) were then spun onto the retrovirus plates for 10 min at 300 g. Activated T cells were transduced overnight, removed from the plates and further cultured in IL-2 containing T-cell media. GFP and mock transduction controls were included in transduction experiments. Cells were typically assayed 10-14 days post-retroviral transduction.

Fig. S1

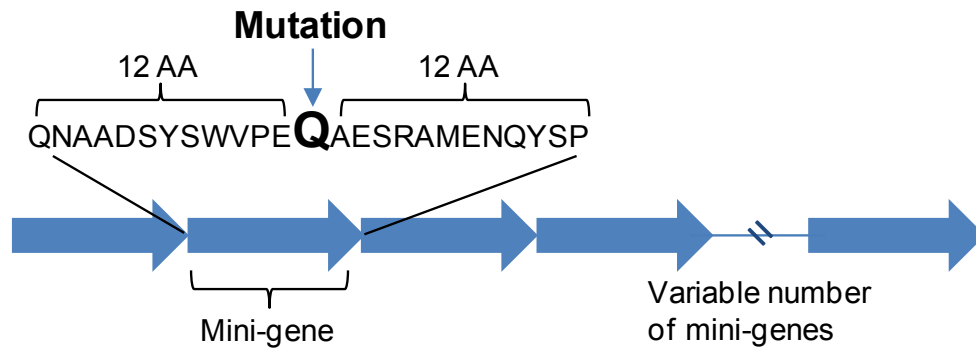


Fig. S1. Basic anatomy of a tandem mini-gene (TMG) construct. The TMG construct consists of a variable number of mini-genes genetically fused together. Each mini-gene encodes for a mutation flanked by 12 amino acids (AA) from the endogenous protein.

Fig. S2

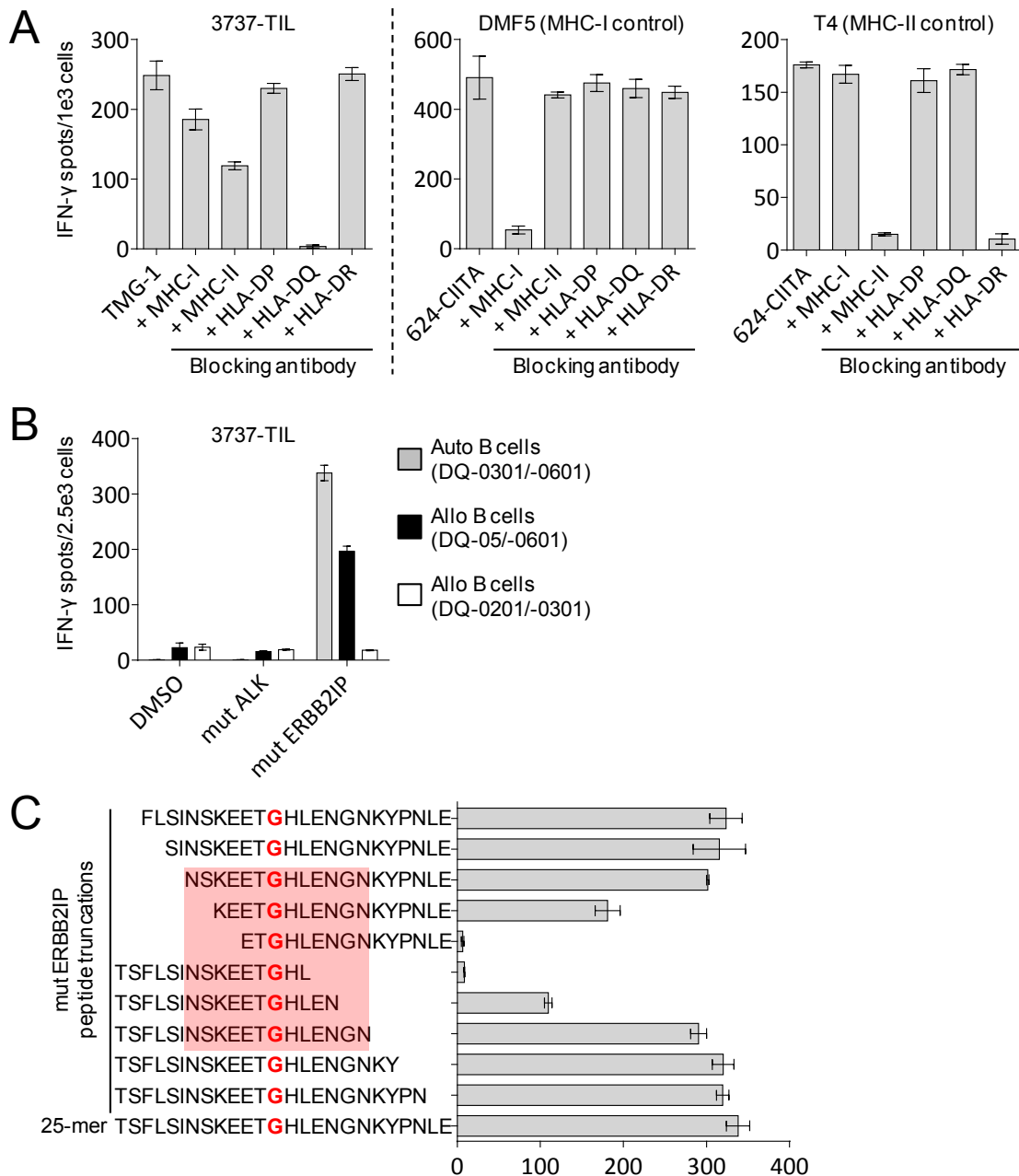


Fig. S2. Molecular characterization of the ERBB2IP-mutation reactive T-cell response. (A) IFN- γ ELISPOT assay at 20 h. 3737-TIL were co-cultured with DCs transfected with TMG-1 that had been pre-incubated with nothing, or the indicated HLA-blocking antibodies (**left**). As controls for antibody blocking, the HLA-A2 restricted MART-reactive T cell DMF5 (**middle**) and the HLA-DR-restricted tyrosinase-reactive T cell T4 (**right**) were co-cultured with the MART and tyrosinase-positive 624-CIITA melanoma cell line that had been pre-incubated with nothing, or the indicated HLA-blocking antibodies. (B) IFN- γ ELISPOT assay at 20 h. 3737-TIL were co-cultured with autologous B cells or allogeneic EBV-B cells partially matched at the HLA-DQ locus

that had been pulsed overnight with DMSO, mutated (mut) ALK or mut ERBB2IP 25-AA long peptides. (C) IFN- γ ELISPOT assay at 20 h. 3737-TIL were co-cultured with autologous B cells that had been pulsed overnight with the mut ERBB2IP 25-AA peptide, or the indicated truncated mut ERBB2IP peptides. All data is representative of 2 independent experiments.

Fig. S3

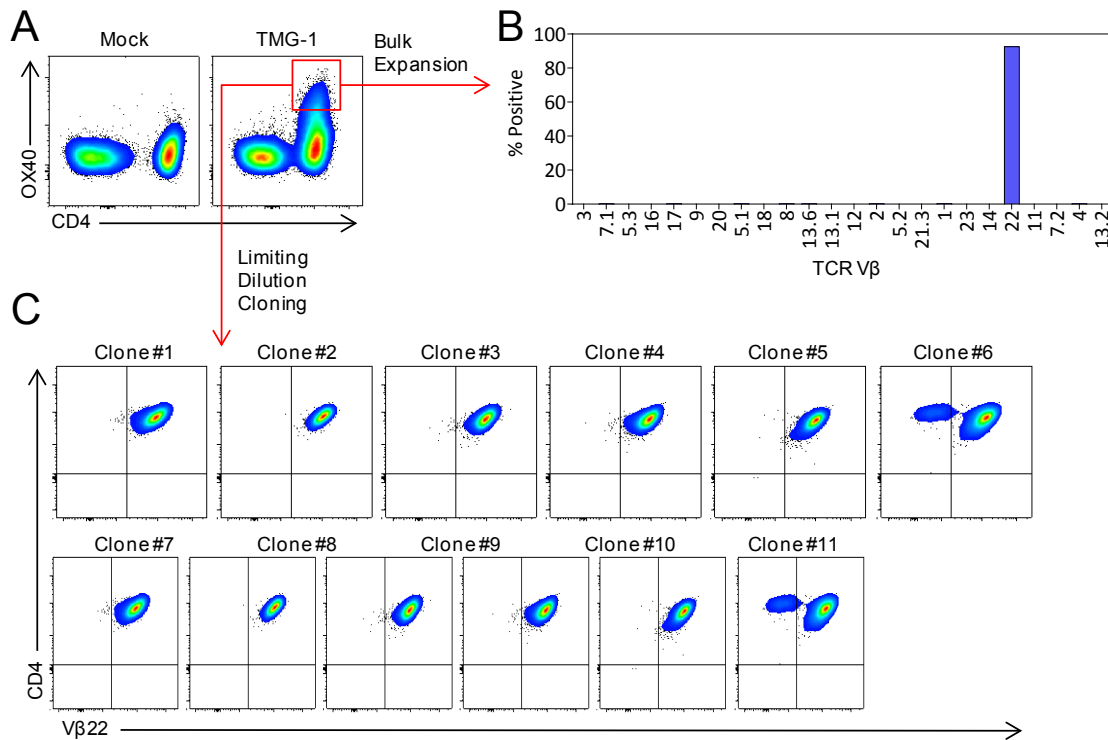


Fig. S3. Assessing clonality of ERBB2IP mutation-reactive CD4⁺ T cells. (A) 3737-TIL were co-cultured with DCs transfected with nothing (Mock) or TMG-1. CD4⁺ T cells that upregulated OX40 at t = 24 h (red box) were sorted by FACS and then expanded in bulk (B) or cloned by limiting dilution (C) using irradiated allogeneic feeders in the presence of anti-CD3 antibody (OKT3, 30 ng/ml) and IL-2 (3000 IU/ml). Flow-cytometry based TCR-V β repertoire analysis was performed on (B) and V β 22-specific staining was done on (C). Data are gated on live CD3⁺ cells.

Fig. S4

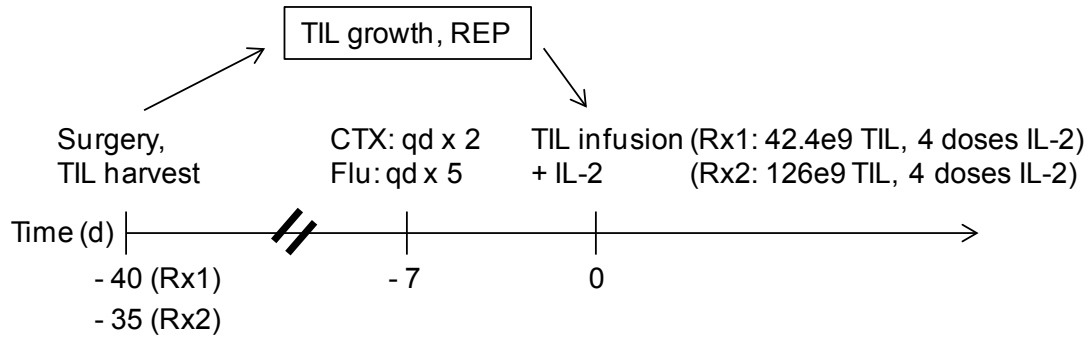


Fig. S4. Patient treatment scheme. For the first treatment (Rx1), patient 3737 underwent a resection of lung lesions. Tumors were then minced into small fragments and incubated with high dose IL-2 to expand tumor infiltrating lymphocytes (TIL). After an initial expansion in IL-2, select TIL cultures were further expanded for 2 weeks using a rapid expansion protocol (REP) consisting of irradiated allogeneic peripheral blood feeder cells, OKT3 and IL-2. Prior to cell infusion, the patient was pre-conditioned with cyclophosphamide (CTX: 60 mg/kg, once a day for two days) followed by fludarabine (Flu: 25 mg/m² for 5 days). 3737-TIL consisted of 42.4 billion TIL containing over 10 billion (25%) ERBB2IP-mutation reactive T cells, and was administered on day 0, followed by IL-2 (Aldesleukin, 7.2e5 IU/kg) every 8 hours. The patient received a total of 4 doses of IL-2. The second treatment (Rx2) was essentially identical as the first, except that the second cell infusion product consisted of 126 billion TIL containing greater than 120 billion (95%) V β 22+ ERBB2IP-mutation-reactive T cells (derived from the first surgery). For both treatments, the side effects were expected with the administration of high dose IL-2 and included malaise and fluid retention but the patient tolerated the treatments well and was discharged on schedule. See Materials and Methods for more details of the treatment.

Fig. S5

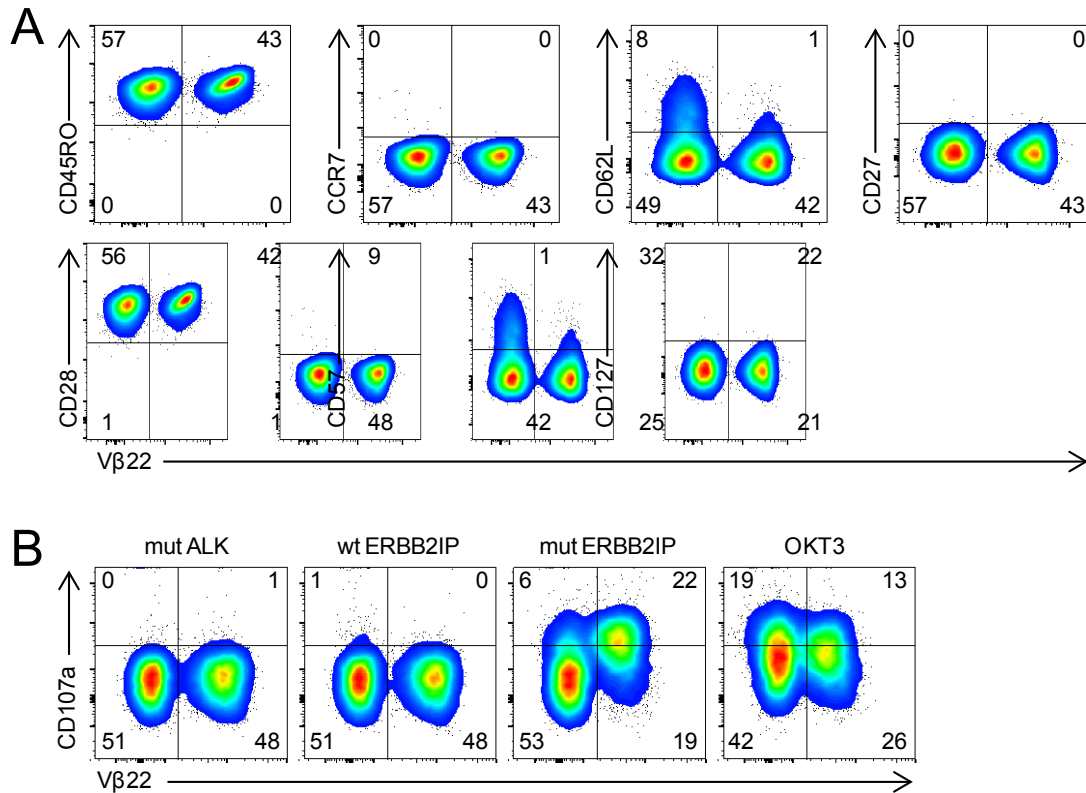


Fig. S5. T-cell differentiation phenotype and cytolytic potential of 3737-TIL. (A) 3737-TIL were assessed for expression of V β 22 (representing ERBB2IP-mutation-reactive T cells) and the indicated T-cell differentiation markers. Data are gated on live CD3+CD4+ cells. Positive and negative quadrant gates were set using isotype stained or unstained cells. Human peripheral blood cells (containing T cells of all differentiation stages) were included in experiments to ensure that the antibodies were working (data not shown). (B) 3737-TIL were co-cultured for 6 h with autologous B cells pulsed overnight with wild-type (wt) ERBB2IP, or mutated (mut) ALK or mut ERBB2IP 25-AA long peptides. Antibodies specific for the degranulation marker CD107a were added at the beginning of the co-culture. Flow cytometry was used to assess expression of V β 22 and to detect cell surface mobilization of CD107a. Data are gated on the CD4+ population. All data are representative of at least two independent experiments.

Fig. S6

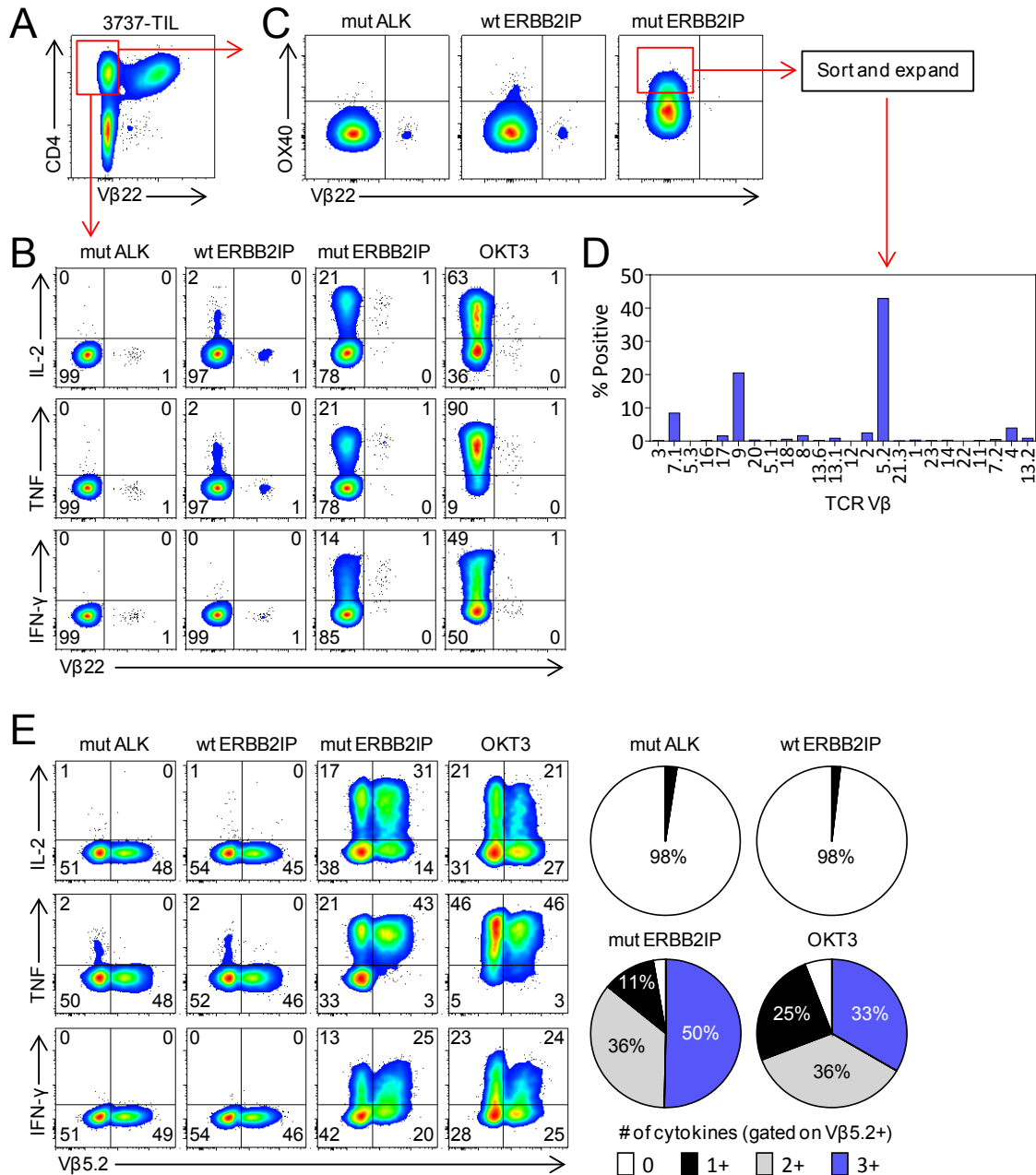


Fig. S6. 3737-TIL contain Vβ22-negative ERBB2IP-mutation-reactive T cells. (A) CD4⁺ Vβ22-negative cells present in 3737-TIL were sorted by FACS (red box). (B-C) These cells were then rested in IL-2 containing media for 2 days prior to being co-cultured with autologous B cells pulsed overnight with wild-type (wt) ERBB2IP, or mutated (mut) ALK or mut ERBB2IP 25-AA long peptides. (B) Flow cytometry was used to assess expression of Vβ22 and to detect intracellular production of IL-2, TNF, and IFN-γ in the CD4⁺ population at 6 h post-stimulation. (C) Flow cytometry was used to assess expression OX40 and Vβ22 in the CD4⁺ population at 24 h post stimulation.

Cells that upregulated OX40 were sorted (red box) and expanded, and the TCR-V β repertoire was analyzed by flow cytometry **(D)**. Data are gated on live CD4⁺ cells. **(E)** The sorted cells described in (D) were co-cultured for 6 h with autologous B cells pulsed overnight with wt ERBB2IP, or mut ALK or mut ERBB2IP 25-AA long peptides. Flow cytometry was used to assess expression of V β 5.2 and to detect intracellular production of IL-2, TNF, and IFN- γ in the CD4⁺ population. Pie charts display the percentage of V β 5.2⁺ cells that expressed the indicated number of cytokines.

Fig. S7

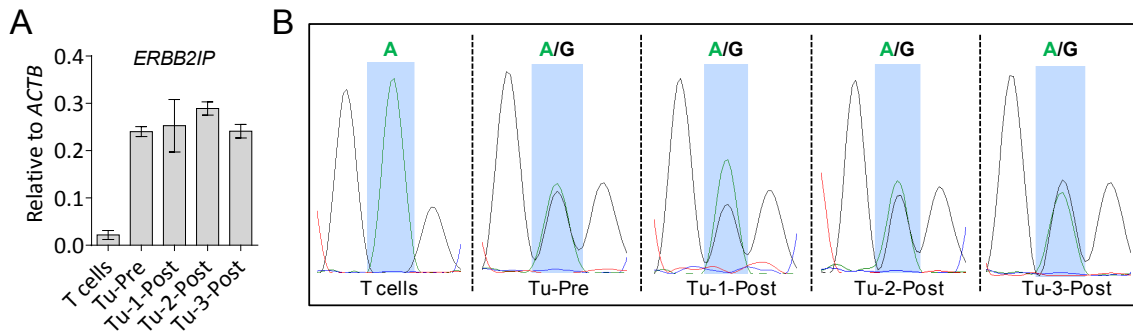


Fig. S7. Expression of wild-type and mutated *ERBB2IP* in normal and tumor tissues. (A) RT-qPCR analysis of *ERBB2IP* expression in 3737-TIL (T cells) and tumors pre-(Tu-Pre) and post adoptive cell transfer. Three separate metastatic lung lesions (Tu-1, -2, -3-Post) were resected approximately 17 months post cell infusion. Data are relative to β -actin (*ACTB*), and are representative of two independent experiments. (B) A 350 bp segment of the *ERBB2IP* gene containing the mutation was PCR-amplified from the cDNA samples described in (A) and Sanger sequenced. The location of the mutation is at nucleotide position 2414 of the coding sequence, corresponding to a change at position 805 of the amino acid sequence.

Table S1. Clinical characteristics of patient 3737

Sex	Age	Primary	Metastatic sites	Prior Therapy	Prior IL-2	Harvest site*	ECOG [†] Status	HLA-I	HLA-II
F	43	Intrahepatic cholangiocarcinoma (poorly differentiated)	Lungs, liver	Cisplatin + gemcitabine, gemcitabine, taxotere	No	Lung	0	A*26 B*38 B*52 C*12	DRB1*0405 DRB1*1502 DQB1*0301 DQB1*0601 DPB1*0401 DPB1*10401

* Harvest site for generation of TIL and for whole exomic sequencing.

[†] Performance status: ECOG, Eastern Cooperative Oncology Group

Table S2. Somatic mutations identified by whole-exome sequencing of a metastatic lung nodule* from patient 3737

Gene Symbol	Gene Description	Transcript Accession	Mutation Position Nucleotide (genomic)	Amino Acid (protein)	Mutation Type	Consequence	% Mutant Reads*
ALK	anaplastic lymphoma receptor tyrosine kinase	CCDS33172.1	chr2_29996620-29996620_C_T	137R>H	Substitution	Nonsynonymous coding	30%
AR	androgen receptor	CCDS14387.1	chrX_66858483-66858483_C	NA	Insertion	Frameshift	31%
CD93	CD93 molecule	CCDS13149.1	chr20_23012929-23012929_C_T	634R>Q	Substitution	Nonsynonymous coding	26%
DIP2C	DIP2 disco-interacting protein 2 homolog C (Drosophila)	CCDS7054.1	chr10_365545-365545_C_T	NA	Substitution	Splice site acceptor	25%
ERBB2IP	erb2 interacting protein	CCDS3990.1	chr5_65385316-65385316_A_G	805E>G	Substitution	Nonsynonymous coding	59%
FCER1A	Fc fragment of IgE; high affinity I; receptor for; alpha polypeptide	CCDS1184.1	chr1_157544227-157544227_G_C	219D>H	Substitution	Nonsynonymous coding	30%
GRXCR1	glutaredoxin; cysteine rich 1	CCDS43225.1	chr4_42590102-42590102_C_T	21A>V	Substitution	Nonsynonymous coding	18%
HLA-DOA	HLA class II histocompatibility antigen, DO alpha chain precursor	CCDS4763.1	chr6_33085209-33085209_C_T	NA	Substitution	Splice site donor	36%
KIF9	kinesin family member 9	CCDS2752.1	chr3_47287859-47287859_T_C	155T>A	Substitution	Nonsynonymous coding	20%
KLHL6	kelch-like 6 (Drosophila)	CCDS3245.2	chr3_184692410-184692413_CAGA_	NA	Deletion	Frameshift	20%
LHX9	LIM homeobox 9	CCDS1393.1	chr1_196164923-196164923_A_	NA	Deletion	Frameshift	21%
LONRF3	LON peptidase N-terminal domain and ring finger 3	CCDS35374.1	chrX_118007666-118007666_A_C	NA	Substitution	Splice site donor	10%
NAGS	N-acetylglutamate synthase	CCDS11473.1	chr17_39440355-39440355_G_A	412R>H	Substitution	Nonsynonymous coding	29%
NLRP2	NLR family; pyrin domain containing 2	CCDS12913.1	chr19_60186650-60186650_G_T	591S>I	Substitution	Nonsynonymous coding	32%
PDZD2	PDZ domain containing 2	CCDS34137.1	chr5_32124833-32124833_A_	NA	Deletion	Frameshift	30%
POU5F2	POU domain, class 5, transcription factor 2	NM_153216	chr5_93102847-93102847_A_C	60V>G	Substitution	Nonsynonymous coding	34%
RAC3	ras-related C3 botulinum toxin substrate 3 (rho family; small GTP binding protein Rac3)	CCDS11798.1	chr17_77584690-77584690_C_A	125T>N	Substitution	Nonsynonymous coding	27%
RAP1GDS1	RAP1; GTP-GDP dissociation stimulator 1	CCDS43253.1	chr4_99532209-99532209_C_A	198L>I	Substitution	Nonsynonymous coding	19%
RASA1	RAS p21 protein activator (GTPase activating protein) 1	CCDS34200.1	chr5_86703757-86703757_C_T	589R>C	Substitution	Nonsynonymous coding	63%
RETSAT	retinol saturase (all-trans-retinol 13;14-reductase)	CCDS1972.1	chr2_85424308-85424308_C_T	553R>K	Substitution	Nonsynonymous coding	11%
SEC24D	SEC24 family; member D (S. cerevisiae)	CCDS3710.1	chr4_119872085-119872085_A_G	901M>T	Substitution	Nonsynonymous coding	18%
SEN3	SUMO1/sentrin/SMT3 specific peptidase 3	ENST00000321337	chr17_7408824-7408824_A_G	292M>V	Substitution	Nonsynonymous coding	33%
SLIT1	slit homolog 1 (Drosophila)	CCDS7453.1	chr10_98753840-98753840_G_C	1280N>K	Substitution	Nonsynonymous coding	45%

TARBP1	TAR (HIV-1) RNA binding protein 1	CCDS1601.1	chr1_232649342-232649342_C_A	655G>V	Substitution	Nonsynonymous coding	18%
TGM6	transglutaminase 6	CCDS13025.1	chr20_2332325-2332325_G_A	398D>N	Substitution	Nonsynonymous coding	51%
TTC39C	tetratricopeptide repeat domain 39C	CCDS32804.1	chr18_19966475-19966475_A_C	503N>T	Substitution	Nonsynonymous coding	24%

* The tumor nodule was estimated to be approximately 70% tumor by pathological analysis of an H&E stained section.

Table S3. Tandem mini-gene (TMG) constructs

TMG	Mutated Gene	Mutated* Mini-gene Amino Acid Sequence	TMG Amino Acid Sequence
1	ALK	RVLKGGSVRKLR HAKQLVLELGEEA	RVLKGGSVRKLR HAKQLVLELGEEA QNAADSYSWVP E Q AESRAMENQYSPTSFLSINSKEET G HLENGNKYPN LEFIPLLVLFAV HT GLFISTQQQVTESDRPRKVRFR I SSHSGRVLKEVYEIYNESLFDLLS AL PYVGPSVTPMTG KKLRDDYLAS LH PRLSIYVSEGYPDIKQELLRCD I ICK GGHSTVTDLQVGTKDLDRDDKD N IERLRDKKLAPI
	CD93	QNAADSYSWVPE Q AESRAMENQYSP	
	ERBB2IP	TSFLSINSKEET G HLENGNKYPNLE	
	FCER1A	FIPLLVLFAV HT GLFISTQQQVT	
	GRXCR1	ESDRPRKVRFR I SSHSGRVLKEVY	
	KIF9	EIYNESLFDLLS AL PYVGPSVTPMT	
	NAGS	GKKLRDDYLAS LH PRLSIYVSEGY	
	NLRP2	PDIKQELLRCD I CKGGHSTVTDLQ	
	RAC3	VGTKDLDRDDKD N IERLRDKKLAPI	
2	RAP1GDS1	VKLLGIHCQNA I TEMCLVAFGNLA	VKLLGIHCQNA I TEMCLVAFGNLANLRKSSPGTSNK CL RQVSSLVLHIELGR LHPCVMAS LKA QSPINLYLTG LLPIHTLDVKST T LPAAVRCSESRLMTDNFGKHYTL K SEAPLYVGGMPVMTMDNFGKHYTL K SEAPLYVGG MPVHDGPFVFAEVN AN YITWLWHEDESQAKEDFS GYDFE T RLHVRIHAALASPAVRPGICPGPD G WRIPLG PLPHEF
	RASA1	NLRKSSPGTSNK CL RQVSSLVLHIE	
	RETSAT	LGRLHPCVMAS LKA QSPINLYLTG	
	SEC24D	LLPIHTLDVKST T LPAAVRCSESRL	
	SLIT1	MTMDNFGKHYTL K SEAPLYVGGMPV	
	TARBP1	AVDVEGMKTQYS V KQRTENVLRIFL	
	TGM6	HDGPFVFAEVN AN YITWLWHEDESRL	
	TTC39C	QAKEDFSGYDFE T RLHVRIHAALAS	
	POU5F2	PAVRPGICPGPD G WRIPLGPLPHEF	
3	SEN3	VAQELFQGS D LG V AEEAERPGEKAG	VAQELFQGS D LG V AEEAERPGEKAGGTATTLTDLTN PLSL THIRIVPGAV S DGRMG SWRAPPTLS VPASPLT LLQSHFRQQARVRHLSQEFGLQITP PGIPV H ESTAT LQHYSSG WAEKSKIL SPDSKIQMVSSSQ KRALLCLIAL LSRKQ TWKIRTCLRRVRQKCF TLLSPQEAGATK DECE GEEGAAGSRDLRSWVT EETGMPNKASKQ GPGSTQ REGSLEEIPGLTNIYKLLTSVWG LLRLVWVG PALAF TCVTSEIAMRLL
	LHX9	GTATTLTDLTN LSL	
	KLHL6	THIRIVPGAV S DGRMG SWRAPPTLS VPASPLTLLQSHFRQQARV	
	AR	RHLSQEFGLQITP PGIPV H ESTAT LQ HYSSG WAEKSKIL	
	PDZD2	SPDSKIQMVSSSQ KRALLCLIAL LSRKQ TWKIRTCLRRVRQKCF	
	HLA-DOA	TLLSPQEAGATK DECEGEEGAAGSRDL RSWVT	
	LONRF3	EETGMPNKASKQ GPGSTQ REGSLEEIPGLTNIYKLLTSVWG LLRLVWVG PALAF TCVTSEIAMRLL	

*Red and bolded denotes mutated amino acids and neo-sequences encoded by point mutations, or nucleotide insertions or deletions. For splice-site donor mutations (*HLA-DOA* and *LONRF3*), we designed mutant mini-gene transcripts that continued into the downstream intron until the next stop codon, based on the assumption that the mutations prevented splicing at that site. The splice-site acceptor mutation in *DIP2C* was not assessed.

Table S4. TCR β V-D-J sequences of V β 22-positive ERBB2IP-mutation-reactive T-cell clones

TCR V β	V-D-J nucleotide sequence (CDR3 highlighted)	V-D-J amino acid sequence (CDR3 highlighted)	Number of V β 22 (TRBV2) clones with indicated V-D-J
V β 22 (TRBV2)	GAACCTGAAGTCACCCAGACTCCCAGCCATCAGGT CACACAGATGGGACAGGAAGTGATCTTGCGCTGT GTCCCCATCTCTAATCACTTATACTTCTATTGGTACA GACAAATCTTGGGGCAGAAAGTCGAGTTTCTGGTT TCCTTTTATAATAATGAAATCTCAGAGAAGTCTGAA ATATTCGATGATCAATTCTCAGTTGAAAGGCCTGAT GGATCAAATTTCACTCTGAAGATCCGGTCCACAAA GCTGGAGGACTCAGCCATGTACTTCTGTGCCAGCA GCCTGGGTGACAGGGGTAATGAAAACTGTTTT TGGCAGTGGAACCCAGCTCTCTGTCTTGG	EPEVTQTPSHQVTQMG QEVILRCVPISNHLFYFYR QILGQKVEFLVSFYNNNEIS EKSEIFDDQFSVERPDGS NFTLKIRSTKLEDSAMYF CASSLGDRGNEKLFFGS GTQLSVL	6/6

Table S5. TCR α V-J sequence of the V β 22+ ERBB2IP-mutation-reactive T-cell clone

TCR V α	V-J nucleotide sequence (CDR3 highlighted)	V-J amino acid sequence (CDR3 highlighted)
TRAV26-2	GATGCTAAGACCACACAGCCAAATTCAATGGAG AGTAACGAAGAAGAGCCTGTTCACCTGCCTTGTA ACCACTCCACAATCAGTGGAAGTATTACATACA TTGGTATCGACAGCTTCCCTCCCAGGGTCCAGAG TACGTGATTCATGGTCTTACAAGCAATGTGAACA ACAGAATGGCCTCTCTGGCAATCGCTGAAGACA GAAAGTCCAGTACCTTGATCCTGCACCGTGCTAC CTTGAGAGATGCTGCTGTGTACTACTGCATCCTG AGACGTCTTAACGACTACAAGCTCAGCTTTGGAG CCGGAACCACAGTAACTGTAAGAGCAA	DAKTTQPNSMESNEEEPVHLP CNHSTISGTDYIHWYRQLPSQ GPEYVIHGLTSNVNNRMA SLAIAEDRKSSTLILHRATLRDA AVYYCILRRRLNDYKLSFGAGTT VTVRA

Table S6. Most frequent TCR β V-D-J sequence of V β 22-negative ERBB2IP-mutation-reactive T cells*

TCR V β	V-D-J nucleotide sequence (CDR3 highlighted)	V-D-J amino acid sequence (CDR3 highlighted)	Number of TOPO- TA clones with indicated V-D-J
V β 5.2 (TRBV5-6)	GACGCTGGAGTCACCCAAAGTCCCACACACCTGAT CAAAACGAGAGGACAGCAAGTGACTCTGAGATGC TCTCCTAAGTCTGGGCATGACACTGTGTCCTGGTAC CAACAGGCCCTGGGTCAGGGGCCCCAGTTTATCTT TCAGTATTATGAGGAGGAAGAGAGACAGAGAGGC AACTTCCCTGATCGATTCTCAGGTCACCAGTTCCT AACTATAGCTCTGAGCTGAATGTGAACGCCTTGTT GCTGGGGGACTCGGCCCTCTATCTCTGTGCCAGCA GCAAAGGCCCGGGAGGCAACTACGAGCAGTACTT CGGGCCGGGCACCAGGCTCACGGTCACAG	DAGVTQSPTHLIKTR GQQVTLRCSPKSGHD TVSWYQQALGQGPQ FIFQYEEEEERQRGNF PDRFSGHQFPNYSSE LNVNALLGDSALYLC ASSKGPGGNYEQYF GPGTRLTVT	3/7

* V β 22-negative cells that upregulated OX40 upon stimulation with mutated ERBB2IP were sorted and expanded. RNA from these cells was then isolated and underwent 5'RACE with TCR- β constant chain primers to identify the expressed TCR-V β sequences. TOPO-TA cloning was performed on the PCR products and individual colonies were then sequenced. Flow cytometry demonstrated that 40-50% of these T cells were V β 5.2 (TRBV5-6). By Sanger sequencing, 3/7 TOPO-TA colonies were V β 5.2 (TRBV5-6) with the indicated sequence above.

Table S7. Immunohistochemistry analysis of T-cell infiltrates pre- and post-ACT*

Tumor Nodule	CD3		CD8		CD4	
	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma
Pre-1A	0-1	1	0-1	1	0-1	1
Pre-2A	0-1	1	0-1	1	0	0
Pre-3A	0	0-1	0	0-1	0	0
Pre-3B	0-1	1	0-1	0-1	0-1	1
Post-1A	1	1	1	1	0-1	1
Post-1B	1	2	1-2	2	1	2
Post-2A	0-1	1	0-1	1	0-1	0-1

*Post-ACT tumors were harvested approximately 17 months after the first ACT.

A positive control (tonsil) was included for all stains.

0, no infiltrate

1, rare to few

2, moderately dense

3, very dense

Table S8. Immunohistochemistry analysis of MHC expression on tumors pre- and post-ACT*

Tumor Nodule	HLA-I	HLA-II (HLA-DR)
Pre-1A	1-2, >50%	0
Pre-2A	1-2, >50%	0
Pre-3A	1, >50%	0
Pre-3B	2, >50%	0
Post-1A	2-3, >50%	0
Post-1B	3, >50%	0
Post-2A	2, >50%	0

*Post-ACT tumors were harvested approximately 17 months after the first ACT.

> 50% denotes greater than 50% of the tumor cells were positive.

A positive control (tonsil) was included for all stains.

0, negative

1, weakly positive

2, moderately positive

3, strongly positive

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