Antigen-Specific Culture of Memory-like CD8 T Cells for Adoptive Immunotherapy

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Abstract

Cytotoxic T cells typically are expanded *ex vivo* in culture with IL2 for adoptive immunotherapy. This culture period leads to a differentiated phenotype and acquisition of effector function, as well as a loss of *in vivo* proliferative capability and antitumor efficacy. Here, we report antigen-specific and polyclonal expansion of cytotoxic T cells in a cocktail of cytokines and small molecules that leads to a memory-like phenotype in mouse and human cells even during extended culture, leading to enhanced *in vivo* expansion and tumor control in mice. *Cancer Immunol Res; 2(9); 839–45. ©2014 AACR.*

Introduction

Adoptive immunotherapy is a promising treatment for hematologic malignancies and solid tumors that consists of infusion of *ex vivo* manipulated immune cells with antitumor specificity (1, 2). This specificity is either an inherent property of cultured tumor-infiltrating lymphocytes (1, 3) or is introduced by antigen-specific expansion (4) or transduction with an antigen receptor gene (5, 6), but, in either case, the T cells are typically stimulated with the potent T-cell mitogen and growth factor IL2. Repeated rounds of stimulation of $CD8^+$ T lymphocytes in the presence of IL2 lead not only to acquisition of effector function and enhanced *in vitro* killing of target cells but also to terminal differentiation and loss of proliferative capacity associated with inferior tumor control (7, 8). Numerous signaling pathways and transcriptional controllers have been identified as enhancing self-renewal capability and memory formation of CD8 T cells, including signaling by common γ-chain cytokines other than IL2 (particularly IL7 and IL21; refs. 9–11), the Wnt–β-catenin pathway (12), and inhibition of the cell growth and metabolism pathways (13–15).

Materials and Methods

T-cell culture

All immune cells were cultured in a T-cell medium consisting of RPMI-1640 with 25 mmol/L of HEPES, supplemented with 10% heat-inactivated fetal bovine serum and 1:100 with penicillin–streptomycin, nonessential amino acids, and sodium pyruvate and 50 μmol/L of β-mercaptoethanol. Mouse CD8 T cells were isolated by pressing mouse spleen and lymph node cells through a 40-μm nylon mesh filter in RPMI-1640 followed by negative selection with a magnetic isolation kit for CD8α T cells (Miltenyi Biotec). For OT-I experiments, mouse CD8 T cells were separately isolated from C57BL/6-Tg(TcraTcrb)1100Mjb/J (hereafter OT-I/Thy1.2) mice (16) and B6.PL-Thy1a/CyJ (hereafter Thy1.1) mice and mixed at a ratio of 1:100. HLA-typed peripheral blood mononuclear cells (PBMC) from cytomegalovirus (CMV)-seronegative donors were obtained from Precision Bioservices. Human CD8 T cells were isolated by separation from freshly thawed PBMCs by negative selection with a magnetic isolation kit for CD8α T cells (Miltenyi Biotec). Antigen-specific cells were enriched as previously described (17) from freshly isolated lymph node and spleen cells (mouse) or overnight-incubated PBMCs (human) after staining with dextramers according to the manufacturer's instructions (Immudex). T cells were incubated and mixed with peptide-pulsed dendritic cells (DC) at a ratio of 2:1 or CD3/CD28 beads (Invitrogen) at a ratio of 1:1 and plated at a density of 10,000 to 20,000 T cells per well of round-bottomed 96-well plates in a volume of 150 to 200 μL per well. Fresh media containing the same concentration of cytokines and drugs were added to each well at half the volume initially plated after 3 to 4 days. Cells were spun over a histopaque-1077 (Sigma-Aldrich) gradient to remove dead cells, counted, and replated with fresh DCs or CD3/CD28 beads once a week.

Generation of DCs

Bone marrow–derived dendritic cells (BMDC) were cultured as previously described (18). C57BL/6 femora, tibiae, humeri, and pelvises were rinsed with RPMI-1640 through a 40-μm nylon mesh, washed, red blood cell lysed with ACK buffer, washed again, and plated in T-cell media supplemented with murine GM-CSF for 7 to 9 days. BMDCs were matured 24 hours before use by the addition of 2 μg of polyinosinic:polycytidylic acid stabilized with SIINFEKL peptide and polyinosinic-polycytidylic acid stabilized with poly-L-lysine (polyICLC, provided by Oncovir) per mL of culture medium.

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Human monocyte–derived DCs (moDC; ref. 4) were generated by isolating monocytes from freshly thawed PBMCs with CD14-positive selection microbeads (Miltenyi Biotec) and culturing these monocytes for 8 to 10 days in T-cell medium supplemented with human GM-CSF and human IL4. moDCs were matured 24 hours before use by the addition of 2 μg of polyICLC per mL of culture medium. For both mouse BMDCs and human moDCs, DCs were coated with cognate-antigen peptide by adding peptide to matured DCs at a concentration of 20 μg/mL and incubating at 37°C for 2 hours. DCs were washed 4 times in RPMI-1640 to remove excess peptide from media before being mixed with T cells.

Cytokines and small molecules

All cytokines except for human IL2 were from PeproTech. Mouse cells were plated in T-cell medium containing 1 ng/mL recombinant murine IL2, or 10 ng/mL murine IL7 and 20 ng/mL murine IL21. Human cells were plated in T-cell medium containing 80 U/mL recombinant human IL2 (R&D Systems), or 10 ng/mL human IL7 and 20 ng/mL human IL21. Human and mouse cells were incubated with 2-deoxyglucose (Sigma) at a concentration of 400 μmol/L, and TWS119 (Selleck Chemicals) at a concentration of 4 μmol/L. For the generation of BMDCs, mouse bone marrow cells were plated in 20 ng/mL of murine GM-CSF. For the generation of moDCs, human monocytes were plated in 100 ng/mL of human GM-CSF and 50 ng/mL of human IL4.

Animals, tumor model, adoptive transfers, peptides, and flow cytometry

Mouse experiments were performed in accordance with the University of Minnesota Animal Care and Use Committee guidelines. C57BL/6J mice, OT-I, and Thy1.1 mice were purchased from The Jackson Laboratory and used at 6 to 10 weeks of age.

Thy1.1 mice were inoculated with 30,000 cells of the SIINFEKL-expressing syngeneic C57BL/6 glioma line Quad-KM3M14 in the ventral striatum as previously described (19). Tumor take and growth were assessed with bioluminescent imaging using a Xenogen IVIS 100 imager. Mice were treated with 75 mg/kg temozolomide (Toronto Research Chemicals) suspended in sterile PBS by oral gavage daily for 5 days starting 5 days after the tumor was inoculated. One day after the last dose of temozolomide, mice were given an adoptive transfer of 750,000 OT-I T cells as an intravenous injection into the retro-orbital venous sinus. Mice were vaccinated with 50 μg of OVA peptide (AnaSpec) and 10 μg of polyICLC via a subcutaneous injection in the thigh near the inguinal lymph node 2 and 5 days after the adoptive transfer. Transferred T-cell levels were measured by flow cytometry using 50 μL of blood extracted from the retro-orbital venous sinus. All antibodies used were from eBioscience. OVA (SIINFEKL), and CMV pp65 (NLVPMVATV) peptides were from AnaSpec. The CDK4 R24C (ACDPHSGHFV) peptide was from Gen-Script. Dextramers were from Immudex. Flow cytometry was performed using BD FACSCanto, LSR II, and LSRFortessa flow cytometers. Cell sorting for RNAseq was performed using a BD FACSAria II. Flow cytometry data were analyzed with Cytobank software.

RNA sequencing and gene expression data analysis

OT-I CD8 T cells were sorted as indicated in the text. Cells were pelleted, and total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies). Each sample was divided into three technical replicates and sequenced to a depth of approximately 40 million paired-end 50 base-pair reads using the Illumina HiSeq 2000 sequencer (Illumina). Fastq files generated from RNA sequencing were mapped to the mm10 mouse genome using TopHat 2.0 (20). Differential expression was calculated using Cuffdiff 2.1.1 (21).

Expression data for 28 mouse samples from the Immunological Genome Project Consortium (http://www.immgen.org/; ref. 22) were downloaded from the Gene Expression Omnibus (GEO; GEO accession no. series GSE15907: specific files GSM605891-6, GSM605898-605911, and GSM920634-920641) for comparison with the gene expression data based in this study. These files were normalized according to the methods described in ref. (23). Expression data described in this study were compared with gene expression data described in the gene list and data provided in Supplementary Table S1 from Best and colleagues (ref. 23; 44 mouse samples). In the microarray data described above, multiple probes for a gene were averaged. Datasets from each gene expression technique (RNAseq and microarray) were separately normalized using the average value of each gene. All datasets were uploaded and analyzed using Genedata Analyst v7.6. Hierarchical clustering was performed with Cluster v3.0 (24) using the Euclidean distance metric and average linkage. Treeview v1.1.3 (25) was used to create heat-maps. To explore functional networks, selected gene expression lists with both fold change of >2 and direction of change were submitted to Ingenuity Pathway Transcriptional Regulators Analysis (www.ingenuity.com). The GEO accession number for this study is GSE54877.

Results and Discussion

The coupling of proliferation and differentiation of CD8 T cells is particularly problematic for antigen-specific cultures, which use multiple stimulations with autologous or artificial antigen-presenting cells pulsed with specific peptides to massively expand antigen-specific cells from very low precursor frequencies in the peripheral blood (4, 26). This approach is appealing because it does not require tumor-infiltrating lymphocytes or tumor-restricted expression of targets for chimeric antigen receptors, and can be adapted to use patient-specific neoantigens identified prospectively by deep sequencing (1, 27–29). As a model system to investigate modifications to antigen-specific cell-culture conditions that would yield less differentiated, memory-like CD8 T cells from patient PBMCs, we mixed congenically marked OT-I CD8 T cells with polyclonal C57BL/6 CD8 T cells at a ratio of ∼1:100, approximating a frequency achievable from the naïve repertoire via peptide–MHC multimer enrichment (17). We screened individual modifications to the culture protocol by mixing SIINFEKL-pulsed BMDCs with the mixed CD8 T cells in media containing either alternate cytokines besides IL2, or IL2 and added drugs and assaying the percentage of memory phenotype CD44$^+$CD62L$^+$ OT-I T cells after 2 weeks in culture. We identified numerous
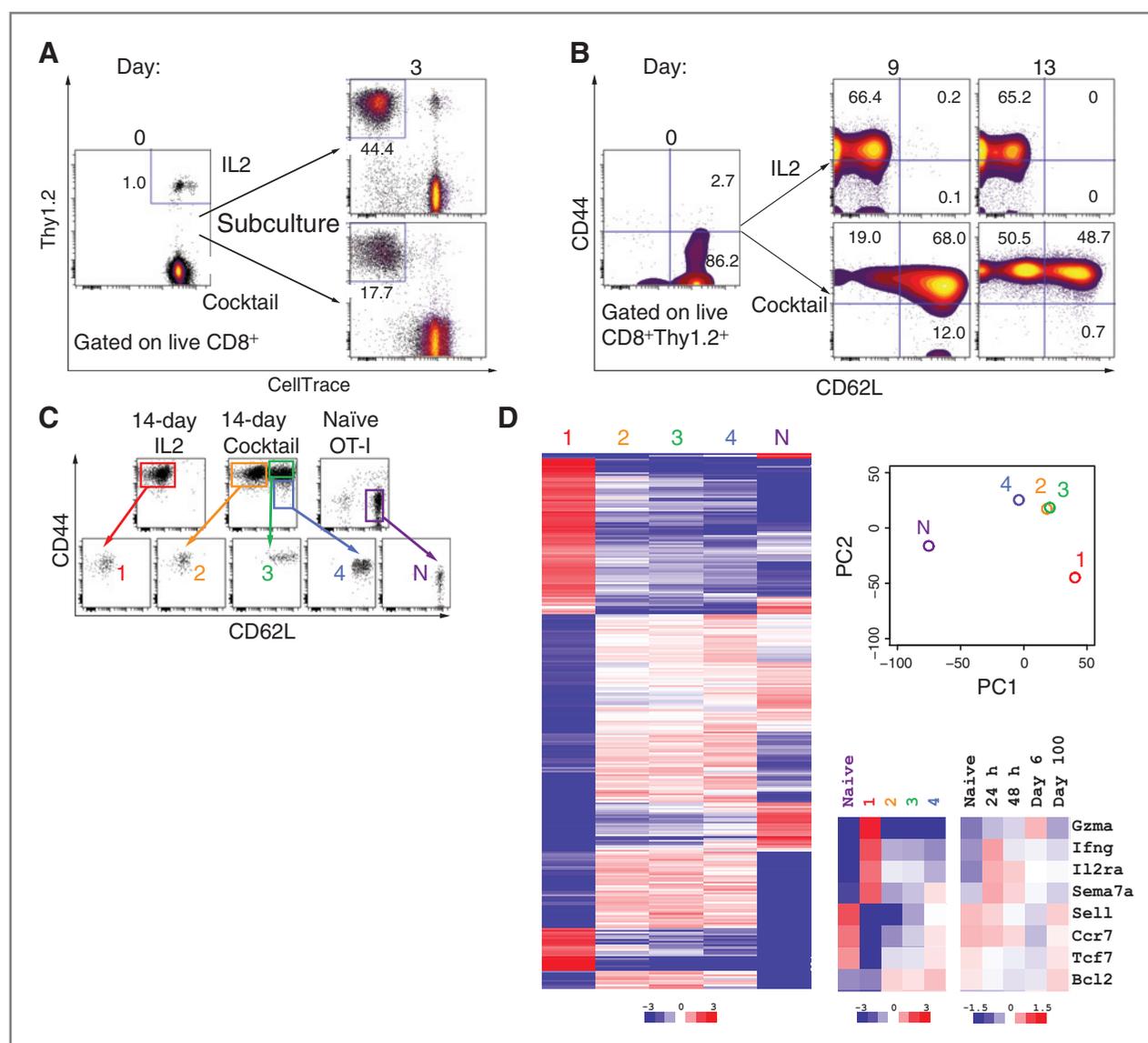
Figure 1. CD8 T cells cultured in a cocktail of cytokines and small molecules resemble memory cells. A, flow cytometric analysis of congenically marked OT-I CD8 T cells mixed at the ratio of approximately 1:100 with bulk CD8 T cells and expanded with SIINFEKL-pulsed BMDCs in IL2 or cocktail of small molecules and cytokines (see Materials and Methods). B, representative plots of expression of the phenotypic markers CD62L and CD44 on Thy1.2+ OT-I CD8 T cells. Plots in A and B are representative of more than five independent experiments. C, plots of pre- and postsort Thy1.2+ OT-I CD8 T cells grown as in A and purified by FACS for RNAseq analysis. D, unsupervised hierarchical clustering of RNAseq of populations sorted as in C showing all genes with a >2-fold difference between groups 1 and 3. Inset, principal component analysis of genes with a >2-fold difference between any two groups for populations depicted in C, and heatmap of selected biologically relevant genes in populations depicted in C and at selected time points of the OT-I response to *Listeria monocytogenes* OVA (from ref. 21).

modified culture conditions that yielded small subpopulations of CD62L+ cells (Supplementary Fig. S1), and when these modifications were combined in a cocktail of memory-inducing factors (IL21 and IL7 instead of IL2, plus 2-deoxyglucose and the GSK3β inhibitor TWS119), we noted an additive effect, with sustained antigen-specific proliferation of OT-I cells (Fig. 1A) in which approximately half of the cells remained CD62L+ (Fig. 1B). Although the accumulation of CellTrace-diluted cells is slower for cells cultured in this cocktail of factors (Fig. 1A), this slower proliferation is balanced by the enhanced survival of cells in this culture, as we observed a greater viability of cells

cultured in the cocktail versus cells grown in IL2 (Supplementary Fig. S2). We enumerated the number of antigen-specific cells grown by this method and observed roughly equivalent numbers of viable antigen-specific cells at various time points in cultures grown with this cocktail of factors as in cultures of cells grown in IL2 (Supplementary Fig. S3), indicating that this method could be used with similar amounts of starting material as in conventional expansion protocols.

To further characterize these cells, we performed RNAseq analysis on sorted OT-I cells derived from naïve (CD44loCD62L+) OT-I mouse spleens, cocktail-cultured cells, and differentiated
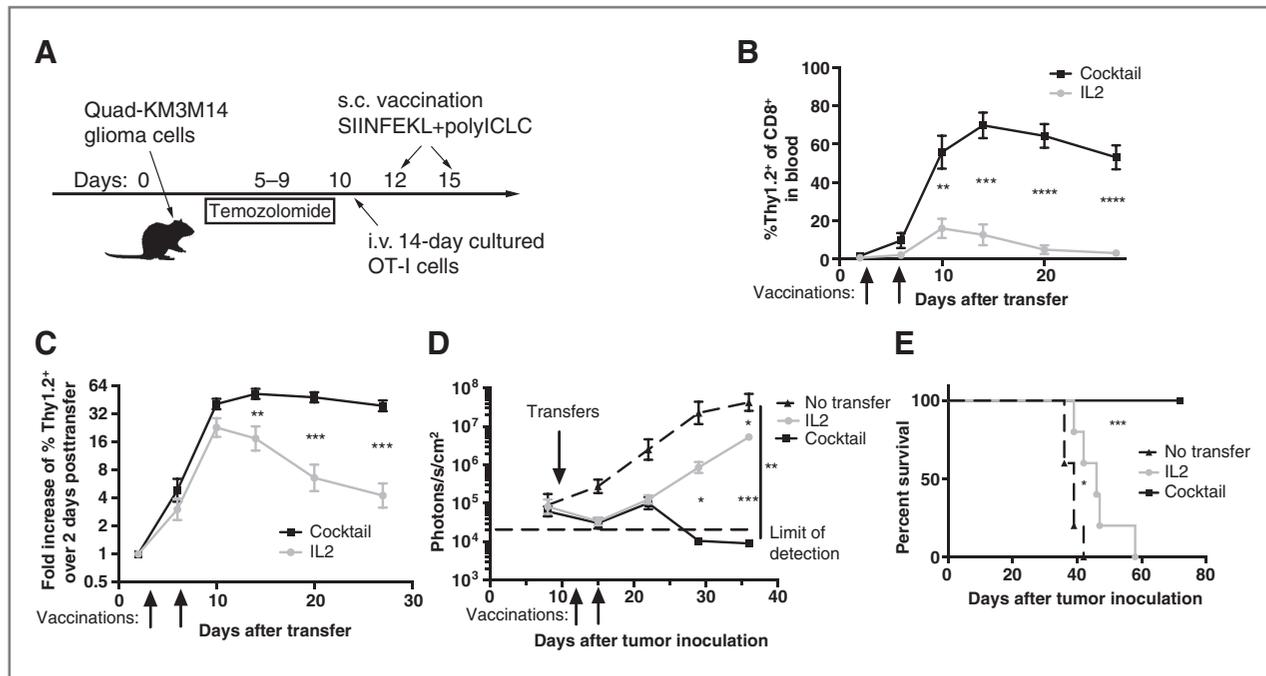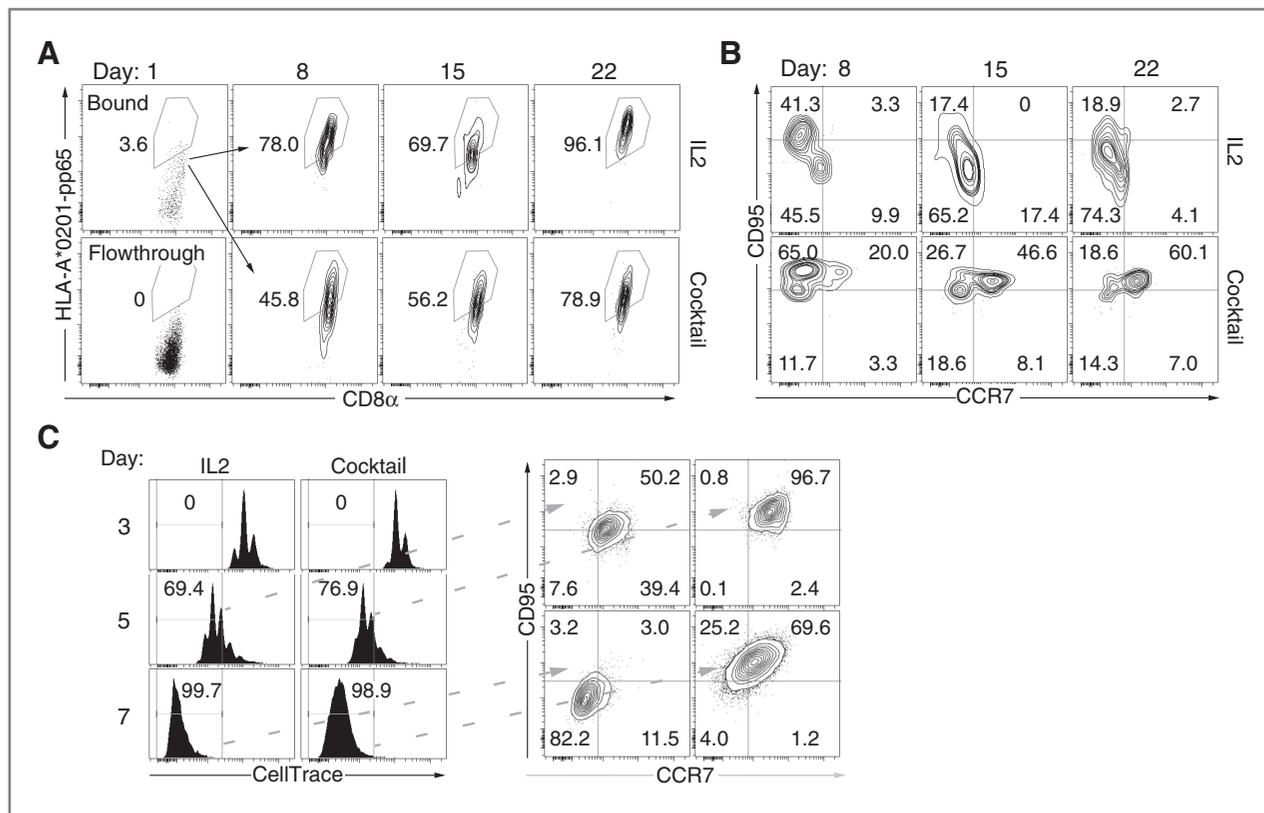
Figure 2. Memory-like antigen-specific cultured CD8 T cells have greater antitumor efficacy than cells cultured in IL2. A, schematic illustration of tumor treatment experiment. Mice were implanted with 30,000 OVA-expressing Quad-KM3M14 syngeneic glioma cells and treated with conditioning temozolomide. A total of 750,000 OT-I cells grown as in Fig. 1A were adoptively transferred and mice were boosted with two s.c. vaccinations with SIINFEKL peptide and polyICLC. B, Thy1.2$^+$ OT-I CD8 T cells as a percentage of total CD8 T cells in blood. $**$, $P < 0.01$; $***$, $P < 0.001$; $****$, $P < 0.0001$, IL2-cultured cells versus cocktail-cultured cells (Student two-tailed t test). C, percentage of Thy1.2$^+$ OT-I CD8 T cells of total CD8 T cells in blood, expressed as fold increase over the percentage of cells 2 days after transfer. $**$, $P < 0.01$; $***$, $P < 0.001$, IL2-cultured cells versus cocktail-cultured cells (Student two-tailed t test). D, bioluminescent tumor signal of mice adoptively transferred with no cells, IL2-cultured cells, or cocktail-cultured cells. $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$, for indicated comparisons. E, survival of mice in D. $*$, $P < 0.05$; $***$, $P < 0.001$, for indicated comparisons (log-rank test). Data depicted are one representative experiment of two separate experiments with similar outcomes.

(CD44^{hi}CD62L$^-$) IL2-cultured cells. Because the extent of differentiation of mouse CD8 T cells has been identified as a determinant of *in vivo* expansion and antitumor efficacy, we sorted the heterogeneous cocktail-cultured cells into CD44^{hi} CD62L$^-$, CD44^{hi}CD62L$^+$, and CD44^{lo}CD62L$^+$ before extracting RNA (Fig. 1C). Principal component analysis and unsupervised non-hierarchical clustering revealed that all three populations cultured in the memory cocktail exhibited gene transcription profiles that clustered close to one another, were highly distinct from cells grown in IL2, and clustered closer to naïve cells than IL2-grown cells (Fig. 1D). To compare our *in vitro* derived cells with *bona fide* memory cells differentiated in response to an infection, we compared the transcriptome of our cultured OT-I cells with OT-I CD8 T cells at various time points of an *in vivo* immune response to *Listeria monocytogenes* OVA described by the Immunological Genome Consortium (23). This analysis revealed that cells grown in IL2 *in vitro* and early effector OT-I cells *in vivo* (1–6 days after infection) showed similar transcriptional profiles, highly expressing genes involved in the effector function (*Gzma, Ifng, Il2ra*, and *Sema7a*). Cocktail-grown cells and late memory cells (100 days after infection) also had transcriptional profiles that were similar to one another, showing levels of expression of effector genes that were intermediate between naïve cells and IL2-grown cells, and higher levels of genes involved in self-renewal and survival including *Bcl2* and *Tcf7* than IL2-grown cells or

early effector cells. (Fig. 1D, Supplementary Fig. S4) Upstream regulator analysis (Supplementary Table S1) identified numerous transcriptional regulators activated in cocktail-grown cells versus IL2-grown cells. These included factors previously described as master regulators of CD8 T-cell memory, including FOXO1 (30) and BCL6 (31), as well as novel transcription factors that have not been described as having a role in CD8 T-cell memory. Tentatively identified novel factors of biologic interest are related to pathways of resistance to aging, stress response, and metabolism such as SIRT1 and FOXM1 (32, 33), as well as factors that have proved critical to other aspects of immune function, such as FOXM1 and MYB in the regulation of proliferation in germinal center B cells (34).

To assay the cocktail-cultured OT-I cells for efficacy in treating a solid tumor, we transferred equal numbers of IL2- or cocktail-cultured OT-I cells intravenously into mice bearing established Quad-KM3M14 gliomas (that express the ovalbumin epitope SIINFEKL) after treatment with the conditioning chemotherapy temozolomide (19). Upon *in vivo* restimulation with a vaccination consisting of the ovalbumin epitope SIINFEKL peptide and polyinosinic–polycytidylic acid stabilized with polyICLC (Fig. 2A), IL2-cultured cells rapidly expanded and then quickly contracted, whereas the cocktail-cultured cells expanded to a greater extent and maintained a significantly higher percentage of CD8 T cells in the blood for several weeks after vaccination (Fig. 2B). Even when normalized to the percentage of CD8 T cells present in

Figure 3. Antigen-specific and polyclonal culture of memory-like CD8 T cells from human PBMCs. A, flow cytometric analysis of CD8 T cells cultured from CMV-seronegative HLA-A*0201+ PBMCs after pulldown with CMV-specific HLA-A*0201-pp65 dextramer. Cells were cultured in IL2 or cocktail of memory-associated cytokines and small molecules. Plots depict dextramer-stained cells at pulldown and after 1, 2, or 3 weeks in culture with autologous antigen-pulsed moDCs. B, representative plots of expression of the phenotypic markers of memory CCR7 and CD95 (Fas) on HLA-A*0201-pp65 dextramer positive gated cells. Plots in A and B are representative of experiments performed with cells from two donors and two separate antigens. C, flow cytometric analysis of MACS-isolated CD8 T cells from human PBMCs expanded with CD3/CD28 antibody-coated beads in IL2- or cocktail-containing media. Cells were labeled with CellTrace violet and divided cells were gated as shown for analysis of phenotypic markers CCR7 and CD95. Plots are representative of five independent experiments with PBMCs from three donors.

blood 2 days after transfer to account for different rates of initial engraftment, the *in vivo* fold expansion and persistence of the cocktail-treated cells were greater (Fig. 2C). This greater expansion and persistence were associated with improved tumor control and enhanced overall survival of glioma-bearing mice that received cocktail-grown cells compared with mice that received IL2-grown cells or control mice that received no transferred cells (Fig. 2D and E).

We next sought to test whether this approach would be feasible using antigen-specific CD8 T cells enriched from the polyclonal repertoire. We stained C57BL/6 splenocytes and lymph node cells with a Kb-Ova–specific dextramer and performed magnetic enrichment with anti-PE microbeads. The bound fraction eluted after performing this enrichment contained naïve antigen-specific cells at frequencies comparable with our OT-I experiments, which could be expanded when cultured with SIINFEKL-pulsed BMDCs in either IL2- or cocktail-containing media (Supplementary Fig. S5A). Similar to the OT-I system, IL2-cultured cells upon expansion were almost uniformly negative for expression of CD62L, whereas cocktail-cultured cells were heterogeneous with a large fraction remaining positive for CD62L after antigen-specific expan-

sion (Supplementary Fig. S5B). To test the applicability of this cocktail to human cells, we performed magnetic enrichment of antigen-specific CD8 cells from naïve human PBMCs by using HLA-A*0201 dextramers specific for both viral (ref. 35; Fig. 3A) and tumor neoantigen (36) epitopes (Supplementary Fig. S6), and cultured those cells with antigen-pulsed autologous moDCs. In both culture conditions, antigen-specific CD8 T cells preferentially expanded upon culture with antigen-pulsed moDCs (Fig. 3A). Antigen-specific CD8 T cells cultured in the cocktail of memory-inducing factors expressed high levels of cell-surface molecules that were differentially expressed between IL2- and cocktail-treated mouse cells, including CD95 (Fas receptor) and CCR7 (Supplementary Table S2), and which serve as markers of a memory phenotype and enhanced *in vivo* persistence upon adoptive transfer in primates (ref. 37; Fig. 3B). To test the applicability of the cocktail for applications requiring polyclonal expansion (e.g., before chimeric antigen receptor transduction), we used CD3/CD28 beads to expand polyclonal human CD8 T cells. We observed a similar degree of expansion in both groups as assessed by the dilution of CellTrace dye, and found that the cocktail-grown cells expressed memory marker cell-surface proteins (Fig. 3C).

The phenotype of these cells seems to correspond to a conventional T_{CM} phenotype and does not seem to be enriched for T_{SCM}, as has been reported (38), as the $CD8^+$ cells expanded with polyclonal stimulation were essentially all $CD45RO^+$ within 3 days of stimulation (Supplementary Fig. S7).

We have demonstrated that naïve CD8 T cells cultured with antigenic stimulation (either in the form of specific peptide–MHC complexes or via anti-CD3) in the presence of a cocktail of memory formation–associated small molecules and cytokines adopt a differential gene expression program relative to cells grown in IL2. In mice, these cells have a greater proliferative potential and persistence *in vivo* and therefore have greater antitumor activity. Coupling this method with a peptide–MHC multimer pulldown allows the rapid (2–3 weeks) culturing of antigen-specific T-cell pools with a less differentiated phenotype than cells grown in IL2. Although these cells retain some naïve-like characteristics (for instance, greater *in vivo* expansion upon adoptive transfer than differentiated effector cells), the predominant phenotypic and transcriptional characteristics of these cells demonstrate activation and expansion by antigen and resemble antigen-specific memory cells expanded after the resolution of a primary infection. The generation of such antigen-specific memory-like CD8 T cells could prove advantageous for cancer immunotherapy, particularly if coupled with prospective bioinformatics-based identification of tumor-specific mutation-derived neoantigens. This combination of factors may also be useful for expanding minimally differentiated T cells before transduction with antigen receptor–expressing vectors, or as a jumping off point for the further optimization of T-cell expansion protocols to limit differentiation. Finally, by analyzing the genetic program instantiated by these less-differentiated cells, we have outlined some of the transcriptional controllers that could be useful targets of future genetic engineering approaches that would seek to enforce maintenance of proliferative capacity in T cells in extended culture before adoptive immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.J. Litterman, S.C. Jameson, D.A. Largaespada
Development of methodology: A.J. Litterman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.J. Litterman, D.M. Zellmer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. Litterman, D.M. Zellmer, R.S. LaRue, S.C. Jameson
Writing, review, and/or revision of the manuscript: A.J. Litterman, D.M. Zellmer, D.A. Largaespada
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.M. Zellmer
Study supervision: D.A. Largaespada

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