

Akt Inhibition Enhances Expansion of Potent Tumor-Specific Lymphocytes with Memory Cell Characteristics

Joseph G. Crompton^{1,2,3}, Madhusudhanan Sukumar¹, Rahul Roychoudhuri¹, David Clever^{1,3}, Alena Gros¹, Robert L. Eil¹, Eric Tran¹, Ken-ichi Hanada¹, Zhiya Yu¹, Douglas C. Palmer¹, Sid P. Kerkar¹, Ryan D. Michalek⁴, Trevor Upham¹, Anthony Leonardi¹, Nicolas Acquavella¹, Ena Wang⁵, Francesco M. Marincola⁵, Luca Gattinoni¹, Pawel Muranski¹, Mark S. Sundrud⁶, Christopher A. Klebanoff^{1,7}, Steven A. Rosenberg¹, Douglas T. Fearon³, and Nicholas P. Restifo¹

Abstract

Adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) results in complete regression of advanced cancer in some patients, but the efficacy of this potentially curative therapy may be limited by poor persistence of TIL after adoptive transfer. Pharmacologic inhibition of the serine/threonine kinase Akt has recently been shown to promote immunologic memory in virus-specific murine models, but whether this approach enhances features of memory (e.g., long-term persistence) in TIL that are characteristically exhausted and senescent is not established. Here, we show that pharmacologic inhibition of

Akt enables expansion of TIL with the transcriptional, metabolic, and functional properties characteristic of memory T cells. Consequently, Akt inhibition results in enhanced persistence of TIL after adoptive transfer into an immunodeficient animal model and augments antitumor immunity of CD8 T cells in a mouse model of cell-based immunotherapy. Pharmacologic inhibition of Akt represents a novel immunometabolic approach to enhance the persistence of antitumor T cells and improve the efficacy of cell-based immunotherapy for metastatic cancer. *Cancer Res*; 75(2); 296–305. ©2014 AACR.

Introduction

Adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) is emerging as a curative therapy for advanced cancer (1, 2). In previous ACT trials for patients with metastatic melanoma, the following features of TIL have been associated with objective response: long telomeres of infused cells, expression of the memory-marker CD27, and persistence of cells in circulation 1 month after transfer (3). These findings suggest that transfer of TIL with features characteristic of memory T cells may improve the efficacy of ACT for advanced melanoma

(4). This notion has also been corroborated by findings from murine models of ACT in which there is a progressive loss of antitumor function as T cells mature toward terminal differentiation (5). Therapeutic TIL isolated for ACT, however, are characterized by a terminally differentiated phenotype that is associated with diminished antitumor activity and poor capacity for long-term persistence (Supplementary Fig. S1; ref. 6). Collectively, these findings suggest that promoting immunologic memory in TIL may enhance antitumor immunity and the curative potential of ACT for advanced cancer.

Recent findings have highlighted the importance of the PI3K–Akt–mTOR pathway in regulating CD8 T-cell differentiation and memory formation (7–11). Akt coordinates transcriptional programs triggered by activation of the T-cell receptor (TCR) and interleukin-2 (IL2) to drive expression of key adhesion and cytolytic molecules that distinguish effector versus memory T cells (12). Despite the canonical role of Akt in controlling glucose metabolism in diverse cell types (13), there is emerging evidence that it is not an obligate regulator of CD8 T-cell metabolism (10, 14). It has been shown that constitutively active Akt promotes cell growth and survival of CD4 T cells, but not CD8 T cells (15–17). More recently, it was shown that loss or reduction of Akt signaling does not compromise T-cell proliferation or survival, but causes differentiated cytotoxic T cells to transcriptionally reprogram from an effector to memory phenotype (10).

The observation that Akt inhibition does not significantly alter metabolism and proliferation of CD8 T cells, but promotes a transcriptional program that drives memory may be therapeutically important because response to ACT not only correlates with features of immunologic memory, but also absolute number of

¹National Cancer Institute (NCI), NIH, Bethesda, Maryland. ²Department of Surgery, University of California Los Angeles, Los Angeles, California. ³Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom. ⁴Metabolon Incorporated, Durham, North Carolina. ⁵Sidra Medical and Research Centre, Doha, Qatar. ⁶Department of Cancer Biology, The Scripps Research Institute, Jupiter, Florida. ⁷Clinical Investigator Development Program, NCI, NIH, Bethesda, Maryland.

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Current address for Nicolas Acquavella: Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Miami, Florida.

J.G. Crompton and M. Sukumar contributed equally to this article.

Corresponding Authors: Joseph G. Crompton, National Cancer Institute, NIH, 10 Center Drive, Bldg. 10, Bethesda, MD 20892. Phone: 443-220-3157; Fax: 301-402-1738; E-mail: joe.crompton@nih.gov; Madhusudhanan Sukumar, sukumarm2@mail.nih.gov; and Nicholas P. Restifo, restifo@nih.gov.

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adoptively transferred TIL. We were therefore interested in exploring whether inhibition of Akt could promote features of memory in tumor-infiltrating cytotoxic T cells without significantly modulating metabolism or cell proliferation.

To evaluate this, we expanded TIL in the presence of a well-characterized allosteric inhibitor of Akt (18) that has previously been used in murine cytotoxic T cells (10). We found that human TIL cultured in Akt inhibitor demonstrate enhanced features of immunologic memory that correlate with improved long-term persistence after adoptive transfer. Although Akt inhibition did not compromise TIL proliferation, we were somewhat surprised to find that it significantly modulated the metabolic profile of TIL. Importantly, in a murine model of ACT, we show that Akt inhibition improved antitumor immunity of cytotoxic T cells. Taken together, these findings support the use of pharmacologic approaches to enhance cell-intrinsic qualities of TIL that may potentially augment antitumor immunity and improve the efficacy of cell-based immunotherapy for advanced cancer (Supplementary Fig. S1).

Patients and Methods

Patient cell samples

Human cells used in this study were isolated from patients with metastatic melanoma receiving treatment under institutional review board-approved clinical protocols (NCT01319565 or NCT00670748) in the Surgery Branch of the National Cancer Institute. Informed consent was obtained from all subjects. Tumor-infiltrating T lymphocyte (TIL) cultures were expanded to clinical-scale as previously described (19). Briefly, tumor fragments or digests were cultured in 6,000 IU/mL IL2 for 2 weeks, and subsequently expanded with a rapid expansion protocol using 30 ng/mL OKT3 (anti-CD3) antibody (Miltenyi Biotec) and 6,000 IU/mL IL2 in the presence of irradiated (50 Gy) allogeneic feeder cells at a 200:1 ratio of feeder cells to TIL. TIL culture media was supplemented with 1 $\mu\text{mol/L}$ Akt inhibitor (PubChem Compound Identification: 10196499, also called Akt inhibitor VIII or Akti-1/2) purchased from Calbiochem. TIL were harvested for myriad assays 30 days after initiation of culture, including FACS analysis, coculture with tumor targets, microarray analysis, adoptive-transfer into NSG mice, and metabolomic analysis.

Mice and tumor lines

Thy1.1 and Ly5.1 Pmel-1TCR-transgenic (Pmel) mice have been described previously (20). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice and C57BL/6 N (B6) mice were purchased from The Jackson Laboratory and NCI Frederick. Mice were housed in the NIH Clinical Research Center vivarium and maintained in compliance with the NIH Animal Care and Use Committee. Mice were excluded from analysis if less than 6 weeks old and not age- and gender-matched with experimental cohort. Mice were randomized to treatment group and investigators blinded when measuring outcomes of tumor size, survival after adoptive transfer, and histopathologic analysis. Splenocytes from Pmel mice were stimulated with hgp100₂₅₋₃₃ peptide (1 $\mu\text{mol/L}$) and 100 IU/mL recombinant human IL2 (rhIL-2; Novartis) in the presence or absence of 1 $\mu\text{mol/L}$ Akti-1/2 (Akti; Calbiochem) and CD8⁺ T cells were harvested at day 5. Secondary stimulation was performed using peptide-pulsed irradiated B6 feeder cells. The B16F10 tumor line (B16) was obtained from the National Cancer Institute tumor repository and tested for mycoplasma contamination.

Gene expression analysis and cytokine production assays

For real-time RT-PCR, RNA was extracted with RNeasy Kits (Qiagen) and cDNA was generated using High Capacity RNA-to-cDNA Kits (Applied Biosystems). Real-time RT-PCR was performed on a CFX96 thermal cycler (Bio-Rad) using primer/probe sets for indicated genes and ACTB (Applied Biosystems). For cytokine production assays, coculture of TIL with autologous tumor was performed and supernatants assessed for the presence of gamma-interferon (IFN γ) by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's protocol.

Adoptive cell transfer

Tumor therapy was performed as described previously (20). Briefly, Pmel splenocytes were stimulated with hgp100₂₅₋₃₃ peptide and expanded in rhIL-2 (1,000 IU/ml) to model clinical protocol of expanding human TIL in high-dose IL2. Expanded splenocytes (2×10^6) were subsequently transferred into irradiated (6 Gy) B6 mice with established subcutaneous B16 melanoma tumor and VVhgp100(1e7pfu) was administered upon transfer. Intraperitoneal injections of rhIL-2 were administered twice daily for 3 days after transfer. To measure engraftment and homeostatic proliferation of murine Pmel CD8⁺ T cells, 1×10^6 cells were adoptively transferred with coadministration of VVhgp100 (1e7pfu) into irradiated (6 Gy) B6 mice after *ex vivo* stimulation (with hgp100₂₅₋₃₃ peptide) and expansion in 100 IU/ml rhIL-2. Transferred cells were enumerated with hemocytometer and FACS staining with conjugated antibodies (all from BD Pharmingen) with specificity against the following: CD8 (catalog number 557654), CD27 (560691), CD62L (553151), Thy1.1 (557266), and Ly5.1 (553775). To measure engraftment and homeostatic proliferation of human TIL, 1×10^7 cells were adoptively transferred into NSG mice after *ex vivo* expansion as described above. Intraperitoneal injections of rhIL-2 were administered twice daily for 3 days after transfer. Transferred cells were enumerated with hemocytometer and FACS staining with conjugated antibodies with specificity against the following: hCD3 (BD Biosciences 557694), hCD4 (Biolegend 317428), hCD8 (BD Biosciences 560179), and hCD62L (Biolegend 304822).

Microarray analysis

Human TIL from 3 patients were isolated and expanded *ex vivo* as described above. After 30 days expansion, T lymphocytes were enriched for the CD8⁺ population by Miltenyi magnetic column separation (order no. 130-096-495) according to the manufacturer's instructions. RNA (100 ng) was extracted from CD8⁺ TILs using Ovation Pico WTA System V2 (NuGEN) according to the manufacturer's instructions. Briefly, first-strand cDNA was synthesized using the SPIA tagged random and oligo dT primer mix in 10- μL reactions after denaturation and incubated at 65°C for 2 minutes and priming at 4°C followed by extension at 25°C for 30 minutes, 42°C for 15 minutes and 77°C for 15 minutes. Second-strand cDNA synthesis of fragmented RNA was performed using DNA polymerase at 4°C for 1 minute, 25°C for 10 minutes, 50°C for 30 minutes, and 80°C for 20 minutes. 5' double stranded cDNA was used as the template for isothermal single-strand cDNA amplification following a cycle of DNA/RNA primer binding, DNA replication, strand displacement, and RNA cleavage at 4°C for 1 minute, 47°C for 75 minutes, and 95°C for 5 minutes in a total 100- μL reaction.

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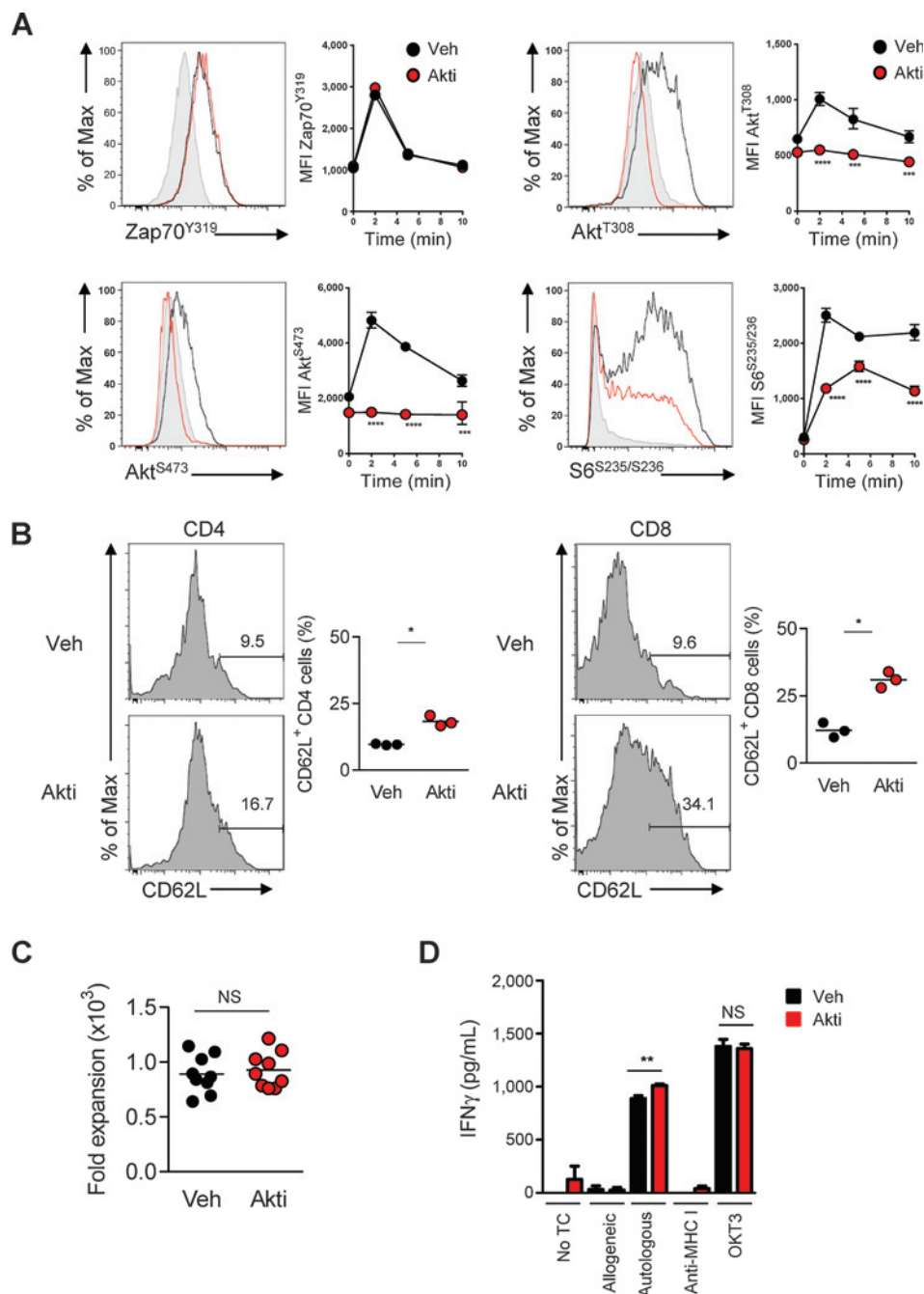


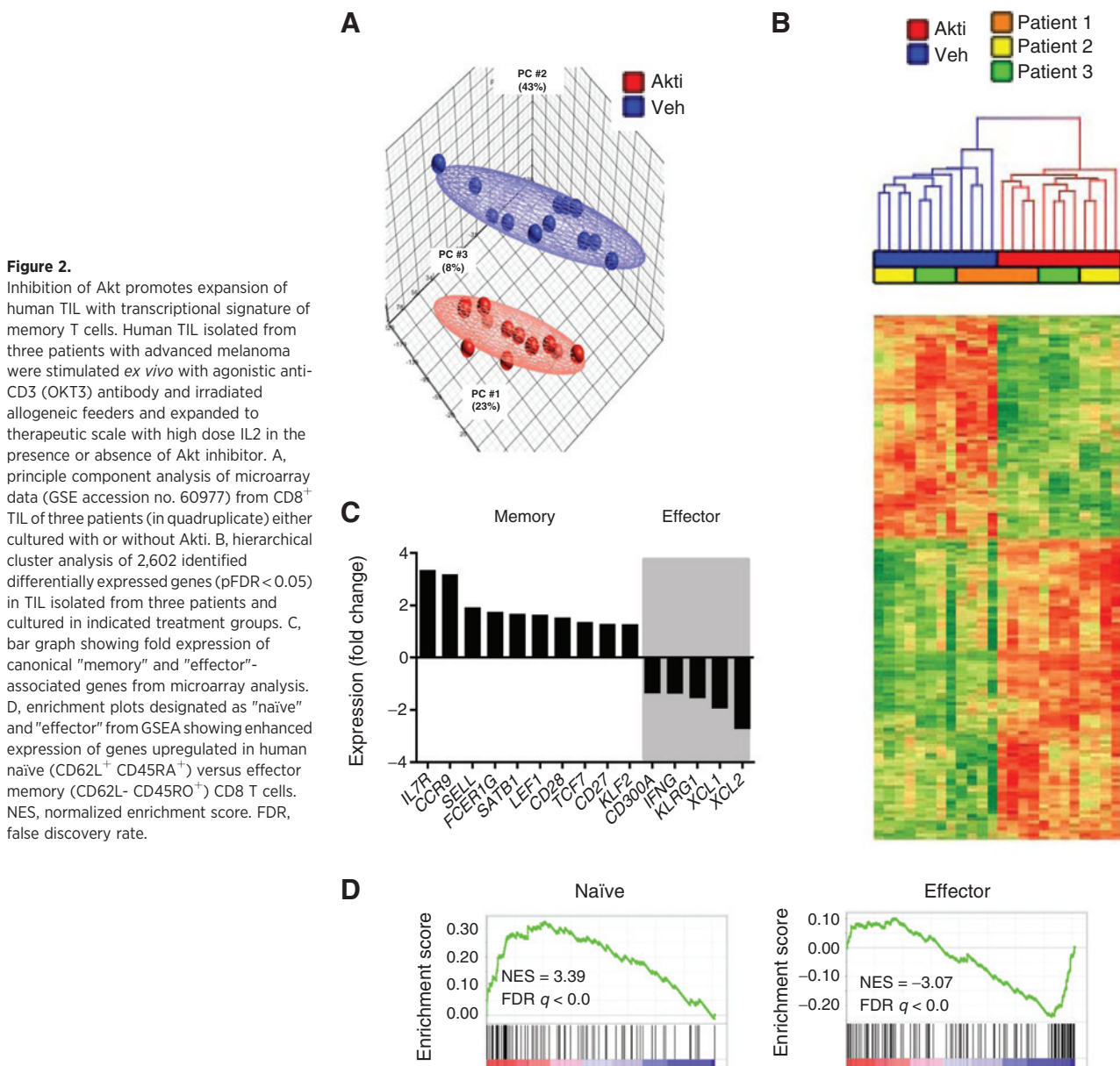
Figure 1. Inhibition of Akt promotes expansion of human TIL with enhanced expression of the memory-marker CD62L. A, FACS histogram and quantification of phosphorylation at indicated residues during acute time points after CD3 stimulation of human TIL either in presence or absence of pharmacologic inhibition of Akt. Gray shading, unstimulated TIL. B, FACS histogram and quantification of CD62L expression on CD4⁺ and CD8⁺ TIL isolated from three patients and expanded *ex vivo* at clinical-scale with agonistic anti-CD3 (OKT3) antibody and irradiated allogeneic feeders with high dose IL2 in the presence or absence of Akt inhibitor. C, scatter plot showing fold expansion at clinical-scale of human TIL from three patients cultured independently in triplicate. D, bar graph showing IFN γ release by ELISA when either Akti-treated TIL or vehicle were cocultured for 12 hours under following conditions: no tumor cells (TC), allogeneic tumor cells, autologous tumor cells, autologous tumor cells with MHC-I blocking antibody (W6/32), and OKT3 alone. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Center bar, mean; error bars, SEM.

Samples were fragmented and biotinylated using the Encore Biotin Module (NuGEN) according to the manufacturer's instructions. Biotinylated cDNA was then hybridized to Human Gene 1.0 ST arrays (Affymetrix) overnight at 45°C and stained on a Genechip Fluidics Station 450 (Affymetrix), according to the respective manufacturer's instructions. Arrays were scanned on a GeneChip Scanner 3000 7G (Affymetrix). Global gene expression profiles were rank ordered by relative fold-change values and analyzed by using Gene Set Enrichment Analysis (GSEA) software (Broad Institute, MIT). P values were calcu-

lated using the Student t test using Partek Genomic Suite after Robust Multiarray Average normalization.

Metabolism assays

Oxygen consumption rate (OCR) was measured at 37°C using an XF24 extracellular analyzer (Seahorse Bioscience) as previously described (21). Briefly, TIL were initially plated with XF media (nonbuffered RPMI-1640 containing 25 mmol/L glucose, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate) and incubated in a non-CO₂ incubator for 30 minutes at 37°C. Using



Seahorse XF-24 proprietary software, we measured OCR under basal conditions and in response to injection port-administration of the following compounds at indicated time points: 1 $\mu\text{mol/L}$ oligomycin, 1.5 $\mu\text{mol/L}$ fluorocarbonyl cyanide phenylhydrazine (FCCP), 100 nmol/L rotenone, and 1 $\mu\text{mol/L}$ antimycin A.

Metabolomics

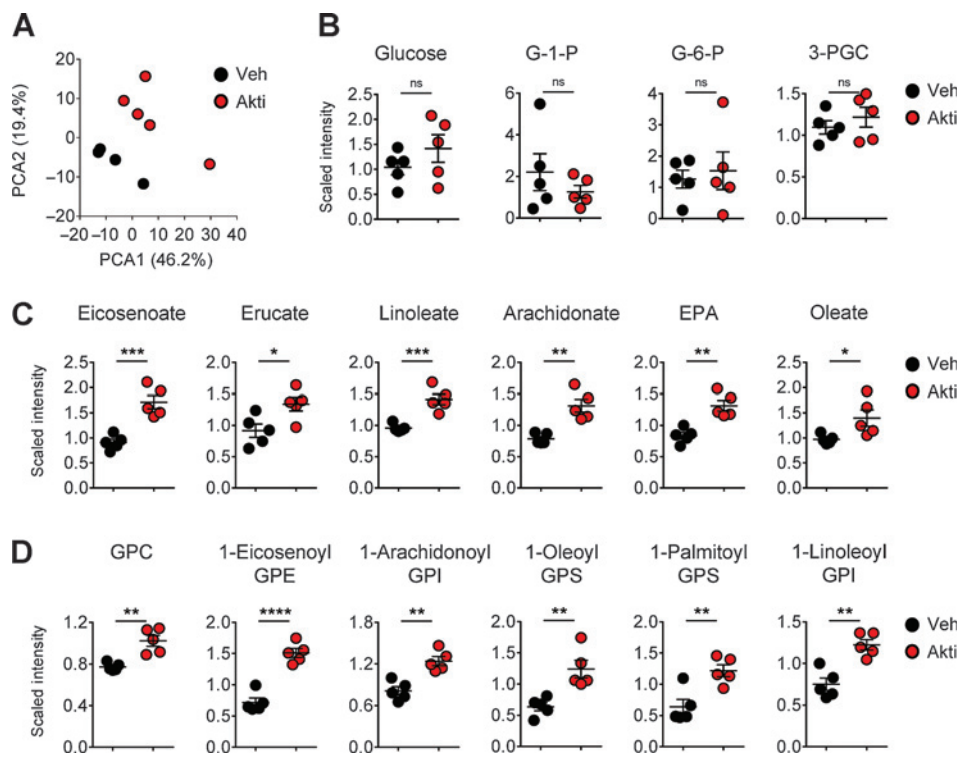
After 30 days expansion, TIL were enriched using Miltenyi magnetic column CD8⁺ separation (order no. 130-096-495) according to the manufacturer's instructions. Five replicates per treatment group (Akti vs. vehicle) from one patient were analyzed on multiple platforms, including gas and liquid chromatography-mass spectrometry with electron ionization according to protocols of Metabolon, Inc. For murine analysis, splenocytes from Pmel mice were stimulated with hgp100₂₅₋₃₃ peptide (1 $\mu\text{mol/L}$) and 100 IU/mL rhIL-2 (Novartis) in the following treatment

groups: 0 $\mu\text{mol/L}$, 1 $\mu\text{mol/L}$, and 2.5 $\mu\text{mol/L}$ Akti-1/2 (Akti; Calbiochem). Splenocytes were harvested on day 10 and CD8⁺ T cells were enriched using a MACS Negative Selection Kit (Miltenyi Biotec order no. 130-104-075).

Statistical analysis

A sample size of five mice per treatment group was used to detect an effect size in all experiments unless otherwise indicated. Data assumed to have a normal distribution and differences between two groups were assessed with unpaired, two-tailed *t* tests. Comparisons involving more than two groups were assessed using an ANOVA. *P* values less than 0.05 were considered significant. The measure of central tendency is mean and variation is SEM unless otherwise stated. All experiments were replicated at least twice in laboratory with the exception of the histopathologic analysis of NSG mice receiving either Akti-treated or conventional CTL.

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**Figure 3.**

Global metabolomic analysis shows that Akt inhibition is associated with enhanced fatty-acid oxidation in human TIL. A, principle component analysis of metabolome (362 biochemicals) of Akti-treated TIL that were stimulated *ex vivo* using agonistic anti-CD3 (OKT3) antibody and irradiated allogeneic feeders and expanded to clinical scale for 30 days with high dose IL2 in the presence or absence of Akt inhibitor. TIL were then isolated to analyze basal metabolic profile (in absence of restimulation) under basal cell culture conditions. B, relative abundance of key metabolites in glycolytic pathway. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; 3-PGC, 3-phosphoglycerate. C, relative abundance of key metabolites involved in lipid metabolism are shown. EPA, eicosapentaenoate 20:5n3. D, relative abundance of lysolipids in Akti-treated TIL versus vehicle. GPC, glycerophosphorylcholine; GPE, glycerophosphoethanolamine; GPS, glycerophosphatidylserine. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Center bar, mean; error bars, SEM.

Study approval

All procedures were approved by the NIH Animal Care and Use Committee and were compliant with the *Guide for Care and Use of Laboratory Animals* (NIH publication no. 85-23; revised 1985). All human subjects were either healthy donors or patients with metastatic melanoma receiving treatment under institutional review board-approved protocols in the Surgery Branch of the National Cancer Institute. Informed consent was obtained from all human subjects.

Results

Inhibition of Akt promotes expression of CD62L in human tumor-specific cytotoxic lymphocytes without compromising cell expansion

Akt is known to control lymphoid homing behavior of T cells through regulation of the adhesion molecule CD62L (10). To test whether Akt inhibition affects expression of CD62L on the surface of human TIL, we used a well-characterized allosteric inhibitor of all three isoforms of Akt (hereafter Akti; ref. 18). We first sought to confirm whether Akti inhibits phosphorylation of Akt and downstream targets in human TIL. When measured at acute time points after TCR stimulation, we found that phosphorylation of Akt is inhibited at both the serine 473 and threonine 308 residues, resulting in decreased phosphorylation of its downstream substrates ribosomal protein S6 (Fig. 1A) and glycogen synthase kinase 3 beta (GSK3 β ; Supplementary Fig. S2). We then isolated TIL from 3 patients with metastatic melanoma as previously described (19), expanded them at clinical-scale in the presence or absence of Akti, and measured surface expression of CD62L on CD4⁺ and CD8⁺ T cells. We found that Akti-expanded TIL had significantly increased expression of CD62L (Fig. 1B). Strikingly,

however, we did not observe an effect of Akt inhibition on the expansion of TIL (Fig. 1C). Finally, we sought to determine if Akt inhibition compromises tumor specificity or capacity to release IFN γ when cocultured with autologous tumor cells. Akti-treated TIL released similar levels of IFN γ compared with conventional TIL that was restricted in an MHC class I-dependent manner (Fig. 1D). Thus, pharmacologic inhibition of Akt enables expansion of TIL expressing elevated levels of CD62L without affecting their expansion or capacity to release IFN γ upon recognition of tumor targets.

Inhibition of Akt in human TIL promotes a transcriptional signature of memory T cells

Expression of CD62L distinguishes antigen-experienced cells with a central memory phenotype (22). In addition to their lymphoid homing capacity, these cells exhibit enhanced capacity for cell survival and proliferation (5, 23, 24). Given elevated expression of CD62L on the surface of Akti-expanded TIL, we asked whether Akt inhibition causes global changes in gene transcription that are characteristic of memory cells. To visualize the transcriptome of CD8⁺ TIL, we performed principle component analysis (PCA) of microarray data and observed segregation of treatment groups among the 3 patients under study (Fig. 2A). Whole-transcriptome analysis revealed differential expression of 2,602 genes ($P < 0.05$) in Akti-treated TIL compared with vehicle and hierarchical clustering analysis of the expression of these genes enabled unsupervised segregation of vehicle- and Akti-expanded TIL (Fig. 2B). It was particularly striking that naïve/memory-associated genes such as *IL7R*, *SELL*, *CD28*, and *CD27* were upregulated with Akt inhibition, whereas effector-associated genes such as *IFNG* and *KLRG1* were suppressed (Fig. 2C; ref. 25). To extend our interpretation

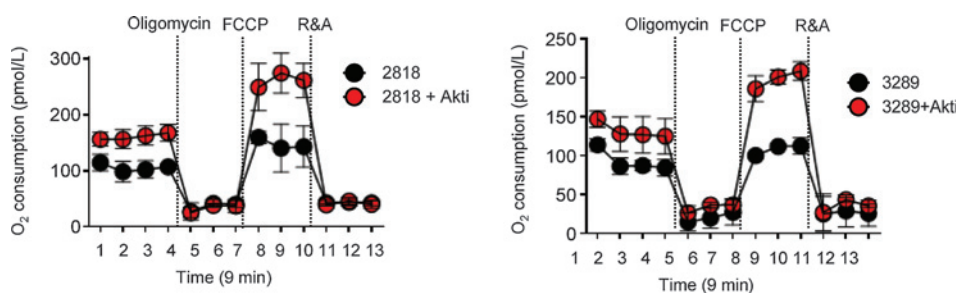


Figure 4.

Therapeutic TIL isolated from patients with melanoma have poor mitochondrial spare respiratory capacity that is augmented with pharmacologic inhibition of Akt. Human TIL isolated from indicated patients were stimulated *ex vivo* using agonistic anti-CD3 (OKT3) antibody and irradiated allogeneic feeders and expanded to clinical-scale for 30 days with high dose IL2 in the presence or absence of Akt inhibitor. An XF24 extracellular analyzer (Seahorse Bioscience) was used to measure OCR of Akti-treated and vehicle TIL in real time under basal cell culture conditions (after 30 days in culture) and in response to indicated inhibitors at acute time points. FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; R&A, rotenone and antimycin A. Spare respiratory capacity is maximal respiration (after FCCP) minus basal respiration. Data are representative of three independent experiments for each patient.

beyond single-gene analysis, we performed GSEA using genes upregulated in human naïve ($CD62L^+ CD45RA^+$) compared with effector memory ($CD62L^- CD45RO^+$) $CD8^+$ T cells isolated from healthy donors. We found that Akti-treated TIL have enhanced expression of naïve-associated genes and decreased expression of effector memory-associated genes (Fig. 2D). Taken together, these findings indicate that pharmacologic inhibition of Akt enables expansion of $CD8^+$ TIL with transcriptional properties characteristic of memory cells.

Akt inhibition is associated with enhanced fatty-acid oxidation in human cytotoxic TIL

There is increasing evidence that memory T cells have metabolic qualities such as reduced glycolysis (26) and enhanced mitochondrial fatty acid oxidation (FAO) that support long-term survival and effector function (27). Having shown that pharmacologic inhibition of Akt in $CD8^+$ TIL promotes a transcriptional profile characteristic of memory T cells, we asked whether Akt inhibition affects metabolism of human $CD8^+$ TIL. Using a well-established gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry-based approach (28–29), we observed that pharmacologic inhibition of Akt modulates several metabolic pathways as evidenced by clear segregation of treatment groups in principal component analysis of more than 360 detected metabolites (Fig. 3A). It has previously been reported in a murine model that Akt is largely dispensable for glucose metabolism of cytotoxic T lymphocytes (10). Consistently, we observed a modest increase in glucose and glucose 6-phosphate in Akti-treated TIL and somewhat diminished 3-phosphoglycerate levels (Fig. 3B), suggesting relatively little impact of Akt inhibition on glycolytic metabolism. With regard to FAO, however, Akti-treated $CD8^+$ TIL showed accumulation of both long-chain and polyunsaturated fatty acids (Fig. 3C) that may be a result of membrane lipid turnover used to fuel FAO. This interpretation is supported by accumulation of phospholipid catabolites and elevated lysolipid levels (Fig. 3D). Another possibility is that enhanced lipid accumulation in Akt-treated TIL was merely due to decreased cell growth and expansion, but this seems less likely because we did not observe any difference in absolute cell numbers after culture (Fig. 1A). Thus, although inhibition of Akt during expansion of TIL does not affect the

abundance of glycolytic metabolites, it results in changes in abundance of metabolites involved in FAO.

Inhibition of Akt augments mitochondrial spare respiratory capacity in human TIL

Spare respiratory capacity (SRC) is a measure of the bioenergetic ability of mitochondria to produce additional energy under conditions of increased stress or work and is thought to be vital for the long-term survival and function of diverse cell types (21, 30–31). Recent work has demonstrated that mitochondrial spare respiratory capacity (SRC) is critical for longevity memory $CD8^+$ T cells (32). Because SRC in memory $CD8^+$ T cells is dependent on mitochondrial FAO, we wondered whether Akt inhibition augments SRC in human TIL. By using an extracellular flux analyzer, we characterized the metabolism of therapeutic TIL in real time by measuring O₂ consumption rates (OCR), an indicator of oxidative phosphorylation (OXPHOS; ref. 21). We found that Akti-treated TIL had slightly higher basal OCR when compared with vehicle (Fig. 4A). To further characterize the bioenergetic profile of TIL, we challenged TIL with a well-established "mitochondrial stress test" in which oligomycin (to block ATP synthesis) is added after measurement of basal OCR, followed by fluorocarbonyl cyanide phenylhydrazone or FCCP (to uncouple ATP synthesis from the electron transport chain, ETC), and finally by coadministration of rotenone and antimycin A (to block complex I and III, respectively, of ETC; refs. 29, 33, 34). Akti-treated TILs demonstrated a considerably higher mitochondrial SRC compared with vehicle controls as indicated by the difference between basal OCR and maximal OCR (after FCCP injection; Fig. 4A). This is consistent with earlier studies in which elevated FAO fuels enhanced SRC in memory T cells (29). Collectively, these findings are consistent with the hypothesis that Akt induces a metabolic program in human TIL similar to that of memory T cells whereby increased FAO supports enhanced mitochondrial SRC.

Inhibition of Akt enhances persistence of human TIL and improves survival of transferred antitumor T cells in a mouse model of ACT

Collectively, we observed that TIL cultured in the presence of Akti possess transcriptional and metabolic characteristics of

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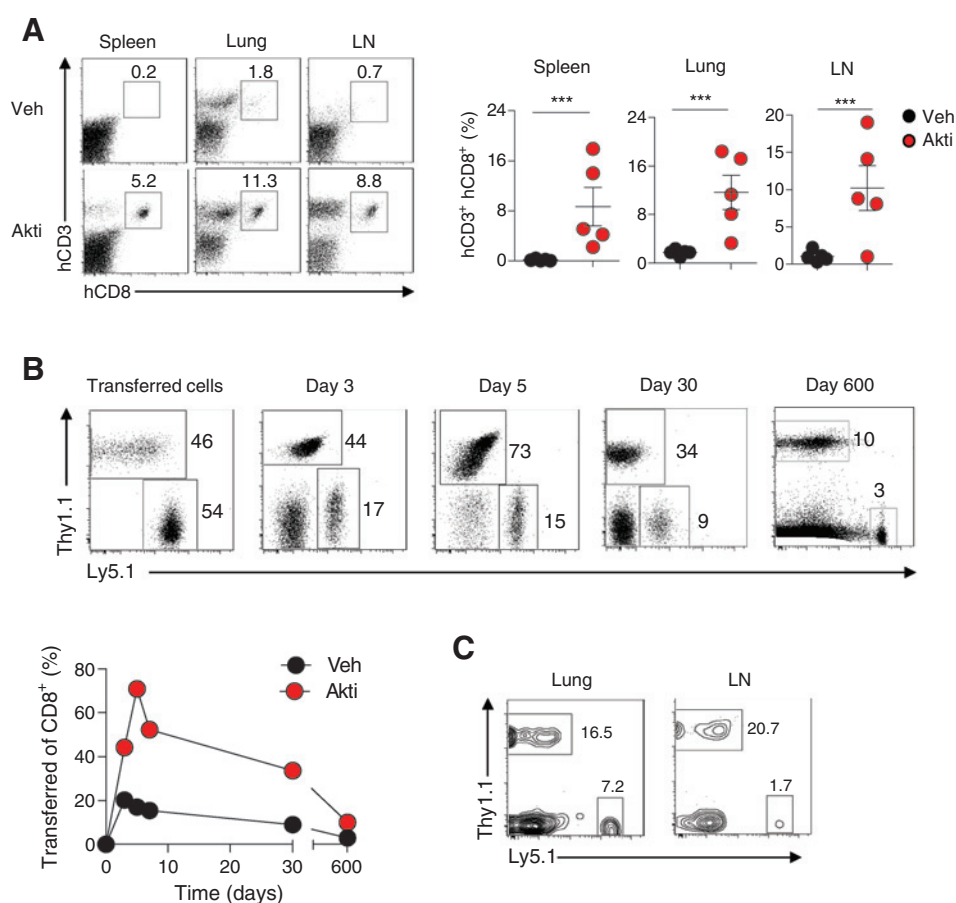


Figure 5. Akt inhibition enhances persistence of human TIL and murine cytotoxic T lymphocytes after adoptive transfer. A, representative FACS analysis and quantification of human Akti-treated and vehicle TIL that had been expanded to clinical-scale *ex vivo* and subsequently adoptively transferred into NSG mice. Human TIL were isolated from indicated lymphoid and nonlymphoid organs of NSG mice 30 days after adoptive transfer. Data are representative of five biologic replicates per treatment group. B, FACS analysis of CD8⁺ T cells from age- and gender-matched Thy1.1 (Akti) and Ly5.1 (vehicle) Pmel1 mice after cotransfer into B6 mice and spleens were harvested at the indicated time points. Data are representative of five biologic replicates for each time point. C, representative FACS analysis of Akti-treated and vehicle T cells in lung and mesenteric lymph nodes (LN) at day 600. ***, $P < 0.001$. Center bar, mean; error bars, SEM.

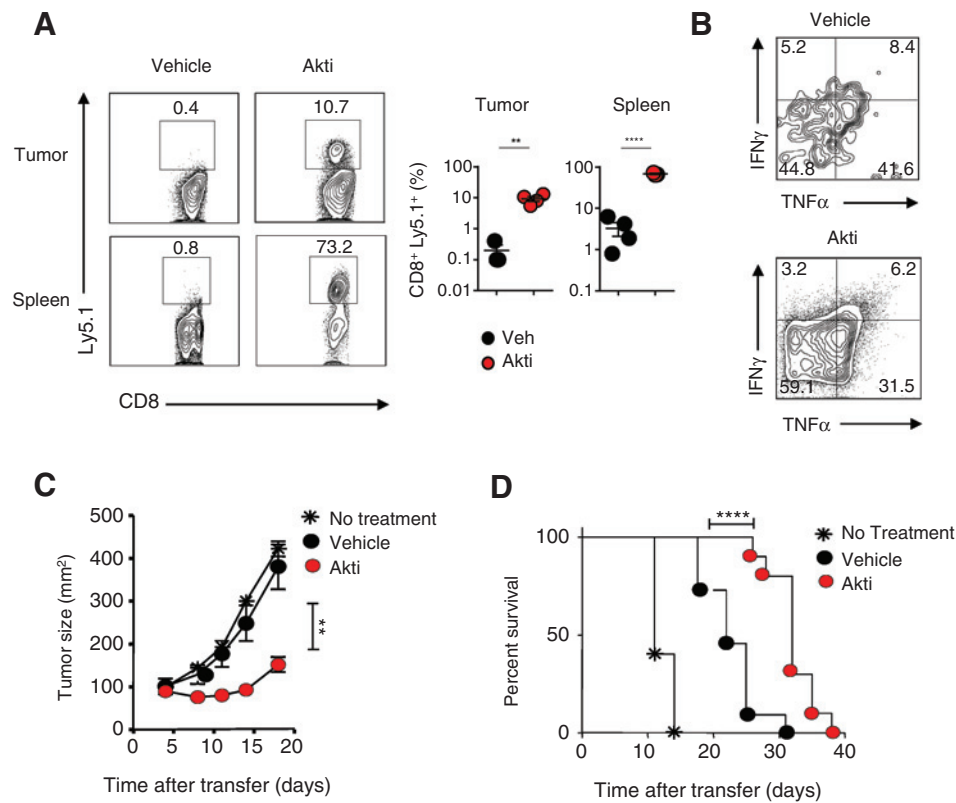
long-lived memory T cells. We therefore hypothesized that Akti-treated TIL may be capable of enhanced long-term persistence. To test this, we performed a clinical-scale expansion of conventional and Akti-treated TIL. At the end of *ex vivo* expansion, both treatment groups were comprised of a similar proportion of CD8⁺ T cells, and Akti-treated TIL had enhanced expression of CD62L (Supplementary Fig. S3). We transferred conventional and Akti-expanded human TIL into NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) mice (Fig. 5A) and observed superior engraftment and persistence of Akti-treated cells 30 days after transfer in both lymphoid and nonlymphoid organs (Fig. 5A). Thus, in a humanized mouse model, Akti-treated human TIL have enhanced persistence and this correlates with phenotypic, metabolic, and transcriptional features of memory.

Previous studies have demonstrated that CD8⁺ T cells with the capacity to persist after adoptive transfer mediate more effective antitumor responses in both mice and humans receiving ACT (3, 23, 26, 35). Accordingly, we endeavored to evaluate the persistence and antitumor function of Akti-expanded tumor-specific T cells using the Pmel-1 mouse model in which transgenic T cells express a T-cell receptor specific for the melanoma-associated antigen, hgp100, widely expressed in B16 melanoma (20). Consistent with our findings in TIL from patients with melanoma, we found that Akt phosphorylation is inhibited at both the serine 473 and threonine 308 residues, resulting in decreased phosphorylation of its downstream target ribosomal protein S6 (Supplementary Fig. S4). Akti-

treated Pmel-1 T cells showed a gene-expression profile (Supplementary Fig. S5) and surface phenotype (Supplementary Fig. S6) reminiscent of long-lived memory T cells. Moreover, global metabolomic analysis showed that Akti-treated Pmel CD8⁺ T cells possessed a distinct metabolic signature characterized by augmented FAO (Supplementary Fig. S7).

To evaluate the effect of Akt inhibition on the cell-intrinsic capacity of tumor-specific CD8⁺ T cells to engraft and persist following adoptive transfer, we cotransferred a 1:1 mixture of vehicle and Akti-expanded Pmel-1 TCR-tg cells that can be distinguished using congenic markers and tracked their kinetics following adoptive transfer into mice. Strikingly, Akti-treated cells expanded to greater numbers and persisted to form a long-lived population of memory cells that could be detected in lymphoid and nonlymphoid organs 600 days following adoptive transfer (Fig. 5B and C).

To test whether Akti-treated CD8⁺ T cells are capable of superior antitumor immunity, we individually transferred vehicle or Akti-expanded Pmel-1 CD8⁺ T cells into sublethally ablated recipients bearing established B16 melanoma tumors and measured tumor growth and survival following transfer. Consistent with human TIL, Akti-treated Pmel-1 T cells exhibited superior persistence and trafficking to the tumor microenvironment (Fig. 6A) and produced similar levels of IFN γ compared with vehicle (Fig. 6B) that correlated with decreased tumor growth (Fig. 6C) and improved survival (Fig. 6D). Thus, consistent with their transcriptional and metabolic characteristics, Akti-expanded cells exhibit enhanced persistence upon adoptive transfer that

**Figure 6.**

Akt inhibition of cytotoxic T cells improves antitumor immunity of adoptively transferred T cells in mouse model of cell-based immunotherapy for melanoma. A, representative FACS analysis and enumeration of Ly5.1 Pmel CD8⁺ T cells isolated from spleen and tumor microenvironment 5 days after adoptive transfer into B16 melanoma-bearing mice. Donor T cells were stimulated *in vitro* with cognate peptide and cultured in IL2. After 5 days, cells were restimulated with cognate peptide and irradiated B6 feeders for an additional 5 days before transfer. Akti was supplemented in media for entirety of *in vitro* culture. Data are representative of four biologic replicates per treatment group. B, representative FACS analysis of IFN γ and TNF α intracellular cytokine staining of adoptively transferred Ly5.1 Pmel CD8⁺ T cells isolated from spleen of tumor-bearing mice. C, treatment response of 2×10^6 Pmel CD8⁺ T cells adoptively transferred (after same *in vitro* culture conditions described above) into mice bearing established B16 melanomas. Recipient mice were pretreated with 6 Gy total body irradiation, adjuvant vaccine, and IL2 in conjunction with cell therapy. Serial tumor measurements were obtained and tumor area calculated. Data are representative of ten biologic replicates per treatment group. D, Kaplan-Meier analysis of survival in tumor-bearing mice receiving adoptively transferred cells treated with Akti (32 days) versus control (22 days; $P < 0.0001$). **, $P < 0.01$; ****, $P < 0.0001$. Center bar, mean; error bars, SEM.

correlates with augmented tumor regression and survival following ACT.

Discussion

The principle aim of this study was to determine if pharmacologic inhibition of Akt promotes features of memory T cells in TIL isolated from patients with advanced cancer. Because there is considerable evidence in animal models and human trials that memory T cells mediate superior regression of tumor (5), we also wanted to evaluate if inhibition of Akt enhances antitumor immunity of adoptively transferred CD8⁺ T cells. We found that Akt inhibition promotes a gene expression signature and metabolic profile characteristic of long-lived memory T cells, and this was associated with enhanced persistence and antitumor immunity. Enhancing "memory" of adoptively transferred TIL has been a long-standing therapeutic goal of cell-based immunotherapy for metastatic cancer. Although the reasons for this observation remain speculative, it is thought that sheer bulk of tumor in patients with advanced cancer requires transfer of TIL with a

capacity—much like long-lived memory T cells—to persist long after their initial encounter with antigen (36, 37).

After antigen activation, diverse signals from the TCR and costimulatory receptors converge on the Akt signaling pathway to drive effector differentiation (8). Cytokine signaling such as IL2 further sustain Akt activity (12), driving CD8 T cells toward terminal differentiation at the expense of memory formation. It has previously been shown in a murine model that exposure to Akt inhibitor 48 hours after TCR activation promotes memory-associated gene expression and lymph node-homing of CD8⁺ T cells (10). Here, we wanted to further characterize the impact of Akt inhibition on *bona fide* cytotoxic T cells isolated from the tumor microenvironment of patients with advanced cancer.

We used a variety of approaches—metabolomic, transcriptomic, and phenotypic—to characterize the global effect of Akt inhibition on human cytotoxic T cells. Consistent with earlier studies in murine virus-specific CD8 T cells (8, 10), we observed that Akt inhibition significantly alters the transcriptome of human TIL and promotes a global signature enriched for genes

in memory and naïve T cells. This finding is especially intriguing in light of recent evidence that CD8⁺ T cells from the tumor microenvironment have been characterized as terminally differentiated and exhausted (6). The finding in this study that Akt-treated TIL demonstrate enhanced expression of naïve-associated genes raises the possibility that characteristically exhausted and senescent TIL (6) may undergo "reprogramming," at least at a population level, that endows a renewed capacity for long-lived persistence.

Akt inhibition is known to promote immunologic memory in virus-specific murine T cells (10). It is also known that memory T cells rely on fatty-acid oxidation (38) and mitochondrial spare respiratory capacity (29) for long-term survival and function. It remains unclear, however, whether Akt itself plays a role in the metabolic fate of antigen-experienced CD8⁺ T cells. In spite of the canonical role of Akt in facilitating glucose metabolism in diverse cell types (39), a recent study in cytotoxic T cells shows that Akt is largely dispensable for T-cell metabolism (10). Consistent with these findings, we observed little difference in key glycolytic metabolites between Akt-treated TIL and controls. We performed a global metabolomic analysis, however, that suggests Akt inhibition modulates fatty acid oxidation in cytotoxic TIL. Further analysis using real-time cellular bioenergetic studies also shows that Akt inhibition enhanced mitochondrial spare respiratory capacity. Taken together, findings from our analysis suggest that pharmacologic inhibition of Akt may modulate metabolic programs of antigen-experienced T cells.

Although the findings suggest that pharmacologic inhibition of Akt may induce considerable plasticity in the metabolic and transcriptional programs of a population of terminally differentiated cytotoxic T cells, future studies using genetic approaches are required to validate and further characterize the role of Akt in T-cell differentiation and metabolism. In spite of these limitations, the results of the present study show that pharmacologic inhibition of Akt promotes a gene expression signature and metabolic profile characteristic of long-lived memory T cells and this is associated with enhanced persistence and antitumor immunity. More importantly, these findings could form the basis for novel immunometabolomic approaches to improve cell-intrinsic features of therapeutic TIL that may enhance the clinical efficacy of cell-based immunotherapy for advanced cancer.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.G. Crompton, M. Sukumar, R. Roychoudhuri, D. Clever, A. Gros, R.L. Eil, L. Gattinoni, P. Muranski, C.A. Klebanoff, S.A. Rosenberg, N.P. Restifo

Development of methodology: J.G. Crompton, R. Roychoudhuri, D. Clever, A. Gros, R.L. Eil, S.P. Kerker, A. Leonardi, N. Acquavella, F.M. Marincola, L. Gattinoni, P. Muranski, M.S. Sundrud, C.A. Klebanoff, N.P. Restifo

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.G. Crompton, D. Clever, R.L. Eil, K. Hanada, Z. Yu, D.C. Palmer, R.D. Michalek, A. Leonardi, F.M. Marincola, L. Gattinoni, C.A. Klebanoff, N.P. Restifo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.G. Crompton, M. Sukumar, R. Roychoudhuri, D. Clever, A. Gros, R.L. Eil, D.C. Palmer, S.P. Kerker, R.D. Michalek, E. Wang, F.M. Marincola, L. Gattinoni, C.A. Klebanoff, S.A. Rosenberg, N.P. Restifo

Writing, review, and/or revision of the manuscript: J.G. Crompton, M. Sukumar, R. Roychoudhuri, E. Tran, S.P. Kerker, E. Wang, L. Gattinoni, P. Muranski, M.S. Sundrud, C.A. Klebanoff, S.A. Rosenberg, D.T. Fearon, N.P. Restifo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.G. Crompton, R.L. Eil, S.P. Kerker, T. Upham, N.P. Restifo

Study supervision: M. Sukumar, D.T. Fearon, N.P. Restifo

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Akt Inhibition Enhances Expansion of Potent Tumor-Specific Lymphocytes with Memory Cell Characteristics

Joseph G. Crompton, Madhusudhanan Sukumar, Rahul Roychoudhuri, et al.

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