SUPPLEMENTAL MATERIALS

Methods

TDL Generation

Single-cell suspensions of surgically harvested tumor tissue were prepared by either enzymatic digestion or mechanical dispersion. Enzymatic digestion was used initially for larger tumor harvests (tumor volume > 1 cm^3), with incubation of tumor in a blend of collagenases (Liberase, Roche) and RPMI 1640 with L-glutamine and gentamicin for up to 2.5 hours at 37^oC on an orbital rotator; the enzymatic reaction was neutralized by addition of RPMI 1640 with 2.5% human serum albumin when approximately 80% or more of the tissue was digested. Mechanical dispersion, initially used on smaller tumors, involved sterile dissection of tissue into 2-4 mm pieces then further dissociated using either Medimachine (BD Biosciences, Bedford, MA) or, later, GentleMACS (Miltenyi Biotec, Auburn, CA) systems. The GentleMACS procedure permitted efficient processing of larger tumor volumes, and enzymatic digestion was discontinued. Single cell suspensions were counted and viability determined with Trypan Blue. If red blood cells exceed 3%, cell suspensions were lysed with ACK lysing buffer (Lonza Walkersville, Inc.) and washed with Hank's balanced salt solution (GIBCO HBSS, Invitrogen Corp.) or phosphate-buffered saline (GIBCO PBS, Invitrogen Corp.). Alternatively, and when viability was <60%, the cell suspension was separated by density-gradient centrifugation with Ficoll-Hypaque (Lonza Walkersville, Inc.) and washed with HBSS or PBS. At least 20 x 10^6 cells were costimulated with anti-CD3/anti-CD28 beads (courtesy of Carl June, University of Pennsylvania, BB-IND 6675), added at a 3:1 bead:T cell ratio, and maintained in culture at a concentration 0.25-1 x 10^6 $CD3^+$ cells/mL in TDL media containing X-VIVO 20 (Lonza Walkersville, Inc, Walkersville, MD), 5% heat-inactivated, filtered, normal-donor Type AB plasma or serum and 100 IU/mL recombinant human IL-2 (Chiron Therapeutics, Emeryville, CA), in Lifecell gas-permeable cell culture bags (Baxter Healthcare Corporation, Deerfield, IL) at 37°C in 5-7% $C0_2$. Culture interval was determined periodic sampling of the TDL culture, with harvest at the time the culture had reached the target yield of 10 x 10^6 cells/kg plus 150 x 10^6 cells for release-criteria and research testing and viable cells were at least 50% $CD3^+$ (T) cells and less than 5% $CD19^+$ (tumor) cells. Early harvest was permitted with at least 1 x 10^6 TNC/kg for clinical infusion if expansion-kinetics decline was observed. Repeat bead-costimulation was permitted to maintain active expansion when mean cell volume decreased to below 500fL, if required to meet release criteria. At the end of culture, the magnetic bead-bearing cells were removed using MaxSep Magnetic Cell Separation System (Baxter: BB-MF 4978), and TDL

products were cryopreserved in Plasmalyte A (Baxter) with 4% human serum albumin, 5% dimethyl sulfoxide (Research Industries, Salt Lake City, UT) and 6% pentastarch (B. Braun, Irvine, CA) and stored in liquid nitrogen under Good Manufacturing Practice conditions.

Release Criteria: The following minimal requirements qualified TDL products for cryopreservation and administration: a minimum of 1 x 10^6 TNC/kg for patient infusion; donor chimerism analysis: at least 90% donor cells by PCR-based tandem repeat analysis; routine bacterial culture: no growth; mycoplasma: PCR negative; endotoxin: < 5 EU/ml by limulus amebocyte lysate testing; viability: > 70% viable cells by 7-amino-actinomycin D-exclusion with flow cytometry or Trypan Blue-exclusion with microscopy; and purity: residual bead-bearing cells < 100 per 3 x 10^6 cells by microscopy; viable cells > 50% $CD3^+$ (T) cells by flow cytometry; and viable cells < 5% tumor cells by clinical pathology (cytology for Hodgkin's Lymphoma; flow cytometry for all other histologies).

TDL Characterization

Harvested tumor-infiltrating lymphocytes and expanded TDL products were phenotypically characterized with multicolor flow cytometry to quantify T-cell subsets and markers of effector function (Figure S2). TDL products were further assessed as cell quantities allowed for clonal populations with PCR for TCR γ-chain gene rearrangements and Vβ spectratyping, and screened for tumor-associated antigen (TAA)-specific $CD8^+$ donor T cells against a MHC/peptide tetramer library, as described below.

Cell Surface Phenotype: As cell numbers permitted, fresh tumor lymphocytes and thawed TDL products were evaluated for $CD4^+$ and $CD8^+$ T-cell subsets and markers of effector function by cell-surface and intracellular flow cytometry, respectively. Antibodies used for cell-surface staining were purchased from BD Biosciences unless otherwise indicated: CD3-Alexa Fluor 700; CD3-fluorescein isothiocyanate (FITC); CD4-APC-cyanin 7 (Cy7); CD4-phycoerythrin (PE); CD8-eFluor 450 (Ebioscience, San Diego, CA); CD8-FITC; CD25-PE-Cy5; CD40L-PE (R&D Systems, Minneapolis, MN); CD27-PE; CD27-APC-eFluor 780 (Ebioscience) and NKG2D-PE (R&D Systems). Cell fixation/permeabilization was performed using the Cytofix/Cytoperm kit (BD Biosciences) and intracellular staining was performed with T-bet-Alexa Fluor 647 (BioLegend, San Diego, CA) and FoxP3-Alexa Fluor 647 (BD Biosciences). Data acquisition was performed on a Gallios 10-color 3-laser flow cytometer using Kaluza 1.1 software (Beckman Coulter, Inc., Brea, CA) and analyzed using FlowJo 9.3.2 (Tree Star, Inc., Ashland, OR).

TCR Gene Rearrangement Analysis: Testing was performed following clinical protocol in the Laboratory of Pathology, Molecular Diagnostics Unit (National Cancer Institute, Bethesda, MD). TDL DNA samples were run in duplicate using 1μg of DNA per assay. A single multiplexed PCR reaction was performed to detect TCR-γ-chain gene rearrangements using primers that interrogate *TRG* rearrangements involving all of the known Vγ family members and the Jγ1/2, JP1/2 and JP joining segments.[1] To allow for fluorescence detection, each joining region primer was covalently linked to a unique fluorescent dye. The products were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA), and electropherograms were analyzed using GeneMapper v3.7 (Applied Biosystems). TRG-PCR is capable of detecting a clonal population compromising a minimum of 2 - 5% of a T-cell population.

Spectratyping Analysis of TDL Product TCR Vβ Repertoire Diversity: Total RNA was extracted from TDL, reverse-transcribed and analyzed for T cell receptor Vβ–chain repertoire diversity as described.[2] In brief, cDNA aliquots were PCR amplified using 23 TCR Vβ-specific forward primers and a constant reverse primer followed by a run-off PCR using nested 6-FAM-labeled constant primer. The PCR product was denatured and DNA fragment length assessed on an ABI 3031 XL capillary-based electrophoresis DNA sequencer. GeneMapper v3.7 software was used for quantitative repertoire analysis within Vβ families.

MHC-Peptide Tetramer Assay for TAA-Reactive $CD8^+$-T Cell Populations: Eleven distinct antigens were selected for analysis on the basis of prior reports demonstrating expression in hematologic malignancies (see list, Figure S2).[3] Soluble HLA-A2 tetramers were prepared with peptides and β2-microglobulin as previously described[4] and conjugated to phycoerythrin (PE) or allophycocyanin (APC), or purchased from Beckman Coulter. Cells were first incubated with PE- or APC-conjugated tetramers at 37°C for 30 minutes in the dark, followed by incubation with CD8-FITC or CD8-APC (Immunotech, Quebec, CAN), CD4-PerCP, CD14-PerCP and CD19 (BD Biosciences) for 30 min at 4°C. Data were collected on a FACSCanto flow cytometer and analyzed using FACSDiva Software (BD Biosciences). For each sample, 300,000 events in the forward-versus-side-scatter lymphocyte gate were collected and analyzed. A negative peptide was used to determine gating, allowing up to 0.03% false-positive events. Using > 0.1% as the threshold, the percentage of tetramer-positive cells among $CD8^+CD14^-CD19^-$ cells was recorded for each peptide.

Cytotoxicity: PanToxiLux (OncoImmunin, Gaithersburg, MD)[5] a fluorescence-based caspase-8 cleavage assay, was used to assess cytotoxic activity. TDL were incubated with the same

patient's CLL in a 10:1 Effector:Target ratio with for one hour at 37 degrees. Prior to incubation, tumor cells were labeled with CD19-APC (BD Biosciences) and a viability stain (Nuclear Fluorescent Label-1, OncoImmunin) to selectively identify tumor cells that were viable when the assay was initiated. For comparison, PBL obtained from the patient's donor were costimulated and expanded *ex vivo* following the identical procedure used for TDL generation; costimulated donor-PBL were incubated with the patient's CLL cells as described above. CLL cells incubated with PanToxiLux alone provided a control for background. Flow cytometry was performed to measure the percentage of previously viable CLL cells showing increased fluorescence due to caspase-8 cleavage.

Anti-CLL CFSE Proliferation: $1x10^6$ TDL were carboxyfluorescein succinimidyl ester (CFSE)-labeled according to manufacturer's instructions (Vybrant, Invitrogen Corp., Carlsbad, CA) and plated onto 24-well plates in 2 mL X-Vivo media plus 5% human AB serum, alone or with $3x10^6$ anti-CD3/anti-CD28 beads, $1x10^6$ PBL from PN7 (94% of lymphocytes residual $CD19^+$ CLL) or $0.2x10^6$ 3rd party, HLA-disparate dendritic cells (DC) matured from peripheral-blood monocytes *ex vivo*.

REFERENCES

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3. Beatty GL, Smith JS, Reshef R, et al. Functional unresponsiveness and replicative senescence of myeloid leukemia antigen-specific CD8+ T cells after allogeneic stem cell transplantation. *Clin Cancer Res*. 2009;15(15):4944-4953.

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5. Liu L, Chahroudi A, Silvestri G, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nat Med*. 2002;8(2):185-189.

Figure S1: Protocol Schema

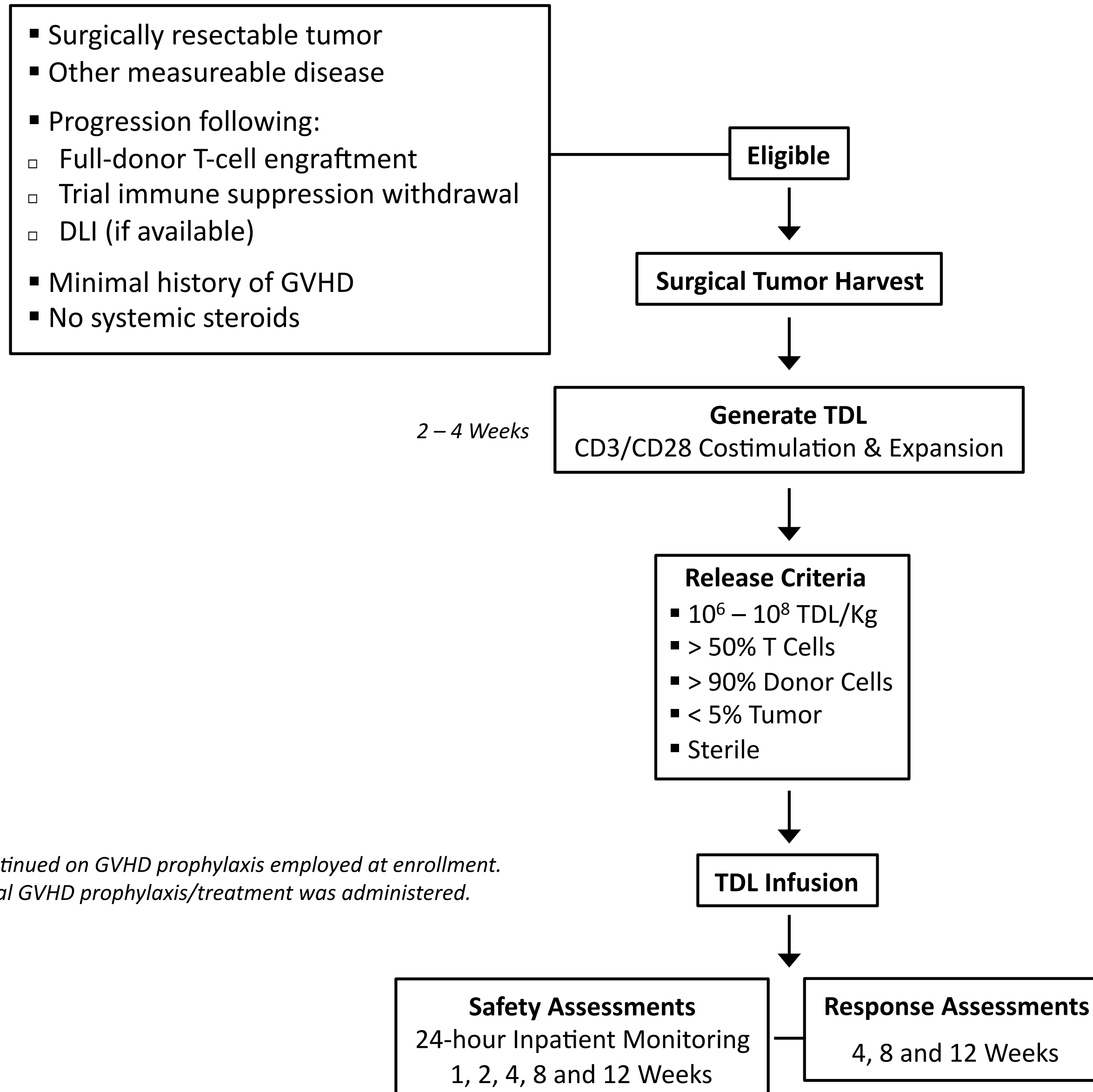
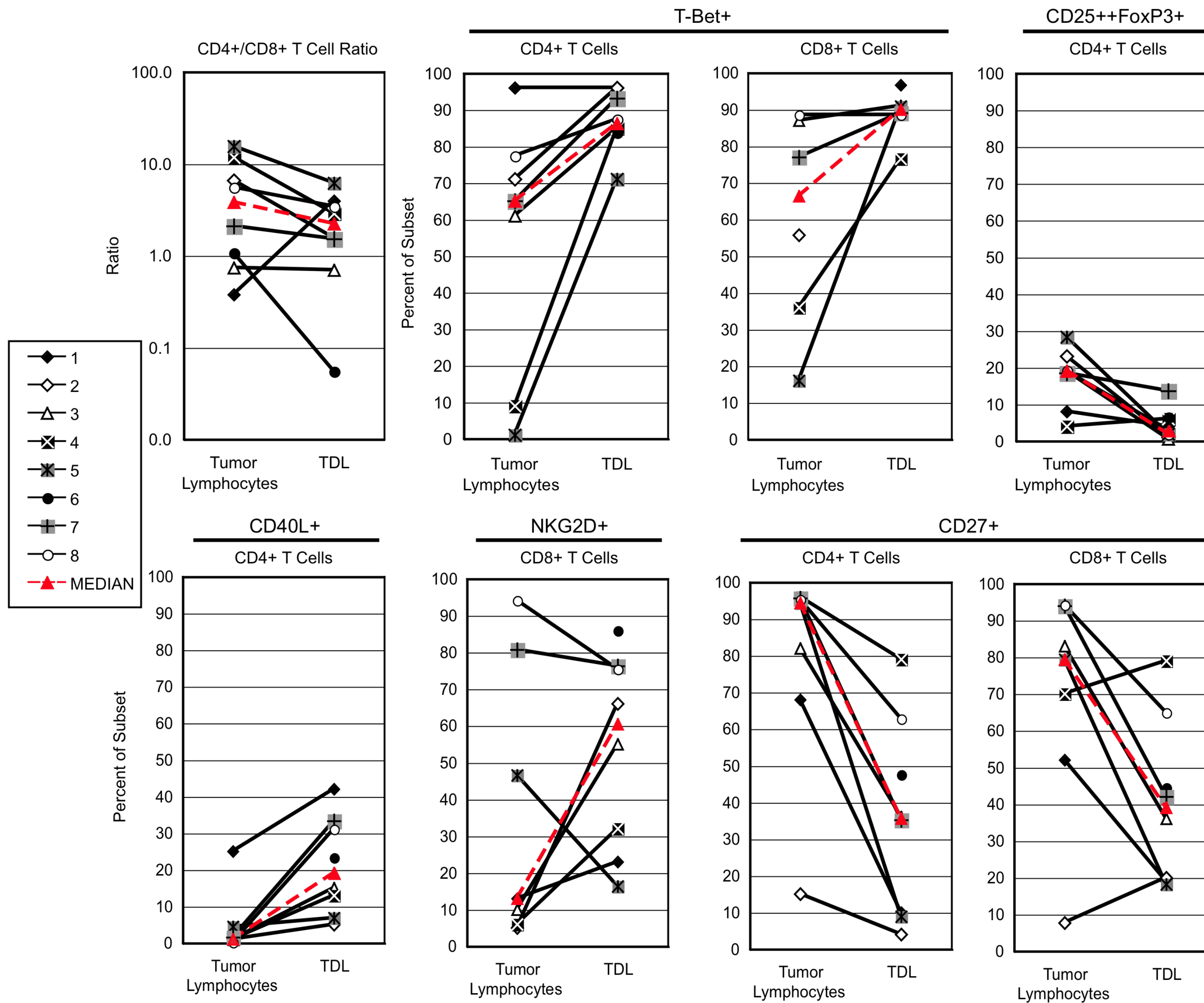


Figure S2: Surface phenotype of fresh tumor lymphocytes and TDL



The lymphocyte phenotype of fresh tumor lymphocyte culture inocula ("Tumor Lymphocytes") and final expanded TDL products ("TDL") was assessed with flow cytometry for each product. Comparisons before and after expansion include the T-cell CD4/CD8 ratio, expression of the effector markers T-Bet, CD40L and NKG2D, the regulatory marker FoxP3, and CD27, lost with replicative senescence.

Figure S3: TDL functional phenotype and activity

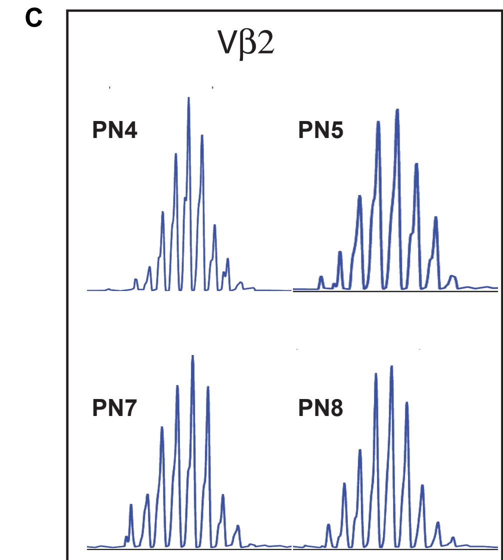
A

Patient No.	TAA-Reactive CTL (MHC-Tetramers)[B]	TCR-γ Repertoire	TCR V-β Spectratype[C]	Cytotxicity *in vitro*[D]	3rd-Party Alloreactivity[D]	Clinical Response	Clinical Reaction
1	N/A	N/A	N/A	N/A	N/A	PD	None
2	N/A	N/A	N/A	N/A	N/A	PD	None
3	N/A	N/A	N/A	N/A	N/A	PD	None
4	N/A	Polyclonal	Polyclonal	N/A	N/A	SD	None
5	N/A	Polyclonal	Polyclonal	N/A	+	NE	Tumor Flare
6	+	Polyclonal	Polyclonal	N/A	N/A	PET-PR	Pain
7	N/A	N/A	N/A	+	+	SD	None
8	+	Polyclonal	Polyclonal	N/A	N/A	PET-PR	CRS

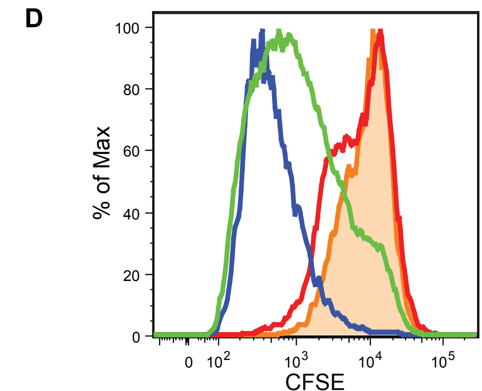
B

		EBV	HoxA9	hTERT	MAGE3	Meis1	NY-ESO-1	PRAME	Prot- 3	SSX-1	Survivin	WT1
PN6	Donor	-	-	-	•	-	-	-	•	-	•	-
	TDL	•	•	•	•	-	-	•	•	-	•	•
	Baseline	•	•	-	•	•	•	•	•	•	•	-
	24hrs	•	•	•	•	-	-	•	•	-	•	•
	Day 7	•	•	•	-	-	-	•	•	-	•	-
PN8	Donor	-	-	•	-	•	-	-	-	-	•	-
	TDL	•	•	•	•	•	-	•	•	-	•	•
	Baseline	•	•	•	•	•	-	•	NP	-	•	•
	24hrs	•	•	•	•	•	-	•	•	-	•	-
	Day 7	•	•	•	•	•	-	•	•	-	•	-

C



D



A. Functional characterization based on sample availability is summarized for each of the TDL products. N/A = sample not available for assay; NE = not evaluable. **B.** PN6 and PN8 were HLA-A2$^+$, permitting testing of donor, TDL and recipient peripheral blood lymphocytes (pre- and 24 hours and 7 days post-TDL infusion) for TAA-reactive CD8$^+$-T cell populations against an HLA-A2/TAA-peptide tetramer library. TAA-Tetramer$^+$ cells detected: ●; No TAA-tetramer$^+$ cells detected: **– ;** NP = not performed**.** Both subjects had EBV-negative HL, with predominantly reactive donor-cell tumor populations. Several TAA-tetramer$^+$ populations were identified in TDL and recipient PBL, including several that were not found in their respective donors. Of note, in PN6, hTERT and WT-1 TAA-tetramer$^+$ populations were identified in TDL and post-infusion PBL, while not found in donor or baseline-recipient lymphocytes. **C.** As sample availability allowed, TDL were assessed for TCR repertoire diversity with spectratyping. Shown are Vβ2 spectratypes for the four tested TDL products, which are representative of the Gaussian distribution observed in all 23 Vβ families tested. **D.** TDL from PN7 was tested for proliferative response to the recipient's PBL, of which 94% were circulating CLL cells. Alloproliferative response (loss of CFSE) was greatest after co-incubation with HLA-disparate 3rd party DC (green), which closely matched proliferative response to positive control (3/28 bead costimulation, blue). Very little proliferation was observed following incubation with HLA-matched PN7 PBL (red), but at culture Days 3, 4 and 5, a minor population of TDL exhibited increased proliferation relative to the majority, which closely matched the rate of spontaneous proliferation exhibited by incubation of TDL alone (orange).