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% CO_2. 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 (Patient 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 minigene (TMG) constructs

The detailed methodology will be published elsewhere, but briefly, for each non-synonymous substitution mutation identified by whole exome sequencing, a "minigene" construct encoding the corresponding amino acid change flanked by 12 amino acids of the wild-type protein sequence was made. Multiple minigenes were genetically fused together to generate a tandem minigene (TMG) construct. These minigene 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/cm^2 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 minigenes 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 $LiCl_2$ 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% CO_2. 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% CO_2. 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 (TSFLSINSKEETGHLENGNKYPNLE); wild-type ERBB2IP (TSFLSINSKEETEHLENGNKYPNLE); 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 FACSAria IIu and BD FACSJazz 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 3.5e4 to 7e4 DCs were used per well of a 96-well flat or round-bottom plate. When B cells were used as APCs, approximately 2e5 cells were used per well of a 96-well round-bottom plate. In ELISPOT assays, 1e3 to 1e4 effector T cells were used per well, and in flow cytometry assays, 1e5 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 μg/ml or 1 μg/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 μg/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 μl of 70% ethanol per well for 2 min, washed 3x with PBS, and then coated with 50 μl of 10 μg/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 μg/ml) and OKT3 (1 μg/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 μl/well of a 0.22 μm filtered 1 μg/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 μl/well of streptavidin-ALP (Mabtech, diluted 1:3000). The plate was then washed 6x with PBS followed by development with 100 μl/well of 0.45 μm 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), Vβ22-PE (clone: IMMU 546), Vβ5.2-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), Vβ22-PE and Vβ5.2-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-PerCPCy5.5 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-Vβ 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-Vβ deep sequencing

TCR-Vβ 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 Vβ22+ 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 Vβ22-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 Vβ22+ ERBB2IP-mutation TCR was done by fusing the Vβ22+ TCR-alpha V-D-J regions to the mouse TCR-alpha constant chain, and the Vβ22+ 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 2e6 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 μg/ml gentamicin (CellGro), 100 U/ml penicillin and 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B (Fungizone) and 2 mM L-glutamine (all from Life Technologies). 2e6 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 Vβ22-positive, ERBB2IP-mutation-specific TCR (1.5 μg/well) and the envelope encoding plasmid RD114 (0.75 μg/well) were co-transfected into the retroviral packaging cell line 293GP (1e6 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 μg/ml, Takara), non–tissue culture–treated 6-well plates at 2,000 g for 2 h at 32°C. Activated T cells (2e6 per well, at 0.5e6 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.