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Materials and Methods^(Rapoport 2015)

NY-ESO-1 SPEAR T-cell and lentiviral vector manufacturing

Cell manufacturing. Manufacture of engineered T-cells occurred initially at the Cell and Vaccine Production Facility at the University of Pennsylvania (Philadelphia, PA), and was then transferred to Progenitor Cell Therapy (PCT, Allendale, NJ), a commercial contractor. Comparability studies were conducted and submitted to the US Food and Drug Administration to demonstrate comparability of the product at PCT. Engineered T-cells were manufactured from CD25 depleted CD4 and CD8 T-cells that were activated and expanded using anti-CD3-CD28 antibody–conjugated paramagnetic microbeads (Life Technologies, Carlsbad, CA) as previously described.^(Levine 1997) T-cells were transduced at a multiplicity of infection of 1 transducing unit (TU) per cell. The manufacturing process lasted for 9-12 days, and release testing occurred over an additional 7-10 days.

Vector. The lentiviral vector, a self-inactivating vector derived from HIV-1,^(Dull 1998) contained the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE), and the EF1 α promoter to drive transgene expression. Lentiviral vector was produced at the City of Hope (Duarte, CA) by transient transfection of 293T-cells using 4 plasmids expressing the transfer vector, Rev, VSV-G, and gag/pol. Clarified supernatant was treated with benzonase, and concentrated by tangential flow filtration and centrifugation. Transduction potency was then measured on primary T-cells.

Assays for gene-modified T-cell persistence, trafficking, and phenotypic analysis

The Translational and Correlative Studies Laboratory at the University of Pennsylvania performed research sample processing, freezing, and laboratory analyses. This laboratory operates under principles of Good Laboratory Practice (GLP), with established standard operating procedures (SOPs) and/or protocols for these procedures. Assay performance and data reporting conformed with MIATA guidelines.^(Janetzki 2009) Assays were transferred to a commercial GLP-compliant laboratory, Cambridge Biomedical (Cambridge, MA), toward the end of the study.

Sample draws and processing. Samples of peripheral blood and bone marrow were collected in K₂EDTA-coated (ie, lavender top) BD vacutainer tubes (Becton Dickinson). Samples drawn at the University of Pennsylvania were delivered to the laboratory within 2 hours of draw; samples drawn at the University of Maryland were delivered to the laboratory by overnight shipment in insulated containers at room temperature. According to established laboratory SOPs, samples were processed within 30 minutes of receipt. Peripheral blood and bone marrow mononuclear cells were purified, processed, and stored in liquid nitrogen.

Quantitative polymerase chain reaction (qPCR) analyses. Whole-blood samples were collected in K₂EDTA coated (ie, lavender top) BD vacutainer tubes (Becton Dickinson). Genomic DNA was isolated from whole blood or bone marrow using QIAamp DNA blood midi kits (Qiagen) and established laboratory SOPs, quantified by spectrophotometer and stored at -80°C until testing. qPCR analyses were performed in bulk using ABI Taqman technology and a validated assay to detect the WPRE sequence present in the lentivirus backbone, using 200 ng genomic DNA per time point for peripheral blood and bone marrow samples and using the methodology described,^(Kalos 2011) including normalizing for

amplification of input DNA using amplifications with the CDKN1A primer/probe set (NF). The WPRE primers: [5' WPRE.227.Forward: 5' CGCAACCCCCACTGGTT 3' (nt 227–244 of the WPRE gene) an antisense primer with the sequence: WPRE.289.Reverse: 5' AAAGCGAAAGTCCCGGAAA 3' (nt 270-289 of the WPRE gene)] amplify a 62-nucleotide sequence from the WPRE coding region that can be detected using a fluorescein amidite (FAM)-labeled probe [5' FAM-TTGCCACCACCTGTC 3']. Calculated values were adjusted by a factor of 0.5 (EF) to account for amplification efficiency of this primer-probe combination and on the basis of flow-cytometric analysis of the infused cell product. Values from this analysis were reported as average transgene copies per cell, calculated as follows: average transgene copies/cell = copies plasmid detected by qPCR/input DNA (ng) × 0.0063 ng DNA/cell × CDKN1A NF × EF. The lower limit of quantification (LLOQ) of the assay was established at 50 copies per µg DNA at the University of Pennsylvania and 100 copies per µg DNA at Cambridge Biomedical. The white blood cell count was used as an amplifier to calculate the number of gene-modified cells in the blood. The assumption of 1 vector copy per cell was based upon the multiplicity of infection of 1 used for cell transduction; however, the gene-modified cell population is likely to contain a subset of cells with multiple integration events.

Quantitative reverse transcription PCR (qRT-PCR) analyses. Total RNA was isolated from whole bone marrow using Ribopure™ blood kits (Ambion) and cDNA was synthesized using iScript cDNA synthesis kits (Bio-Rad). qPCR assays were performed using the inventoried and recommended ABI-based primers-probes (indicated in parentheses) to detect and quantify the relative abundance of LAGE-1 (Hs00535628_m1), NY-ESO-1 (Hs00265824), and CD138 (Hs00896423_m1) transcripts. Each data point (sample, standard curve) was evaluated in triplicate with a positive Ct value in 2/3 replicates with % CV less

than 15% required for all reported values. To control for the quality and quantity of interrogated cDNA, a parallel amplification reaction was performed using a primer-probe for the housekeeping gene *PP1B* (Hs00168719_m1), and an inventoried ABI Taqman assay. RNA isolated from the melanoma cell line A375 (positive for NY-ESO-1 and LAGE-1) generated cDNA that was used as a reference sample. Data were reported as the relative quantity (RQ) value.

Persistence of NY-ESO-1 SPEAR T-cells. Persistence of NY-ESO-1 SPEAR T-cells was measured by qPCR performed on DNA isolated from peripheral blood mononuclear cells (PBMCs) and was expressed as copies of the WPRE sequence per μg genomic DNA. The WPRE sequence is constitutive of the lentiviral vector used to transduce T-cells, but is absent from the human genome, and therefore accurately reflects the level of transduced cells among PBMCs.

Memory phenotype of persisting NY-ESO-1 SPEAR T-cells. Memory T-cell subsets were identified following hierarchical gating. Total PBMCs were gated for single viable CD3^+ T-cells and further separated based upon expression of CD4 and CD8. Within these parental CD4^+ and CD8^+ subsets, gene-modified T-cells were identified using pentamer staining. Memory T-cell subsets were further identified based upon their surface expression of CD45RA and CCR7 and CD95; central memory (CM) cells were $\text{CCR7}^+\text{CD45RA}^-$, effector memory (EM) cells were $\text{CCR7}^-\text{CD45RA}^-$, effector memory RA (EMRA) cells were $\text{CCR7}^-\text{CD45RA}^+$ (also known as terminally differentiated effector memory cells re-expressing CD45RA, TEMRA), stem cell memory (SCM) cells were $\text{CCR7}^+\text{CD45RA}^+\text{CD95}^+$, and naïve cells were $\text{CCR7}^+\text{CD45RA}^+\text{CD95}^-$.

T-cell receptor (TCR) clonotype analyses. TCR clonotype analysis was performed by Adaptive Biotechnologies (Seattle, WA) and high-throughput next-generation sequencing of the TCR V β complementarity-determining region 3 (CDR3) using the Illumina HiSeq/MiSeq platform-based immunoSEQ assay v.5. For these analyses, approximately 1000 ng (approximately 16,000 genome equivalents) of genomic DNA isolated from bone marrow samples were subjected to combined multiplex PCR and sequencing followed by algorithmic analyses to quantify individual TCR V β CDR3 sequences in samples, as described previously.^(Robins 2009)

Multiparametric flow cytometry. Cells were stored in liquid nitrogen vapor phase and thawed prior to evaluation by flow cytometry on the same day. Approximately 1×10^6 cells/condition were stained and acquired using a BD LSR II cytometer (BD Biosciences). Compensation values were established using single antibody stains and BD compensation beads (BD Biosciences). Data were analyzed using FlowJo v9.6.2 (Treestar), R v3.3.1 (R Foundation for Statistical Computing),^(R Core Team 2014) Pestle (NIH), and Simplified Presentation of Incredibly Complex Evaluations (SPICE; NIH) software packages.

Flow cytometry detection reagents. The following antibodies were used for T-cell phenotyping: CD8-BV650 (BD), CD4-BV605 (BD), CD3-Alexa Fluor700 (BD), CD95-BV711 (BD), CD45RO-PerCPCy5.5 (BD), CD25-APC-Cy7 (BD), CD127-BV421 (BioLegend), CCR7-PE-Cy7 (BioLegend), CD45RA-ECD (Beckman Coulter), PD-1-BV785 (BioLegend), LAG3-FITC (R&D Systems), and TIM-3-APC (R&D Systems). The following antibodies were used for intracellular cytokine staining (as phenotyping unless stated): CD3-V450 (BD), CD45RA-APC-Cy7 (BioLegend), IL-2-AlexaFluor700 (BioLegend), TNFa-APC

(BD), IFN γ -BV711 (BioLegend), and Ki-67-FITC (BD). The dead cell exclusion stain (Live/Dead Aqua) was purchased from Invitrogen. To detect transduced NY-ESO-1^{e259}TCR-expressing cells, PE-conjugated pentamer reagents specific for the HLA-A*02:01 SLLMWITQC complex (ProImmune; Oxford, UK) were used at the manufacturer's recommended concentrations.

T2 cells pulsing with peptide. T2 cells were cultured at 1×10^6 cells/mL in RPMI-10% FBS in the presence of the NY-ESO-1 (9V) peptide (SLLMWITQV, Peptide Protein Research) at 1 μ M, or with vehicle only (DMSO 0.027% volume/volume). After an overnight incubation at 37°C, 5% CO₂, non-pulsed or pulsed T2 cells were centrifuged and the supernatants were discarded. Non-pulsed T2 and pulsed T2 cells were resuspended in RPMI-10% FBS and were used in the ICS assay.

Intracellular cytokine staining assay with pentamer staining. On the day of the assay, cryopreserved PBMC from patients enrolled in the clinical trial, as well as PBMC from a healthy donor control and NY-ESO-1 TCR transduced cells, were thawed and counted. Pentamer staining was performed before the 6-hour stimulation period. Briefly, PBMC (1.5×10^6 cells per well) was first incubated with the NY-ESO-1 pentamer in 50 μ L of 2% FBS for 10 minutes at room temperature. The cells were then washed in RPMI-10% FBS, and the NY-ESO-1 pulsed T2 cells (7.5×10^5 cells per well) were added in RPMI-10% FBS. The Golgi Plug/Golgi Stop reagent was added and the incubation was resumed for an additional 5 hours at 37°C and 5% CO₂. The cells were washed and were surface stained at 4°C for 30 minutes (Live/Dead Aqua, CD4, CD8, CCR7, and CD45RA) followed by fixation and permeabilization and intracellular staining (Ki67, TNF- α , IL-2, CD3, and IFN- γ) at 4°C for

30 minutes. Labeled cells were acquired on an LSR II flow cytometer using the FACS DiVa software (BD Bioscience).

Note: For the phenotyping, flow cytometry gating was used to select T-cells carrying the engineered TCR from within the whole T-cell population. During functional testing, the entire T-cell population was exposed to peptide-bearing T2 cells, but responses were only observed in T-cells carrying the engineered TCR; no response was observed to unpulsed T2 cells or in samples without the NY-ESO-1 SPEAR TCR.

References (supplemental)

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Lenalidomide Maintenance Therapy Criteria

Prior to lenalidomide maintenance phase, all study participants must be registered into the mandatory RevAssist[®] program, and be willing and able to comply with the requirements of RevAssist[®]. At around day 100 post-transplant, after completion of post-transplant immunological assessments and myeloma restaging studies, patients will be eligible to receive low-dose lenalidomide for maintenance therapy (10 mg/day) until progression of myeloma or development of intolerance. Because of the increased risk of thromboembolic events, patients will also receive low-dose aspirin (81 mg/day or 325 mg/day). Preparations for lenalidomide maintenance therapy should be made at least 7-10 days before the day 100 visit in order to minimize delay in starting it. In addition, at 1 year post-transplant, patients may receive a standard 23-valent pneumococcal polysaccharide vaccine in accordance with the recommendations of the American Society for Blood and Marrow Transplantation. Prior to starting lenalidomide, patients should have adequate bone marrow function defined as: platelet count >50,000/ μ l and absolute neutrophil count >1000.

Supplemental Table 1: Hematologic and non-hematologic adverse events occurring at toxicity grade 3 or 4 by preferred term (ITT population)

Preferred term	N = 25 N (%)	
	Grade 3	Grade 4
Hematologic toxicities		
Febrile neutropenia	15 (60)	2 (8)
Anemia	14 (56)	0 (0)
Neutropenia	4 (16)	7 (28)
Leukopenia	1 (4)	13 (52)
Lymphocyte count increased	1 (4)	0 (0)
Lymphopenia	0 (0)	2 (8)
Thrombocytopenia	0 (0)	18 (72)
Non-hematologic toxicities	Grade 3	Grade 4
Diarrhea	10 (40)	0 (0)
Hypophosphatemia	6 (24)	0 (0)
Hypocalcemia	4 (16)	1 (4)
Graft versus host disease	3 (12)	0 (0)
Hypokalemia	3 (12)	0 (0)
Rash	3 (12)	0 (0)
Stomatitis	3 (12)	0 (0)
Abdominal pain	2 (8)	0 (0)
Fatigue	2 (8)	0 (0)
Hyponatremia	2 (8)	0 (0)
Hypoxia	2 (8)	0 (0)
Mucosal inflammation	2 (8)	0 (0)
Neutropenic colitis	2 (8)	0 (0)
Nausea	2 (8)	0 (0)
Esophagitis	2 (8)	0 (0)
Pain in extremity	2 (8)	0 (0)
Atrial fibrillation	1 (4)	1 (4)
Hypotension	1 (4)	1 (4)
Pneumonitis	1 (4)	1 (4)
Acute kidney injury	1 (4)	0 (0)
Arthralgia	1 (4)	0 (0)

Asthenia	1 (4)	0 (0)
Autoimmune disorder	1 (4)	0 (0)
Colitis	1 (4)	0 (0)
Decreased appetite	1 (4)	0 (0)
Deep vein thrombosis	1 (4)	0 (0)
Dehydration	1 (4)	0 (0)
Ejection fraction decreased*	1 (4)	0 (0)
Erythema multiforme	1 (4)	0 (0)
Failure to thrive	1 (4)	0 (0)
Gastrointestinal pain	1 (4)	0 (0)
Hematuria	1 (4)	0 (0)
Hepatic infection	1 (4)	0 (0)
Hydronephrosis	1 (4)	0 (0)
Hypercalcemia	1 (4)	0 (0)
Hypertension	1 (4)	0 (0)
Joint range of motion decreased	1 (4)	0 (0)
Lower gastrointestinal hemorrhage	1 (4)	0 (0)
Malnutrition	1 (4)	0 (0)
Muscular weakness	1 (4)	0 (0)
Myoglobin urine	1 (4)	0 (0)
Oropharyngeal pain	1 (4)	0 (0)
Periodontal disease	1 (4)	0 (0)
Pneumonia	1 (4)	0 (0)
Pulmonary edema	1 (4)	0 (0)
Radiculopathy	1 (4)	0 (0)
Skin lesion	1 (4)	0 (0)
Staphylococcal infection	1 (4)	0 (0)
Supraventricular tachycardia	1 (4)	0 (0)
Syncope	1 (4)	0 (0)
Tinea infection	1 (4)	0 (0)
Unresponsive to stimuli	1 (4)	0 (0)
Upper respiratory tract infection	1 (4)	0 (0)
Viral upper respiratory tract infection	1 (4)	0 (0)
Blood creatine phosphokinase increased	0 (0)	1 (4)

Hip fracture	0 (0)	1 (4)
Hyperglycemia	0 (0)	1 (4)
Hypomagnesemia	0 (0)	1 (4)
Neutropenic sepsis	0 (0)	1 (4)

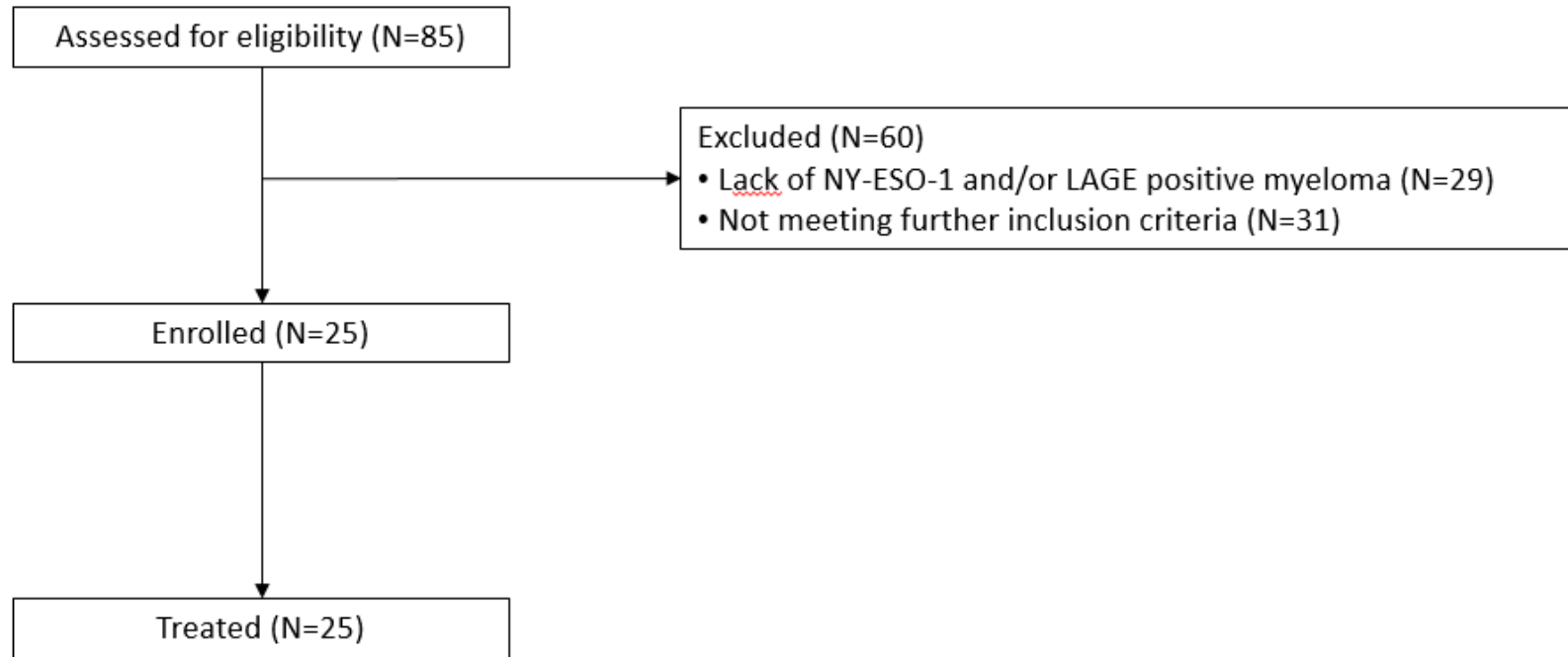
Observation period: administration of high-dose melphalan at day -2 to end of intervention phase.

Adverse events were coded using MedDRA Version 20.0. Patients were counted once for each preferred term.

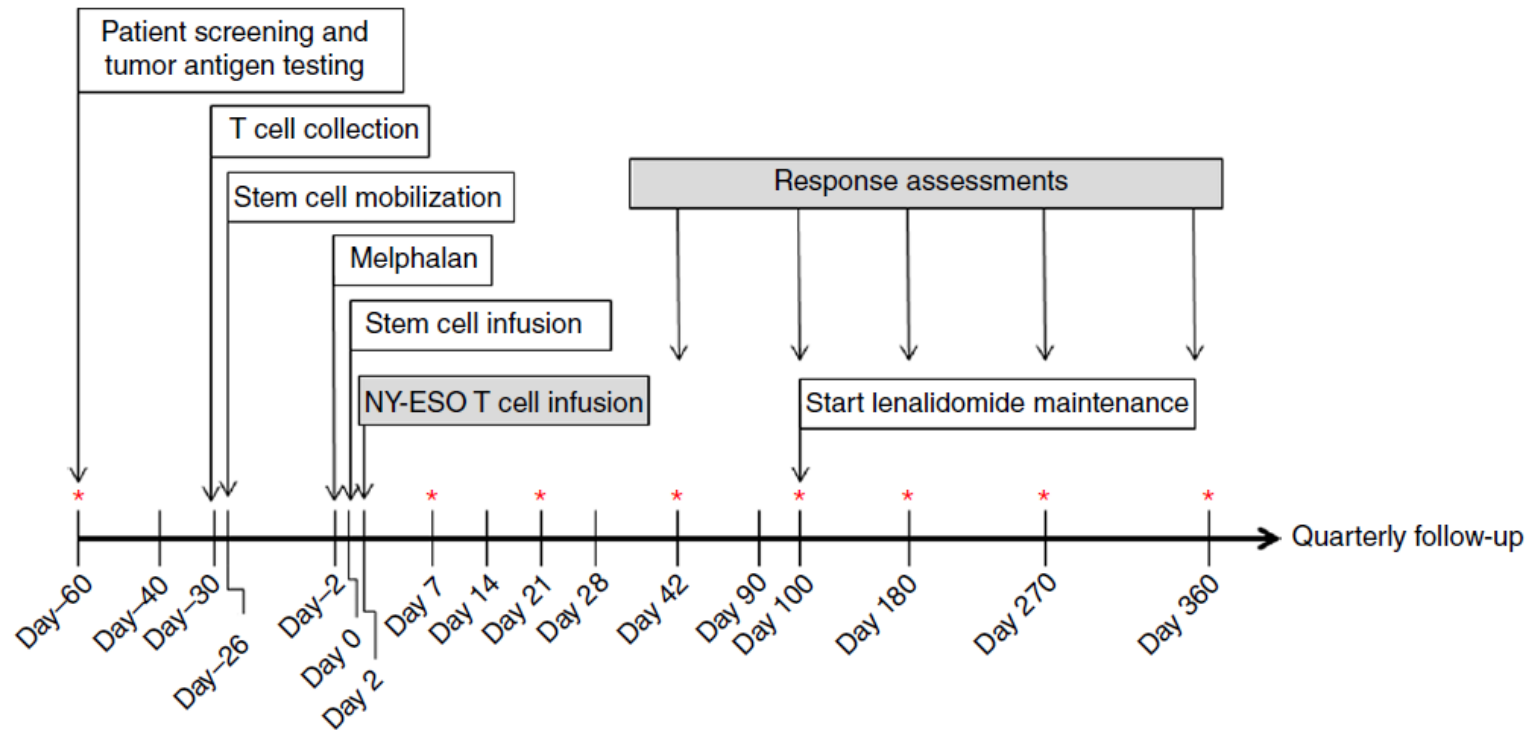
Adverse event data are listed in descending order of frequency of Grade 3 events.

*Adverse event “ejection fraction decreased” did not resolve within the observation period.

Supplemental Figure 1. Study CONSORT diagram



Supplemental Figure 2. Overview of clinical study. (Rapoport et al, *Nat Med.* 2015)



Legend: Patient screening, including HLA testing and tumor antigen testing, and apheresis scheduling requires 2–4 weeks. Manufacture of gene-modified cells takes 3–4 weeks. Patients received high-dose melphalan 2 d before stem cell infusion and 4 d before T cell infusion. Response was assessed at days 42, 100 and 180, and quarterly thereafter. Asterisks indicate optional bone marrow biopsies. For eligible patients, maintenance lenalidomide was given starting at day 100. Once off-study, patients are monitored for up to 15 years for delayed adverse events in accordance with FDA guidance.

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