Supporting Methods

Clinical Case Histories. PCD Patient 1: A 74 year old woman (patient NA-06-03) with a distant history of breast cancer in 1977 treated with modified radical mastectomy and chemotherapy (CMF, tamoxifen and BCG) developed ascending numbness in all four extremities in February 2005. By 9/05, the patient had developed a partial left foot drop, malaise with weakness, and falls and was found to be Yo antibody positive (Athena Diagnostics, confirmed in our laboratory by Western blot against cdr2 fusion protein; data not shown). Further workup revealed a CA125 > 2000 and CT of the abdomen/pelvis showing a 5 x 2.7 cm mass and retroperitoneal lymphadenopathy, found by needle biopsy to be a poorly differentiated papillary serous carcinoma. Neurologic examination in 3/06 revealed some distal leg weakness with trace knee jerks, absent ankle jerks, decreased pinprick and vibratory sensation in all four extremities, and a flat footed proprioceptive gait in which she was able to walk only three or four steps independently. At this time, samples were obtained for this clinical study, and she was treated with tacrolimus, 0.3 mg/kg/d, and prednisone, 60 mg/d (1), and showed significant improvement over the course of 10 days, with improved subjective sensation, and an improved gait that was independent, including the ability to turn independently. She also underwent chemotherapy with carboplatin and taxol. On 7/06 the patient underwent a TAH/BSO; no tumor cells were found on careful pathologic examination, and follow-up PET scan on 8/06 was negative. At last follow-up in 12/06 the patient remained without evidence of tumor, and with continued improvement in symptoms; she was not dysarthric, had trace nystagmus on lateral gaze, 2/5 left dorsiflexor weakness but otherwise intact segmental motor exam, mild decrease in pinprick sensation in all four extremities with dropped reflexes, no evident appendicular dysmetria or dysdiadokokinesia, and was able to walk slowly independently with a slightly wide gait.

PCD Patient 2: A 59 year old woman (patient NA-03-18) presented with sudden onset of falling forward, vertigo and vomiting on August 17, 2003. This resolved after 3 d, but returned 9/03, worsening over 2 d, with difficulty ambulating. Workup revealed Yo antibodies (confirmed in our laboratory), which prompted a cancer search, which showed

a left adnexal mass, leading to a diagnosis of ovarian cancer, stage IIIc. The patient was seen at the Rockefeller University Hospital 11/03, and found to have square wave jerks, moderate nystagmus, no dysarthria, normal sensation and reflexes except for absent ankle jerks, and mild dysdiadokokinesia, and a slow, wide-based gate, with imbalance on turns and the inability to tandem gait. On 2/04 the patient developed worsening appendicular and gait ataxia; at this time samples were obtained for this clinical study, and she was treated with tacrolimus, 0.25 mg/kg/d, and prednisone, 60 mg/d, with subtle gait improvement and stabilization of symptoms. Her neurologic course was characterized by slow progression over the next 2 years, with symptomatic exacerbations in 7/04, 12/04 and 9/05, and she was treated with tacrolimus/prednisone on each occasion. She has been stable since 09/05; as of 12/06, she has moderate dysarthria and appendicular ataxia that is moderate on the left and mild on the right, and she is unable to walk without assistance; she remains free of evidence of tumor with a CA-125 of 14.

iTopia Peptide Screen. Jerini Peptide Technologies (Berlin, Germany) generated a human cdr2 peptide library (peptides ≥ 80% purity). iTopia screening assays were performed according to the manufacturer's instructions (Beckman Coulter Immunomics). Data were analyzed with the iTopia^{TM} software using Prism (GraphPad).

Synthetic Peptides. Synthetic peptides used for all other experiments were obtained from Invitrogen or American Peptide Company and were ≥90% pure. The sequences of the peptides used in this study are as follows: human cdr2 (289-297) SLLEEMFLT, murine cdr2 (289-297) SLLEEMFLA, human cdr2 (290-298) LLEEMFLTV, human cdr2 (289-298) SLLEEMFLTV, and FluM1 (58-66) GILGFVFTL (2).

Cells. EA2K^b (EL4-A2/K^b, generously provided by Linda Sherman, The Scripps Research Institue, La Jolla, CA) is a stable transfectant of the murine thymoma EL4 (H-2^b haplotype) that expresses a chimeric MHC class I molecule A2/K^b (3). T2, HEK293, MCF7, and SKBR3 cell lines were obtained from the American Type Culture Collection. The AAD plasmid (4), B16.AAD, MC38.AAD, and COV413 cells were generously provided by Victor Engelhard (University of Virginia, Charlottesville, VA). The AAD-

expressing cell lines, HeLa.AAD and MCF7.AAD, were generated by transfecting HeLa and MCF7 cells, respectively, with the AAD plasmid by using Fugene6 (Roche) according to the manufacturer's instructions, followed by selection with G418 (Geneticin; Invitrogen). The HeLa.A2.1 cell line was generated by transfecting HeLa cells by the same method with pA2.1 plasmid (generously provided by Paul Robbins NIH/NCI, Bethesda, MD). PBL used for electroporation were cryopreserved leukopheresis samples of donor blood.

Primary murine kidney epithelial cell cultures (KECs) were made by mashing mouse kidneys with the back of a syringe, pipetting until a single-cell suspension was obtained, and passing the suspension over a 70 μM cell strainer. After washing, cells were cultured in D-10 medium (DMEM with 10% FBS) in 10-cm tissue culture dishes. Cells were fed by replacing medium on days 4 and 7. Between day 7 and day 9, 30 units/ml recombinant murine IFNγ (R & D Systems) was added to the cells to increase surface MHC class I expression. At 24 h, 10^9 pfu of purified adenovirus was added to each plate. The next day, cells were washed three times with PBS and harvested with trypsin EDTA for use in the ELISPOT assay.

Mice. A2.1 transgenic HHD mice were generously provided by François Lemonnier (Pasteur Institute; Paris, France). They are derived from a strain deficient for mouse β2-microglobulin and H-2D^b molecules and transgenic for a chimeric MHC class I molecule, HLA-A201/D^b, linked to human β2-microglobulin (5). AAD mice, which express the α1 and α2 domains from the HLA-A2.1 molecule and the α3 domain from the murine H2-Db molecule (6), and AAA mice, which have fully human A2.1 molecules (7), were obtained from the Jackson Laboratory. Mice were maintained in specific pathogen-free facilities at the Rockefeller University and all protocols were approved by the Institutional Animal Care and Use Committee at the Rockefeller University.

Recombinant Adenovirus. Recombinant adenovirus (E1/E3-deleted) encoding full-length human cdr2 (Ad-hcdr2) and full-length murine cdr2 (Ad-mcdr2) were constructed by using the AdEasy vector system as described (8). Adenovirus was purified either by

cesium chloride (CsCl) density centrifugation according to published procedures (9), or by using a commercially available membrane-based ion exhange system (Adenopure kit; Puresyn Inc.), titered by plaque assay, and frozen at -80°C until use.

Western Blot Analysis. Tissue culture cells were lysed in Passive Lysis Buffer (Promega) according to the manufacturer's instructions. Cell proteins were separated by 10% SDS/PAGE and transferred by semidry blotting onto an Immobilon P PVDF membrane (Millipore). The membrane was blocked for 1 h at room temperature with 10% nonfat milk in PBS + 0.1% Tween (PBST) and then incubated with PCD patient antiserum (1:3,000 in blocking solution) overnight at 4ºC. After washing with PBST, the membrane was incubated with rabbit anti-human-HRP (Jackson ImmunoResearch) at a dilution of 1:10,000 at room temperature for 1 h and washed again prior to detection by an enhanced chemiluminescence kit (Perkin Elmer Life Sciences, Inc.) according to the manufacturer's instructions.

For stripping and reprobing with γ-tubulin antibody, the membrane was washed extensively in PBST, incubated shaking in stripping buffer [62.5mM Tris, pH 6.8/2% SDS/150mM β-mercaptoethanol; (Sigma)] for 30 min at 50ºC, then washed again in PBST prior to incubation with γ-tubulin antibody (GTU 88, Sigma) and detection as described above.

Generation of cdr2 Peptide-Specific HLA-A2.1-Restricted CTL. Eight- to 10-week-old female mice were immunized intradermally in the flank with 10^9 pfu of recombinant adenovirus. As an adjuvant, 400 ng of pertussis toxin (Sigma-Aldrich) was administered intraperitoneally at 0 and 48 h. Twelve days after immunization, spleens were removed, RBCs were lysed with ACK (Biofluids Inc.), and cells were plated at 3.5 x 10^6 cells/well in a 24-well plate in R-10 media [RPMI 1640 containing 10% fetal bovine serum (FBS; HyClone), 100 μM non-essential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, 2 mM Glutamax (Invitrogen), 50 μM 2-mercaptoethanol, and 50 μg/ml gentamicin] along with 0.5 μM free peptide. After 7-10 d of culture, and every 9-14 d thereafter, 10^5 CTL were restimulated with 3.5 x 10^6 peptide-pulsed (0.5 μM) irradiated (3000 rad) RBC-

depleted splenocytes in 24 well plates in R-10 media supplemented with 10 IU /ml recombinant human IL-2 (Chiron Corp.).

Cloning of Murine cdr2-Specific HLA-A2.1-Restricted TCR-α and TCR-β cDNA.

Total RNA was extracted with the RNeasy kit (Qiagen) according to the manufacturer's instructions from 2 x 10^5 CD8-purified (MACS, Miltenyi Biotech) cdr2-290 clone 11 and clone 12 T cells. Total RNA (1 μg) was used to clone the TCR cDNAs by RACE PCR (GeneRacer kit; Invitrogen). 5'-RACE was performed using the 5' GeneRacer primer and 3' primer of gene-specific primer TCR-CαRev (5'-ACT GGA CCA CAG CCT CAG CGT CAT-3'); TCR-Cβ1Rev (5'-TGA ATT CTT TCT TTT GAC CAT AGC CAT-3'); or TCR-Cβ2Rev (5'GGA ATT TTT TTT CTT GAC CAT GGC CAT-3') as 3' primers for the TCR α– β1- or β2-chain respectively. Results were run on a gel and appropriately sized bands were excised and cloned into pCR4-TOPO vector (Invitrogen). For each of the CTL clones, plasmid DNAs were prepared from 16 individual clones from TCR α-chain cDNA, and 16 clones from TCR β-chain cDNA. Full-length insert of all 64 plasmids was confirmed by sequencing in both directions. Two independent 5'RACE PCR reactions were performed for each T cell clone.

In vitro **TCR RNA Transcription and Expression in PBL.** Gene-specific oligonucleotide primers were generated for the production of *in vitro* transcribed RNA encoding GFP from pEGFP-N1 (Clontech) and TCR α and β chains. The 5' primers included sequence for T7 RNA polymerase binding and transcription, followed by a Kozak sequence, a start codon and the next 16-17 bp of Vα or Vβ region for each TCR gene or EGFP: cdr2-TCRα fwd 5'-TAA TAC GAC TCA CTA TAG GGA GAG CCA CCA TGG ACA AGA TTC TGA CAG C-3'; cdr2-TCRβ fwd 5'-TAA TAC GAC TCA CTA TAG GGA GAG CCA CCA TGT CTA ACA CTG TCC TCG C-3'; EGFP fwd 5'-TAA TAC GAC TCA CTA TAG GGA GAG CCA CCA TGG TGA GCA AGG GCG AGG-3'. 3' primers included 64 T residues and 16-25 bp of the relevant α or β constant region sequence or EGFP sequence. Reverse primers were Cα: 5'-(64)T TTA ACT GGA CCA CAG CCT CAG CGT C-3'; C2β: 5'-(64)T TTA GGA ATT TTT TTT CTT

GAC CAT GGC C-3' or EGFP: 5'-(64)T TTA CTT GTA CAG CTC GTC C-3'. The subcloned cDNA in pCR4-TOPO was used to generate PCR products for *in vitro* transcription using the above primer sets. Resulting bands were gel purified and used for a second round of PCR amplification, followed by purification on DNA Clean & Concentrator columns (Zymo Research). T7 mMESSAGE mMACHINE High Yield Capped RNA transcription Kit (Ambion) was utilized to generate *in vitro*-transcribed RNA, which was purified by using an RNeasy Mini Kit (Qiagen) and resuspended in RNase-free water at 1-3 mg/ml.

In preparation for electroporation, donor PBLs obtained by leukopheresis were stimulated *in vitro* at a concentration of 10^6 PBL/ml with 50 ng/ml OKT-3 and IL-2 (300 IU/ml, Chiron Corp.) in a 1:1 mix of Stemline T cell Expansion medium (STEM; Sigma-Aldrich): RPMI 1640 culture medium plus 5% FBS, Glutamax (Invitrogen), and gentamicin for 3 days. After the stimulation, the cells were enriched for CD8+ T cells by MACS separation (Miltenyi Biotec). The purified CD8+ T cells were cultured for an additional 10-17 d in IL-2-containing medium before electroporation. For TCR electroporation, 2.0 μg of RNA were used per 1 x 10^6 cells in Opti-MEM serum-free medium (Invitrogen), using an ECM 830 Electro Square Porator (BTX Instrument Division) with settings as described (10). After electroporation, cells were rested for 6-8 h without IL-2 before use in FACs staining or coculture experiments.

Electroporated T Cell Cytokine Release Assays. RNA-electroporated CD8+ T cells were tested for specificity in cytokine release assays. Responder cells (1 x 10^5) and 1 x 10^5 stimulator cells (peptide-pulsed T2 or Ad-transduced KECs) in were incubated for 18-20 h in a 0.2-ml culture volume in individual wells of 96-well plates. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay using an IFN-γ ELISA kit (Pierce Endogen) according to the manufacturer's recommendations.

IFN-γ ELISPOTs Murine and Human. Enzyme-linked immunospot (ELISPOT) assay was used to quantify antigen-specific IFN-γ-producing effector cells. Briefly,

nitrocellulose-bottomed 96-well plates (MultiScreen HA, Millipore) were coated overnight at 4°C with anti-IFN-γ mAb (clone AN18 at 5 μg/ml for mouse; Pharmingen; clone 1-DIK at 10 μg/ml for human; Mabtech). Wells were washed three times with PBS and blocked for 2 h with R-10 culture medium at 37°C. For direct *ex vivo* mouse ELISPOTs, CD8+ T cells were isolated by positive selection from spleens using MACS purification (Miltenyi Biotec) and 2 x 10^5 CD8+ T cells were cocultured with 5 x 10^4 stimulator cells. For ELISPOTs with CTL lines or clones, 1 x 10^4 CD8+ T cells, purified by negative selection using a mouse CD8 isolation kit (Miltenyi Biotec), were cocultured with 5 x 10^4 stimulator cells. For human ELISPOTs with RNA electroporated human PBL, 1x10^5 CD8+ T cells were cocultured with 5 x 10^4 irradiated stimulator cells. After incubation for 18 h at 37°C, plates were washed six times with PBS + 0.05% Tween-20. Biotinylated IFN-γ mAb (clone R4-6A2 for mouse; Pharmingen; clone 7-B6-1 for human; Mabtech), the conjugate (avidin-peroxidase complex; Vectastain avidin-biotin complex method Elite Kit; Vector Laboratories) and AEC substrate (Sigma-Aldrich) were then used for spot development. All conditions were performed in triplicate wells unless otherwise indicated. Colored spots represent IFN-γ-releasing cells are reported as spot-forming-cells (SFCs) per 10^6 cells. The ELIPSOT plate evaluation was performed in a blinded fashion by an independent evaluation service (ZellNet Consulting) using an automated ELISPOT reader (Carl Zeiss) with KS Elispot 4.8 software.

Tetramer Staining. PE-labeled cdr2(290-298)/HLA-A2.1, cdr2(289-297)/HLA-A2.1, FluM1(58-66)/HLA-A2.1, PSMA(4-12)/HLA.A2.1 and negative tetramer/HLA-A2.1 were purchased from Beckman Coulter Immunomics and used according to the manufacturer's recommendations. Human cells were stained in a FACS buffer made of PBS, 1% fetal bovine serum, and 1% pooled human serum. For tetramer staining of human PBMC or electroporated CD8+ T cells, 1.0 x 10^6 cells were incubated with 1:20 dilution of tetramer for 20 minutes at room temperature. Antibody to CD8 was then added for an additional 10 minutes. Topro-3 (Molecular Probes) vital dye was added to each tube and dead cells were excluded by analyzing only cells that were negative for the dye. For tetramer staining of murine CTL, CD8+ T cells were purified by negative selection (MACs; Miltenyi Biotec) and incubated for 20 min on ice in Fc block (BD

Pharmingen) in a FACS buffer made of PBS, 5% FBS (Hyclone), 5% normal goat serum, and 1% pooled human serum. For tetramer staining 2 x 10^5 to 1.0 x 10^6 murine cells were incubated with tetramer for 20 minutes in FACS buffer at room temperature. Where indicated, antibody to CD8 was then added to the cells during the final 10 minutes. All human and murine cell samples were washed and analyzed immediately. Data was collected on a FACScaliber (Becton Dickinson) and analyzed using Flowjo software (Treestar).

CD107a Assay. Tumor cells (10^5) were placed into one well of a 24-well plate along with 10^5 TCR-electroporated or mock-electroporated CD8+ T cells in a total volume of 1 ml. FITC-conjugated CD107a antibody and GolgiStop (Becton Dickinson) were added to the well according to the manufacturer's instructions. The cells were incubated for 4 h at 37°C, washed, and analyzed by flow cytometry.

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