IL6 Fuels Durable Memory for Th17 Cell–Mediated Responses to Tumors

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ABSTRACT

The accessibility of adoptive T-cell transfer therapies (ACT) is hindered by the cost and time required for product development. Here we describe a streamlined ACT protocol using Th17 cells expanded only 4 days in vitro. While shortening expansion compromised cell yield, this method licensed Th17 cells to eradicate large tumors to a greater extent than cells expanded longer term. Day 4 Th17 cells engrafted, induced release of multiple cytokines including IL6, IL17, MCP-1, and GM-CSF in the tumor-bearing host, and persisted as memory cells. IL6 was a critical component for efficacy of these therapies via its promotion of long-term immunity and resistance to tumor relapse. Mechanistically, IL6 diminished engraftment of FoxP3+ donor T cells, corresponding with robust tumor infiltration by donor effector over regulatory cells for the Day 4 Th17 cell product relative to cell products expanded longer durations in vitro. Collectively, this work describes a method to rapidly generate therapeutic T-cell products for ACT and implicates IL6 in promoting durable immunity of Th17 cells against large, established solid tumors.

Significance: An abbreviated, 4-day in vitro expansion method licenses Th17 cells to confer long-lived immunity against solid malignancies via induction of systemic IL6 in the host.

See related commentary by Fiering and Ho, p. 3795

Introduction

Adoptive T-cell transfer therapy (ACT) is a personalized treatment effective for patients with aggressive, end-stage malignancies. Administration of peripheral T cells modified with Chimeric Antigen Receptors (CAR) has resulted in remissions of up to 90% of treated patients with hematologic malignancies (1), leading to the first FDA approval for personalized gene therapy in 2017. While CAR T cells are effective in hematologic disease, T-cell receptor (TCR)-based products, such as tumor-infiltrating lymphocyte (TIL) therapies, have had greater success in patients with solid tumors and are advantageous for their ability to target intracellular antigens (2). Transfer of ex vivo expanded TIL has generated up to 50% response rates in metastatic melanoma (3) in addition to complete durable responses in metastatic breast (4) and gastrointestinal (5) cancers among others.

For both CARs and TILs, however, the oppressive solid tumor microenvironment limits T-cell immunity, making development of novel and safe methods to bolster antitumor potential of the transferred cell paramount. Efficacy of ACT therapies is often correlated with higher number of T cells transferred (3, 6); therefore, protocols expand T cells in vitro over long periods of time to large magnitudes (6, 7). Yet, such protocols paradoxically drive T cells to a state of terminal differentiation, yielding large numbers of T cells with poor quality and persistence individually (8). Longer expansion also drives higher cost and treatment delays, which are not feasible for many patients with aggressive diseases. Thus, novel methods for generating potent T cells after shorter expansion could improve efficacy, reduce costs, and permit treatment of patients more rapidly.

We used two tumor-targeting models to determine whether shortening T-cell expansion could overcome these limitations. We hypothesized that the CD4+Th17 cell subset—poised with potent immunity and stemness qualities versus cytotoxic CD8+ T cells (9–12)—would be the candidate subset to address this concept. By transferring either murine TCR-specific Th17 cells into syngeneic hosts or human CAR-engineered Th17 cells against human tumors in NSG mice, we report that Th17 cells expanded only 4 days (termed Day 4 Th17 cells) improved antitumor responses despite obtaining fewer cells. Day 4 Th17 cells transferred into immunocompetent animals elicited profound cytokine release, including a unique induction of IL6 relative to Th17 cells expanded longer term. IL6 modulated the balance between Th17 and Treg cells to promote the superior antitumor responses of Day 4 Th17 cells. Herein, we describe how a shortened T-cell expansion protocol with CD4+Th17 cells improves treatment outcomes and how induction of IL6 fosters durable memory responses for Th17 cells against solid tumors.

Materials and Methods

Study design and rigor

Sample size: Animal experiment sample sizes were selected on the basis of power analysis while minimizing use of animals per the Medical University of South Carolina’s (MUSC) Institutional Animal
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Care and Use Committee (IACUC) policies. For survival experiments, power calculations for the log-rank test were based on the anticipated effect sizes (differences in survival percentages) at the end of the experiments. To achieve minimum power of 80% with two-sided α = 0.05 for anticipated survival differences of 55% and 40%, sample sizes of n = 12 and n = 20 were necessary. For other continuous measures, power was based on the Student t test. Our target minimum standardized effect size of 2.0 required group sizes of n = 5 to achieve 80% power. Stopping data collection: experimental endpoints were determined prior to study execution. Primary antitumor efficacy was conducted over 30–40 days. Rechallenge experiments were conducted up to 100 days. Data inclusion/exclusion criteria: with experiments herein, data points were only removed for two scenarios established prospectively: (i) if injection of T cells was unsuccessful, the animal was withdrawn from study, or (ii) if tumor placement resulted in an intractable tumor in contrast to a subcutaneous tumor, the animal was removed from tumor analysis as tumor burden cannot be accurately measured. Outliers: all data points are reported without removal of outliers. Randomization: for all C57BL/6 tumor experiments, female mice were randomly assigned to treatment group by tumor size, but treatment group assignment. For NSG experiments, male and female mice were randomly assigned to treatment group by tumor size, but housing of animals was not altered. Tumor sizes were measured as L x W by calipers and reported as tumor area. Assessment of T-cell engraftment in peripheral blood was performed on all animals within the study when feasible. For experiments where animals were euthanized prior to endpoint for tissue distribution analysis, mice were selected using random number generation. Blinding: all tumor measurements and T-cell injections were performed by a lab member blinded to treatment group.

Mice and tumor lines

C57BL/6, tyrosinase-related protein 1 (TRP-1) TCR transgenic mice (Rag$^{-/-}$-B6-TRP-1 TCR), and NOD/SCID/gamma chain knock-out (NSG) mice were purchased from the Jackson laboratories and bred in house at the MUSC Hollings Cancer Center comparative medicine department. C57BL/6 CD45.1 mice were purchased from NCI Frederick laboratories for indicated studies. Tumor experiments were conducted with mice ages 6–10 weeks. NSG mice were housed in microisolator cages to maintain specific pathogen-free conditions and provided bottle access to autoclaved water and food. All housing and experiments were conducted in accordance with MUSC’s IACUC procedures and with the supervision and support of the Division of Laboratory Animal Resources. All studies and procedures were IACUC approved prior to execution. B16F10 (H-2b) melanoma cell line was obtained from Nicholas P. Restifo and M108 mesothelioma was a gift of the Carl H. June lab and used for in vivo tumor studies. Cell lines were validated prior to transfer and were confirmed pathogen and Mycoplasma free via PCR screen most recently in March 2020. B16F10 cells were passaged less than two times and M108 were passaged less than four times after thaw prior to injection.

T-cell cultures

TRP-1 cells

TRP-1 transgenic T cells were activated in the presence of irradiated (10 Gy) feeder splenocytes pulsed with 1 μmol/L TRP-1,16,18,19 peptide (SGHNCGTCRPGWGRGAACNQLTVTR). Feeder cells were added in a 1: 5 T-cell ratio. Cells were seeded at a concentration of 1.5 × 10^6/mL and polarized to Th17 phenotype using the following cocktail of cytokines: 100 ng/mL recombinant human (rh) IL6 (NIH preclinical repository), 100 ng/mL rhIL-21 (Shenandoah), and 30 ng/mL hTGFβ1 (Biologend), 10 ng/mL rhIL-1β (Shenandoah), and 10 μg/mL each of anti-mouse (rm) IFNγ (clone XMG1.2), antiIL-4 (clone 11B11), and antiIL-12 (clone I5E6-1A12). On days 2 and 3 of culture, IL23 and IL2 were added in concentrations of 20 ng/mL and 50 IU/mL, respectively. From day 4 and onward, cells were split to a density of 0.8 × 10^6/mL and fresh media added with 100 IU IL2/mL as needed. Th0 cells were expanded in IL2 only.

Human normal donor peripheral T cells

Peripheral blood from healthy donors (deidentified) was purchased as auffy coat (Sylvan N. Goldman Oklahoma Blood Institute). Lymphocytes were isolated from buffy coat using Lymphocyte Separation Medium (Mediatech). CD4⁺ T cells were isolated via untouched magnetic bead Isolation Kit (Dynabeads, Invitrogen) and rested overnight at 37°C in media containing 20 IU IL2/mL. CD4⁺ T cells were then activated using magnetic beads decorated with antibodies targeting CD3 and ICOS at a 1 bead:5 T-cell ratio, and polarized to the Th17 phenotype using the following cocktail of cytokines: 10 ng/mL hIL1b, 10 ng/mL rhIL6, 20 ng/mL hIL23, 5 μg/mL rhIL4, and 5 μg/mL rhIFNγ. CD4⁺ T cell cultures were maintained with 100 IU IL2/mL over duration of expansion. On day 2 after activation, cells were transduced with a CAR containing an anti-mesothelin single-chain variable fragment and a TCR CD3ζ domain with a 4-1BB costimulatory domain (13). Meso-CAR was a gift of the Carl H. June lab.

ACT

C57BL/6

A total of 0.4 × 10^6 B16F10 cells were resuspended in sterile PBS and injected subcutaneously on the abdomen of mice. Tumors grew from 5 to 12 days to target size prior to treatment. One day prior to ACT, all mice received 5 Gy total body irradiation (TBI). For experiments with antibody blockade, 100 μg antibody/mouse in sterile PBS was injected intraperitoneally every other day starting one day post ACT for a total of five cycles. In vivo blocking or neutralizing antibodies were purchased from BioXCell: anti-IL6, clone MP5-20F3; anti-IL6R, clone 15A7; anti-IL17A, clone 17F3; anti-IFNγ, clone XMG1.2; anti-CD4, clone GK1.5; anti-CD8, clone 2.43; Isotype controls: IgG1 clone HRPN, IgG2b clone LTF-2, mouse IgG1 clone MOPC-21.

NSG

M108 was injected subcutaneously in Matrigel (Corning) 47 days prior to ACT. A total of 1.5 × 10^6 M108 cells were injected per animal in a 1:1 mix of Matrigel. Meso-CAR Th17 cells were resuspended in sterile PBS and transferred via tail vein injection.

Cytokine multiplex assay

Murine serum samples were frozen and stored at −80°C prior to analysis. Serum was assayed with the 31-plex Mouse Cytokine Array Discovery Assay (Eve Technologies).

Tissue distribution

Peripheral blood was collected from the mandibular vein into 0.125 mol/L EDTA, subjected to red blood cell lysis (Biolegend), and assayed via flow cytometry. Spleens, lymph nodes, and tumors were taken from animals and processed into single cell suspension by mechanical dissociation over a 70 μm filter. M108 tumors were minced and digested in 1 mg/mL collagenase type IV (Worthington) at 37°C for 1 hour. Skin was minced and incubated in buffer containing 3 mg/mL collagenase IV (Worthington Biochemical), and 0.2 mg/mL DNase (Sigma) in Hank’s balanced salt solution at 37°C for 45 minutes with stirring (14). Digestion was neutralized with RPMI containing...
Flow cytometry
Flow cytometry was performed using BD FACSVerse or BD Fortessa X-20 instruments and analyzed using FlowJo software (BD Biosciences). For extracellular staining, samples were suspended in FACS buffer (PBS + 2% FBS) and incubated with antibodies for 20 minutes. Transcription factor staining was conducted using the FoxP3/Transcription factor kit according to manufacturer's instructions (eBioscience). A complete list of flow antibodies can be found in Supplementary Materials (Supplementary Table S1).

ELISA
TRP-1 T cells used for ELISA kinetics were washed and plated each indicated day at 0.2 × 10^6 cells/200 μL in fresh media and activated with 1 μmol/L TRP-1 peptide and feeder cells at a 1:2 feeder: T-cell ratio for 18 hours. Supernatant was collected and frozen prior to analysis using DuoSet ELISA Kits (R&D Systems) as per manufacturer's instructions.

Microarray
RNA isolation was conducted using Qiagen RNeasy Mini kit for both unactivated CD4⁺ TRP-1 T cells and expanded/polarized TRP-1 Th17 cells. TRP-1 CD4⁺ T cells were combined from 6 TRP-1 transgenic animals. Naïve CD4⁺ T cells were isolated using mouse CD4⁺ negative isolation Kit (Dynabeads, Invitrogen) and used as baseline comparison. Th17 polarization was conducted as described previously for cells expanded 4, 7, and 12 days. RNA was sent to the University of Chicago genomics core and sequenced with Illumina MouseRef-8. Heatmaps were constructed in GraphPad Prism (v7.0) as log 2 fold change over baseline. Microarray data are available through Gene Expression Omnibus (GEO) accession number GSE149330.

Statistical analysis
Kaplan–Meier survival curves were compared between treatment group pairs using the log-rank test. Comparisons of continuous measures between two groups were made using Mann–Whitney U test or Student two-tailed t test as indicated, dependent on whether t-test assumptions were met. Comparisons made were primarily between Th17 treatment groups (day 4 vs. day 7, day 4 vs. day 14). Given the data are exploratory in nature and it was desired not to be overly restrictive, tests performed were minimized and hypothesis tests were not adjusted for multiple comparisons. P values less than 0.05 were considered significant. Plots display mean values and error bars represent SDs.

Data and materials availability
All data are available upon reasonable request. Microarray data are available through GEO accession number GSE149330.

Results
Th17 cells expanded four days demonstrate enhanced tumor immunity
Given the robust antitumor properties of CD4⁺ Th17 cells compared with Th1 or Th2 cells (9, 10, 12, 15), we hypothesized that few Th17 cells obtained using a shortened expansion protocol could effectively treat melanoma. To address this question, CD4⁺ T cells expressing a TCR against TRP-1 were used (10). TRP-1 cells were polarized to a Th17 phenotype and expanded from as little time as 12 hours up to 14 days post TCR activation, where the latter time point models clinical protocols for rapid TIL expansion (16; Fig. 1A). The therapeutic quality of the cell product acquired at each time was assessed, accounting for yield by dividing total number of cells at the end of culture equally among mice. This resulted in doses ranging from 10,000 to 10 × 10^6 cells/mouse. Th17 cells were infused into mice bearing established B16F10 tumors preconditioned with 5 Gy TBI (Fig. 1B).

We discovered that 4 days was the minimum time, after which, Th17 cell products were able to eradicate tumors (Fig. 1C). A low number of Day 4 Th17 cells (0.4 × 10^6) could regress small tumors (~50 mm²) in mice as effectively as those treated with more Th17 cells expanded longer (Fig. 1C). Th17 cells expanded less than 4 days were unable to control melanoma growth.

We next questioned if mice with larger tumors could benefit from Day 4 Th17 cell therapy. We postulated that the low dose of cells yielded with 4 days of expansion would be insufficient to treat larger tumors (median ~140 mm²). Despite administering low numbers of cells, Day 4 Th17 cells were still able to ablate large tumors (Fig. 1D). Unexpectedly, three-fold more Day 7 Th17 cells showed impaired antitumor responses and reduced survival (Fig. 1D and E). Day 14 Th17 cells were able to regress large tumors, but 25-fold more cells were infused and an extended expansion protocol was needed.

We next questioned whether our findings in murine Th17 cells would be recapitulated in human Th17 cells redirected with a CAR. To address this, human mesothelioma was established in NSG mice for a median tumor size of 100 mm² prior to treatment (Fig. 1F). Human Th17 cells were transduced with a 4-1BB anti-mesothelin CAR (13) and expanded for 4 or 7 days. We obtained sufficient T cells to infuse mice with 1 × 10^6 Day 4 CAR Th17 cells versus 3 × 10^6 Day 7 CAR Th17 cells (~30% transduction efficiency). Day 4 CAR Th17 cells regressed tumor in 9/10 animals (one mouse died mid experiment). In contrast, day 7 CAR Th17 cells regressed tumor in only one mouse (Fig. 1G and H). These findings reveal that Th17 cells expanded 4 days ex vivo elicit antitumor responses in both murine TCR and human CAR ACT models.

Four-day expanded Th17 cells promote superior antitumor immunity on a per cell basis
We posited that in equal number, Day 4 Th17 cells would control melanoma more effectively than Day 7 or Day 14 Th17 cells. To test this concept, we infused only approximately 0.3 × 10^6 Th17 cells from each group into lymphodepleted mice (Fig. 2A). At equal doses, Day 4 Th17 cells elicited greater antitumor responses and improved survival versus longer expanded Th17 cells (Fig. 2B and C). Similar results were observed with higher doses spanning 0.8 × 10^6–10^6 cells/animal (Supplementary Fig. S1A and S1B). Day 4 cells engrafted in higher frequencies (Fig. 2D) one week post transfer and persisted longer than Day 7 or Day 14 cells (Supplementary Fig. S1C). Notably, we observed similar results in the human meso-CAR T-cell model (Fig. 2E–H). At equal numbers, Day 4 CAR Th17 cells regressed tumors (Fig. 2F and G) and persisted more effectively at the tumor compared with Day 7 CAR Th17 cells (Fig. 2H). Collectively, our findings suggest that Day 4 Th17 cells possess greater antitumor activity with longer persistence compared with long-term expanded Th17 cells in two aggressive solid tumor models.

Day four Th17 cells exhibit an activated phenotype
We speculated that Th17 cells expanded for shorter duration would be less differentiated in vitro than Th17 cells expanded for one to two
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Figure 1.
Four-day ex vivo expansion yields Th17 cells with potent antitumor efficacy. A, TRP-1 Th17 cells were expanded from 12 hours to 14 days. B, ACT schematic. B16F10 tumors were established in mice from 7 days (tumor \(-50 \text{ mm}^2\)) to 12 \(-7\) days (tumor \(-140 \text{ mm}^2\)). Animals were irradiated 5 Gy one day prior to ACT. C, Tumors established 7 days in mice were treated with Th17 cells. \(n = 5-10\) mice/group, representative of two experiments. D, Mice bearing B16F10 established 12 days were treated as in B, \(n = 10\) mice/group; no treatment, NT. Representative of two experiments. E, Survival of mice treated in D, combination of two experiments. \(n = 22\)/group; \(n = 17\) no treatment (NT). F-H, Human CD4\(^+\) T cells were polarized to Th17 phenotype and transduced with meso-CAR. Human M108 was inoculated 47 days pre-ACT. G, Waterfall plot relating tumor size 33 days post ACT to start. H, Serial measurements. \(n = 8-9\) mice/group, one experiment. Statistics: Log-rank test (E), \(*, P < 0.05; **, P < 0.01\).

weeks. However, regardless of expansion time, these Th17 cells possessed an effector memory profile (CD44\(^{+}\)CD62L\(^{lo}\)) in \textit{vitro} (Fig. 3A). Gene array substantiated these findings, demonstrating low mRNA expression of Cd27 and Sell versus naïve CD4\(^+\) cells (Fig. 3B).

Th17 cells expanded 4 days had a more blast-like phenotype than those expanded longer (Fig. 3C); therefore, we postulated these cells would be highly activated and functional. Four days after TCR activation, Th17 cells expressed high levels of CD25, ICOS, and OX40, which diminished over time (Fig. 3D and E). CD28, CD69, CD95, and PD-1 expression similarly diminished with culture time (Fig. 3D and F). Th17 cells had a dynamic functionality during \textit{in vivo} expansion; Day 4 Th17 cells produced copious IL17A and IL22 relative to Day 7 or Day 14 Th17 cells, while Day 7 and Day 14 cells expressed higher levels of IFN\(\gamma\) and IL2 (Fig. 3G and H). Day 4 and Day 7 Th17 cells both elicited IL6 after peptide restimulation, which was detected at low levels after 2 weeks of expansion (Fig. 3H). Complementing IFN\(\gamma\) production, Th17 cells expanded for more than a week had a cytotoxic profile expressing transcripts for granzymes and perforin (Fig. 3G). Our kinetic analysis underscores the heterogeneity of Th17 cells as they are expanded \textit{in vitro} and supports prior literature concerning acquisition of cytotoxic properties by Th17/Tc17 cells (9, 11, 17). Collectively, Th17 cells are highly activated and predominantly produce helper cytokines 4 days...
after stimulation, while longer in vitro expansion drives a cytotoxic cytokine repertoire.

**Day four Th17 cells elicit systemic cytokine release**

Because expanded Th17 cells have diverse ability to produce cytokines, we suspected that the cytokine profiles in the blood of treated mice would be distinct comparing Th17 cell therapies. Therefore, we transferred TRP-1 Th17 cells expanded from 12 hours to 14 days into mice with B16F10 melanoma (as in Fig. 1A) and assessed serum cytokine concentration one week post ACT. Day 4 Th17 cells not only secreted more IL17 but also induced heightened expression of multiple factors within the host including IL6, MCP-1, G-CSF, and GM-CSF (Fig. 4A and B). Strikingly, this inflammatory response resulted from transfer of only 0.4 × 10^6 Day 4 Th17 cells, while, in contrast, the cytokine response was diminished in animals treated with 25-fold more Day 14 Th17 cells (Fig. 4B). Consistent with an acute inflammatory response, animals treated with few Day 4 Th17 cells grew to endpoint in control treatment-naïve animals, mice cured with Day 4 Th17 cells mounted rapid recall responses against secondary challenges with donor cell frequency (Fig. 4D).

Th17 programming is important to cytokine induction as unpolarized CD4^+ T cells (Th0) expanded 4 days did not induce IL6 or other cytokines in mice (Supplementary Fig. S2A and S2B). Yet, IL17 was not required for antitumor responses unlike IFNγ, as reported previously (10; Supplementary Fig. S2C–S2I). Overall, few Day 4 Th17 cells induce robust cytokine release in mice versus ample Th17 cells expanded longer or Th0 cells expanded 4 days, and concentration of IL6 in circulation correlated with engraftment of donor Th17 cells.

**CD4^+ T cells are required for sustained antitumor responses**

We reproducibly observed that Day 4 Th17 cells elicited durable and long-term antitumor responses in mice (Fig. 5; Supplementary Figs. S1–S5). We next addressed if mice previously cured with this therapy were protected against tumor rechallenge (Fig. 5A). While tumors grew to endpoint in control treatment-naïve animals, mice cured with Day 4 Th17 cells mounted rapid recall responses against secondary challenges.

Figure 2.

Four-day expanded Th17 cells have greater potency on a per cell basis. A, ACT schematic. B, Average dose of 0.29 × 10^6 TRP-1 Th17 cells were transferred; n = 8 mice/group for each experiment. C, Survival of mice from experiments in B; results compiled from two experiments. D, Frequency of Th17 cells (VB14^+ CD45.2^+) in spleen and tumor-draining lymph nodes (dLN) 7 days post treatment; n = 13 mice/group; n = 6 no treatment (NT). Representative of two experiments. E–H, Human M108 tumors were established 47 days prior to ACT. F, Waterfall plot, percent change in tumor size day 33 versus starting tumor size. G, Serial measurements of animals treated in E, H, Frequency of CAR^+ cells 46 days post transfer. n = 6–9 mice/group from one experiment. Statistics: Log-rank test (C); Mann-Whitney U test (D and H). *, P < 0.05; **, P < 0.01; ****, P < 0.0001. Mean and SD shown.

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Figure 3.
Day 4 Th17 cells exhibit an activated phenotype. A, Memory phenotype of TRP-1 Th17 cells. n = 7. B, Gene array heatmaps display (±) log₂-fold change of memory markers versus naive TRP-1 CD4⁺ T cells compiled from 6 animals. C, FSC and SSC of TRP-1 cells prior to peptide activation/polarization (D0) versus 4, 7, and 14 days post activation. n = 5 independent cultures; n = 3 for D0. Connect lines indicate paired biological replicates. D, Heatmap of (±) log₂-fold change of mRNA expression versus naive, unpolarized TRP-1 CD4⁺ T cells. Compiled from 6 animals. E, Cytokine production 18 hours post peptide stimulation. Four independent cultures, representative of two experiments. Statistics: One-sample t test of differences (D and H); ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Mean and SE shown in H.

Because Day 4 Th17 cells induced multiple cytokines and expressed more chemokine transcripts than Day 7 or Day 14 Th17 cells (Supplementary Fig. S3A), we next posited that Day 4 Th17 cells may orchestrate host lymphocytes to regress tumors. Yet, host cells, including CD8⁺, CD4⁺, and B cells, infiltrated the tumor at similar frequencies regardless of Th17 cell therapy (Supplementary Fig. S3B). As Th17 cells can cooperate with CD8⁺ T cells (18), we depleted CD8⁺ T cells to determine whether Day 4 Th17 cells could influence preexisting CD8⁺ T-cell immunity to melanoma. CD8⁺ T cells were not required for tumor regression (Supplementary Fig. S3C–S3E) or to protect against tumor rechallenge (Supplementary Fig. S3F and S3G). Instead, depletion of CD4⁺ T cells resulted in relapse upon rechallenge (Supplementary Figs. S3F, S3H, and S3I), supporting that donor CD4⁺ Th17 cells are more critical for durable treatment outcome.

As few Day 4 Th17 cells regress tumors unlike Day 7 or Day 14 Th17 cells, we posited that Day 4 Th17 cells may more rapidly acquire T resident memory (T RM) phenotypes in the tumor (Supplementary Fig. S4A and S4B). Just prior to tumor regression, however, less than 1% of Th17 cells expressed CD103, CD69, or CLA (Supplementary Figs. S4C and S4D). Although T RM phenotypes were not prominent early after transfer, we discovered that donor CD4⁺ T cells persisting long term (>100 days) in animals cured with Day 4 Th17 cell therapy expressed T RM phenotypes at higher levels in the skin relative to cells persisting
elsewhere (Supplementary Fig. S4C–S4F). Therefore, Day 4 Th17 cells—able to clear tumor at low doses—persisted at the tumor site and expressed a surface signature resembling skin TRM cells (14).

IL6 promotes long-term tumor immunity

The mechanism behind durable immunity elicited by Day 4 Th17 cells remained unknown. As Day 4 Th17 cells induced elevated IL6 relative to long-term expanded Th17 cells, we hypothesized that in vivo, IL6 may be important for their efficacy. After transfer of Day 4 Th17 cells, we blocked IL6 with either an IL6R antibody or IL6 neutralizing antibody (Fig. 5D; Supplementary Fig S5A). Note that IL6 induction was only transient, thus antibody was administered up to 10 days post ACT (Supplementary Fig. S6). Nearly all mice cured with Day 4 Th17 cells survived relapse free (Fig. 5E and F). However, IL6R blockade significantly impaired long-term immunity as indicated by a higher incidence of tumor relapse and reduced overall survival.
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(Fig. 5E and F). Similar results were obtained with IL6 neutralization (Supplementary Fig. S5). IL6 blockade did not significantly impact release of other cytokines, nor did blockade greatly mitigate acute weight loss (Supplementary Fig. S6). Our results reveal, for the first time, that IL6 promotes durable and long-lived Th17-mediated tumor immunity for ACT.

Systemic IL6 dampens engraftment of donor T regulatory cells

IL6 is important for the differentiation of CD4+ T cells to the Th17 phenotype (19). In addition, IL6 can reprogram Tregs toward a Th17 signature by downregulating FoxP3 (20). On the basis of this foundational work, we posited that IL6 may preferentially maintain Th17 biology while blunting the outgrowth of Tregs in the tumor.

We first assessed the overall frequency of donor Th17 cells and Tregs in the tumor after treatment with Th17 cells expanded 4, 7, or 14 days (Fig. 6A). Day 4 Th17 cells were present in the highest frequency at the tumor; yet, the frequency of host Tregs was similar among groups (Fig. 6B). This finding corresponded with the highest ratio of Th17 to Tregs at the tumor for Day 4 Th17 cell therapies (Fig. 6B), but suggested that host Tregs may not be implicated in relapse for day 7 or day 14 cell therapies.

However, we discovered that a small fraction of tumor-specific Tregs remain in the Th17 cell product prior to transfer (Fig. 6C). Thus, we hypothesized that IL6 may promote the engraftment of donor Th17 cells over donor Tregs. While Day 4 Th17 cell products may have a slightly greater frequency of FoxP3⁺ cells ex vivo, this relationship was the opposite in vivo (Fig. 6D). Specifically, donor products expanded for one or two weeks had a greater frequency of FoxP3⁺ cells infiltrating the tumor (Fig. 6D). This corresponded with a heightened ratio of TRP effector to regulatory cells in tumors for Day 4 Th17 cell therapies (Fig. 6D). Interestingly, the frequency of FoxP3⁺ donor cells at the tumor was inversely correlated with serum IL6 (Fig. 6E). Therefore, we next addressed whether IL6 was modulating the balance of Th17 and Treg donor cells (Fig. 6F). In both the tumor and tumor-draining lymph nodes, IL6 blockade augmented the frequency of
FoxP3⁺ donor T cells (Fig. 6G). Collectively, we propose a model where IL6 limits the engraftment of donor Treg populations, enabling Th17 cells to thrive post transfer (Fig. 6H).

In summary, few Th17 cells—expanded only 4 days ex vivo—confer durable and long-term immunity, while Th17 cells expanded longer lose individual potency against solid tumors (Fig. 7). Short-term expanded Th17 cells possessed a heightened ability to engraft and persist in a transiently lymphodepleted host. Efficacy of this therapy appeared to be direct, as depletion of CD4⁺ but not CD8⁺ T cells compromised tumor control. Intriguingly, IL6 was critical for long-term resistance to tumor relapse. IL6 diminished engraftment of donor Tregs in the cellular product, promoting robust engraftment of cytotoxic donor Th17 cells. These findings shed a new perspective on the number of cells needed to ablate large tumors and highlight an unappreciated role for IL6 in fostering long-lived protective immunity to tumors.
**Discussion**

ACT therapy mediates remarkable responses in patients with aggressive cancers; yet, accessibility is limited because of its personalized nature and the advanced technology required to produce a cellular product (21). Ex vivo TIL expansion requires at least one month to obtain a product suitable for rapid expansion to large magnitudes over an additional several weeks (16). Such a treatment delay for patients with aggressive disease can limit the feasibility of this approach. CAR generation via peripheral T-cell modification is naturally quicker, taking up to 2 weeks, although generating CAR T-cell products that are consistently effective against solid tumors has been challenging (2). Our data suggest that shortening ex vivo expansion licenses enhanced antitumor efficacy against solid tumors for either CAR or TIL therapies using a highly potent T-cell subset: the Th17 cell. Short-term expansion of this lymphocyte population directly elicited prolonged responses against solid tumors in part by driving heightened immune activation and release of IL6 in the host, enriching effector Th17 over Treg cells in the tumor. As briefly expanded Th17 cells could overcome some limitations of ACT, our team is designing an early-phase clinical study for safety and efficacy of this therapy.

Th17 cells play a controversial role in tumor immunity, mediating either protumor or antitumor responses in patients, which likely depends on their ability to recognize tumor antigens or their interaction with host immune cells (22). Indeed, given the induction of chemokines and cytokines by early Th17 cells, it is possible that Th17 cells orchestrate host immune cells with either protumor or antitumor function. Endogenous Th17 cells have been associated with myeloid-derived suppressor cells (MDSC), where either MDSCs can promote Th17 polarization via IL6/IL23/IL1β and nitric oxide (23), or where Th17 cells can bolster the suppressive nature of MDSCs, thereby fueling cancer growth (24). IL6 is also a key regulator of MDSC mobilization, and in the chronic setting, drives carcinogenesis (25).

ACT of early Th17 cells might imply cross-talk with MDSCs particularly given IL6 induction in the host; yet, the transient nature of peak IL6 coupled with tumor eradication imply, at least in this context, that Th17 cells or IL6 are not likely influencing MDSCs to foster tumor growth.

In terms of immunity, transferred CD4+ T cells can be more efficacious at tumor clearance than CD8+ T cells owing to their ability to cooperate with other immune cells (26). Tumor-specific Th17 cells elicit robust immunity compared with Th1 or Th2 cohorts, partially due to their stem-like features and self-renewal properties, which permit long-term persistence (9–12). Th17 cells are plastic and can evolve into Th1-like cells, expressing IFNγ, a property deemed critical to their antitumor efficacy (9, 10). Th17 cells also promote cytotoxic T-cell activity against tumors (18). Here, we showed that briefly expanded Th17 cells could directly control tumors, while CD8+ T cells were not required for primary immunity or protection against tumor rechallenge. Yet, our work does not rule out that other host cells—including NK cells, B cells, or granulocytes among others—are not contributing to the treatment outcome. A comprehensive set of studies defining how tumor-specific Th17 cells interact with host immune cells should be conducted and may clarify the debate on protumor versus antitumor effects of Th17 cells.

Our work is the first to show that shortening Th17 ex vivo expansion bolsters in vivo antitumor efficacy via induction of IL6 in the host, which acts to dampen the regulatory characteristics of the transferred cells while instilling their durable antitumor properties. Preclinical models evaluating the role of IL6 in ACT are lacking; as efficacy of human CAR T cells are explored in immunodeficient mice, these systems either fail to induce IL6 or require additional manipulation to promote its release (27–29). Therefore, our observation of multiple cytokine release in an animal with an intact immune system is important and clinically relevant given that autologous transferred cells can interact with host immune cells.
However, stimulating a robust immune response with a cell product may come with risks via toxicities. We remain intrigued that few Day 4 Th17 cells could induce high levels of cytokines in the host, reminiscent of clinical cytokine release syndrome (CRS). CRS, seen in patients receiving cell or antibody-based cancer therapies, corresponds with heightened IL6, IFNγ, GM-CSF, G-CSF, IL2, and MCP-1 (among others) in circulation, and presents with flu-like to life-threatening symptoms (30). To circumvent toxicity, physicians commonly use tocilizumab, an FDA-approved mAb targeting the IL6 receptor, aiming to mitigate adverse events while putatively preserving productive antitumor immunity.

CRS is a common thread relating many T-cell engaging immunotherapies, yet the role of IL6 in antitumor responses is debated (30–33). Blocking IL6 can improve tumor regression in combination with anti-PD-1/PD-L1 checkpoint inhibition immunotherapies (34). Conversely, IL6 induction has been reported in therapeutic preclinical TCR-specific ACT models (35, 36) and clinically is associated with enhanced CAR T-cell expansion (31, 37). In fact, CAR T cells of responsive patients possessed a STAT3 signature associated with IL6, IL17, and IL22 production (37). IL6 is currently considered dispensable for cell therapy as patients who receive tocilizumab for CRS may still experience complete responses, though prospective studies directly addressing this question are lacking (38, 39). Notably, IL6 promotes CD4+ T-cell memory responses to influenza virus (40, 41) and suppresses development of antigen-specific Treg cells post vaccination (42), two qualities that could contribute to long-lived immunity and could be desirable in a cell therapy setting.

Our report of systemic IL6 release is novel to Th17 ACT therapy, and importantly, we describe a role for IL6 in regulating the balance between Th17 and Treg cells to promote durable antitumor immunity. The programs for Th17 cells and Tregs are closely regulated to control immune homeostasis (19, 43). IL6 and TGFβ are critical mediators of Th17 differentiation from naïve CD4+ T cells (19, 44). TGFβ alone promotes FoxP3 expression in naïve CD4+ T cells (45); yet, IL6 can downregulate FoxP3 via activating STAT3 and promoting RORγt to drive the Th17 program (19). We discovered that IL6, induced by Day 4 Th17 cells but not by Th17 cells expanded longer-term, was required to elicit durable resistance to tumor relapse. IL6 was the critical mediator that diminished engraftment of donor tumor-specific Tregs from the cell product, thereby promoting a more inflammatory tumor microenvironment. These findings harmonize with other reports showing that IL6 promotes Th17 immunity via suppression of Treg cells (42). We further reveal that early Th17 cells persist in the skin of animals after melanoma clearance, and these cells acquire phenotypes reminiscent of Th17 cells. However, it remains unknown if IL6 plays a role in acquisition of this particular memory phenotype. Our findings are clinically meaningful as they uncover that IL6 imprints infused antitumor Th17 cells with long-lived and protective responses against metastasis and aggressively growing solid malignancies.

Previous reports indicate that reduction of ex vivo expansion time for CAR CD8+ T cells improves their efficacy against CD19+ malignancies (46). Our work broadens applicability of this concept by demonstrating that few CD4+ T cells can ablate hard-to-treat solid tumors and highlights a previously unappreciated role for cell therapy–induced IL6 in generating long-lasting memory responses to cancer. Yet, the efficacy of antitumor Th17 cells has yet to be explored in clinical trials. Theoretically, polarizing cytokines could be used to generate these cells or naturally arising Th17 cells (CD4+CCR4+CCR6+ T cells; ref. 47) could be isolated to prepare TIL, TCR, or CAR T-cell products. Given our observation of high systemic IL6 in this model of Th17 cell therapy, cytokine levels should be monitored closely in patients.

Because we describe a beneficial role for IL6 in Th17 cell therapy, yet acknowledge that IL6 blockade protects patients against toxicity, we advocate for methods where IL6 could be leveraged locally to foster durable immunity without compromising the patient’s health. More broadly, understanding how IL6 directs memory responses for other cancer immunotherapies is important to improve patient outcomes. Studies addressing timing of IL6 blockade or levels of IL6, which are harmful rather than immunologically beneficial, are warranted. Targeting other cytokines, like IL1 (28, 29) MCP-1, or GM-CSF could differentially affect toxicity or efficacy, thus studies on the influence of specific cytokines on ACT outcomes should be conducted.

Moving forward, it is important to consider the impact of ACT expansion protocols on treatment outcomes in patients with cancer. While cellular therapies have been administered for decades, clinical protocols have not adopted shorter expansion methods (3, 4, 6). The approach detailed herein rapidly generates potent antitumor T cells despite lower yield, which could improve feasibility for treating patients with aggressive disease. Our findings have implications for translating ACT therapies to patients worldwide.

Disclosure of Potential Conflicts of Interest
H.M. Knochelmann reports grants from NIH and Melanoma Research Foundation during the conduct of the study; in addition, H.M. Knochelmann has a patent for ex vivo expansion of T-cells for ACT pending. A.S. Smith reports grants from Medical University of South Carolina during the conduct of the study. J.S. Bowers reports grants from NIH during the conduct of the study; in addition, J.S. Bowers has a patent for modified T cells and uses thereof issued. M.H. Nelson reports grants from American Cancer Society during the conduct of the study. G.B. Lesinski reports other funding from ProDa Biotech, LLC, as a consultant and research funding via sponsored research agreement through Emory University from Merck and Co., Inc., Vaccinex, Inc., Boehringer Ingelheim, Inc., and Bristol Myers Squibb, Inc. outside the submitted work. Z. Li reports being a scientific advisory board member for Alphamab, Heat Biologics, and Hengxenix outside the submitted work. C.M. Paulos reports a provisional patent pending related to this work. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
H.M. Knochelmann: Conceptualization, resources, data curation, formal analysis, writing—original draft, writing—review and editing. C.J. Dwyer: Data curation, formal analysis, investigation, methodology, writing—review and editing. M.M. Wyatt: Data curation, formal analysis, supervision, methodology, writing—review and editing. A.S. Smith: Data curation, formal analysis, investigation, methodology, writing—review and editing. J.S. Bowers: Data curation, investigation, methodology, writing—review and editing. M.M. Wyatt: Data curation, formal analysis, supervision, investigation, methodology, writing—review and editing. M.H. Nelson: Conceptualization, data curation, supervision, methodology, writing—review and editing. G.O. Rangel Rivera: Data curation, investigation, methodology, writing—review and editing. J.D. Horton: Data curation, writing—review and editing. C. Krieg: Supervision, investigation, writing—review and editing. K. Armeson: Formal analysis, writing—review and editing. G.B. Lesinski: Supervision, investigation, writing—review and editing. M.P. Rubinstein: Supervision, investigation, writing—review and editing. Z. Li: Supervision, investigation, writing—review and editing. C.M. Paulos: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, visualization, writing—original draft, project administration, writing—review and editing.

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