Supplementary Methods

Details on TIL expansion from tumor fragments and the rapid expansion protocol (REP) used to generate the final TIL infusion product

Multiple tumor fragments (1 per well in 24-well plates) were placed in culture with TIL culture medium (RPMI-1640 supplemented with 10% human AB serum, 10 mM HEPES pH 7.4, 100 Units/ml Penicillin G, 100 μg/ml Streptomycin, 50 μg/ml Gentamicin and 50 μM β-mercaptoethanol) and 6,000 IU/ml of IL-2 (Proleukin^{TM}; Prometheus, San Diego, CA). The cells were cultured for up to 5 weeks. Cultures with visible TIL were allowed to grow to confluence and sub-cultured by splitting 1:1 with TIL culture medium containing 6,000 IU/ml of IL-2. Viable cell counts were performed using Trypan Blue exclusion with a hemocytometer or a Cellometer™ automated cell counting instrument (Bethesda, MD). TIL obtained after 5 weeks in culture from tumor fragments were designated as "pre-REP" TIL. The pre-REP TIL were cryopreserved and tested for sterility after testing for anti-tumor reactivity (see below). These cells were thawed for secondary expansion to treat patients.

Patients with initial total pre-REP TIL outgrowth of at least 48 x10^6 from all cultured tumor fragments were eligible to receive ACT after secondary large-scale expansion of the cells using a rapid expansion protocol (REP). This allowed for a minimum of 40 x 10^6 cells to be expanded in the REP following removal of 8 x 10^6 cells for quality control (sterility) analysis. When a patient was to be treated, up to 50 x 10^6 pre-REP TIL (depending on the yield after cryopreservation and thawing) were expanded using the REP, as previously described (1-3). Briefly, the TIL (1 x 10^6 cells per flask) were activated using anti-CD3 antibody (Orthoclone OKT-3, Abbott Labs), 6,000 IU/ml IL-2 (ProLeukin™), and irradiated PBMC feeder cells (200:1 feeder-to-TIL ratio) obtained from pooled normal donor G-CSF-mobilized apheresis products (3-6 normal donor products pooled) obtained from the Dept. of Stem Cell Transplantation and

Cellular Therapy at MD Anderson Cancer Center. The TIL were expanded with periodic feeding with culture medium and IL-2 for 14 days to a final volume of 20 to 60 L. On day 14, volume reduction was achieved by washing and concentrating the cell product down to an approximate volume of 500 ml infusion product in saline using Cobe 2991 cell washers (Gambro). A portion of the final product (0.5-1.0%) was frozen down and used for lab correlative studies.

Melanoma cell lines and measurement of anti-tumor cell reactivity of pre-REP TIL

We attempted to generate an autologous melanoma cell line for each patient from the tumor sample used for pre-REP TIL outgrowth. Briefly, leftover tumor nodule tissue was digested using a cocktail of enzymes consisting of collagenase, hyaluronidase Type V, and DNase I Type IV (Sigma-Aldrich, St. Louis, MO) and the tumor cells enriched after centrifugation over a Ficoll gradient, as described previously (4, 5). The tumor cells were plated into 6-well plates and then transferred to T-25 and T-75 flasks as the cells expanded. The lines were expanded for 5 weeks and used as autologous tumor targets to measure pre-REP TIL anti-tumor reactivity. We were successful in 17/31 cases to generate an autologous tumor line for testing. TIL from successfully expanded tumor fragment cultures (at least 48 x 10^6 TIL) were tested for anti-tumor reactivity by measuring IFN-γ production using ELISA on culture supernatants collected from triplicate 24-h co-cultures of 1 x 10^5 TIL with 1 x 10^5 cells from autologous melanoma lines in 96-well plates. In cases where an autologous tumor line was not available, cells from an allogeneic a tumor cell line that matched at least one HLA-A loci from our melanoma cell line bank were used as targets to determine TIL reactivity. In some cases, targets from both a matched allogeneic tumor cell line target and an autologous tumor cell line were used. At least one HLA-A-unmatched (at both loci) tumor cell lines were used as a negative control. A positive control for IFN-γ production consisted of wells with TIL alone treated with PMA (50 pg/ml) and Ionomycin (2 μg/ml). IFN-γ

ELISA was performed done using ELISA kits (Pierce, St. Louis, MO). An average ≥100 pg/ml after subtraction of the HLA-A-unmatched control was considered as positive.

Determination of clinical responses using irRC

The methods to derive the irRC (6) overall responses are shown in Table S1 (Supplementary Data on-line). All measurable lesions, up to a maximum of five lesions per organ and ten lesions in total were identified as *index* lesions to be measured and recorded on the medical record at baseline. Lesions were selected based on their size (lesions with the longest diameters), their suitability for accurate repeat assessment by imaging techniques, and how representative they were of the patient's tumor burden. Lesions that were accurately measured in two perpendicular diameters, with at least one diameter ≥10 mm, were defined as measurable lesions. In the case of cutaneous lesions, ≥ 5 mm in diameter were considered measurable. The area for each index lesion was calculated as the product of the largest diameter with its perpendicular and a sum of the products of diameters (SPD) for all *index* lesions was calculated and considered the baseline sum of the products of diameters. Measurable lesions, other than *index* and all sites of non-measurable disease were identified as *non-index* lesions. *Non-index* lesions were recorded on the medical record and evaluated at the same assessment time points as the *index* lesions. Clinical responses were determined complete assessment of evaluable lesions with physical examination of visible lesions (e.g., subcutaneous lesions near the skin surface) and CT scans taken at baseline, 6 weeks (+/- 7 days), 12 weeks (+/- 7 days), and 16-20 weeks, as well as every 3 months, after TIL infusion. Immune-Related Best Overall Response (irBOR) was the best confirmed irRC overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local surgery or palliative radiotherapy for painful bone lesions) for the individual subjects in the study. For

assessment of irBOR, all available assessments per subject were considered. If a lesion was surgically resected or treated with definitive radiosurgery, the size of the lesion prior to the definitive local therapy was included in the calculated irBOR. Complete responses and progressions were confirmed by a second, consecutive assessment at least four week apart.

Flow cytometry staining methodology

Briefly, the samples were thawed in pre-warmed complete media (RPMI-1640 supplemented with 10% human AB serum, 10 mM Hepes, 100 Units/ml Penicillin G, 100 µg/ml Streptomycin, 50 µg/ml Gentamicin and 50 µM β-mercaptoethanol), washed in PBS, and first stained with a fixable viability dye (LIVE⁄DEAD® Fixable Aqua Dead Cell Stain, Invitrogen) for 30 min at 4°C. Cells were then washed twice in FACS Wash Buffer/FWB (D-PBS, 1% BSA), re-suspended in FACS staining buffer/FSB (D-PBS, 1% BSA, and 10% goat serum) and stained with antibody mixtures at the manufacturer's recommended concentration or otherwise experimentally optimized concentrations for 20 minutes in the dark, at room temperature. The Perforin intracellular staining was done according to manufacturer's instructions. Samples were subsequently washed twice in FACS wash buffer and fixed in 1% paraformaldehyde solution in PBS containing 0.2% ethanol.

Detection and measurement of telomere length in TIL by Southern blotting

TIL genomic DNA was isolated using a Qiagen DNA Prep kit following the manufacturer's instructions. Genomic Southern blotting was performed using a TeloTAGGG Telomere Length Assay kit (Roche Diagnostics, Indianapolis, IN). Briefly, 2 µg genomic DNA was digested by restriction enzymes HinfI and RsaI and then separated on a 1% agarose gel. Known telomere repeat lengths ranging from 21.4 to 1.9 kb were run along side the digested samples. The

fractionated DNA was transferred overnight onto Nytran supercharged membranes using the Turbo blotter system with 20X SSC buffer (Schleicher and Schell Bioscience). Following 3 hours of hybridization at 42ºC with the digitoxigenin (DIG)-labeled telomere repeat probe supplied with the kit, the membrane was washed and incubated with anti-DIG-alkaline phosphatase solution for 30 min at room temperature. The telomere length signal was detected by chemoluminescence reagents (Pierce, St. Louis, MO) and the blots exposed to BioMax Light chemoluminescence film. The exposed films were imaged using a Perfection V700 Flatbed Scanner (Epson) and saved as TIFF image files for analysis. The telomere lengths were determined using the ImageJ analysis program downloaded from the National Institutes of Health (NIH, Bethesda, MD) website *(*rsbweb.nih.gov/ij/). A standard curve of migration distance from the first and longest telomere standard (21.4 Kb) to the last and shortest telomere standard (1.9 Kb) was established, with the 21.4 kb band designated as 0 mm and a migration distance assigned to each subsequent standard in mm from this point. All telomeres detected were within this range. The position of the maximal telomere signal density in each sample lane was determined and this position was then converted into telomere size in kb by matching this same location in the lanes with the standards and then converting this mm length to actual telomere length in kb using the standard curve. This highest frequency telomere length in the each sample (i.e., the telomere length in the highest frequency of the cells in the sample) was used as the overall telomere length used to compare responders and non-responders. This approach is consistent with that used in the telomere research field to determine and compare telomere lengths between samples (7-12).

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