Engraftment of human central memory-derived effector CD8$^+$ T cells in immunodeficient mice

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In clinical trials of adoptive T-cell therapy, the persistence of transferred cells correlates with therapeutic efficacy. However, properties of human T cells that enable their persistence in vivo are poorly understood, and model systems that enable investigation of the fate of human effector T cells (T_E) have not been described. Here, we analyzed the engraftment of adoptively transferred human cytomegalovirus pp65-specific CD8$^+$ T_E cells derived from purified CD45RO$^+$CD62L$^+$ central memory (T_{CM}) or CD45RO$^+$CD62L$^-$ effector memory (T_{EM}) precursors in an immunodeficient mouse model. The engraftment of T_{CM}-derived effector cells ($T_{CM/E}$) was dependent on human interleukin-15, and superior in magnitude and duration to T_{EM}-derived effector cells ($T_{EM/E}$). T-cell receptor Vβ analysis of persisting cells demonstrated that CD8$^+$ $T_{CM/E}$ engraftment was polyclonal, suggesting that the ability to engraft is a general feature of $T_{CM/E}$. CD8$^+$ $T_{EM/E}$ proliferated extensively after transfer but underwent rapid apoptosis. In contrast, $T_{CM/E}$ were less prone to apoptosis and established a persistent reservoir of functional T cells in vivo characterized by higher CD28 expression. These studies predict that human CD8$^+$ effector T cells derived from T_{CM} precursors may be preferred for adoptive therapy based on superior engraftment fitness. (*Blood*. 2011;117(6): 1888-1898)

Introduction

Durable reconstitution of immunity to viral pathogens by the adoptive transfer of virus-specific effector T cells (T_E) can be reproducibly achieved in a variety of clinical settings.[1,2] The adoptive transfer of Epstein-Barr virus (EBV)-specific T cells to target EBV-associated malignancies also leads to persistent engraftment of transferred T cells and tumor regressions.[3] The persistence of transferred tumor-reactive T cells, either isolated from the blood or tumor infiltrate, has been less consistent, even if the host undergoes lymphodepleting chemotherapy before T-cell transfer and receives interleukin-2 (IL-2) after T-cell transfer.[4] In T-cell therapy of viruses, the T_E cells are typically derived from virus-specific memory T cells isolated from immune donors; however, studies in which virus-specific T-cell clones or gene-marked polyclonal T-cell lines have been adoptively transferred have shown that not all T_E cells derived from memory precursors are capable of durable engraftment.[5,6] It is now possible to engineer any T cell to be tumor-reactive through the introduction of genes that encode T-cell receptors or chimeric antigen receptors that are specific for tumor antigens. Thus, defining characteristics of T cells that predict their persistence after adoptive transfer has important implications for future applications of T-cell therapy for cancer.

The repertoire of memory T cells in mice and humans is heterogeneous with respect to phenotype and function.[7,8] Conventionally, CD8$^+$ memory T cells are divided into effector memory T cells (T_{EM}), which are prevalent in the blood and peripheral tissues and are capable of rapid effector function after engaging antigen; and central memory T cells (T_{CM}), which express CD62L and CCR7 and, as a consequence, reside predominantly in lymph nodes where they are capable of extensive proliferation and differentiation on antigen reencounter. Thus, it is conceivable that the cell-intrinsic programming of these distinct memory T-cell subtypes may dictate divergent fates of their derived effector cells, including the ability to survive in vivo after adoptive transfer. We recently demonstrated a clear dichotomy in the engraftment potential of T_{CM}- and T_{EM}-derived CD8$^+$ cytotoxic T lymphocyte clones using a nonhuman primate model system in cynomolgus macaques that closely recapitulates the cell culture methods used in clinical T-cell therapy trials.[5] Virus-specific effector cytotoxic T lymphocytes derived from T_{CM}, but not T_{EM}, established persistent, functional T-cell immunity after adoptive transfer to lymphoreplete healthy macaques. Remarkably, an infusion of T_E cells derived from a single T_{CM} restored pools of both T_{CM} and T_{EM} in vivo that mounted a recall response to subsequent viral antigen challenge.[5] A more recent study in which T_E clones were derived from T_{CM} or T_{EM} elicited by prior vaccination and adoptively transferred to rhesus macaques did not observe a consistent difference in engraftment, with both subsets displaying poor persistence in the peripheral blood.[9]

Here we investigated the relative engraftment fitness of human CD8$^+$ cytomegalovirus (CMV)-specific T_E cells derived from CD62L$^+$ and CD62L$^-$ memory T-cell precursors using methods for ex vivo activation and expansion that are used commonly in clinical trials. To assess engraftment, we developed a NOD/*Scid* IL-2RγC^{null} (NOG) mouse model in which human IL-15, a

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nonredundant cytokine that is essential for $CD8^+$ memory cell survival,[10-12] was produced at low levels. Our data demonstrate that human T_E derived from T_{CM} precursors have superior engraftment compared with T_E derived from T_{EM}, and provide superior antitumor activity. The engraftment of T_{CM}-derived T_E cells is polyclonal, suggesting that the cell-intrinsic characteristics that enable survival are a general property conferred by their T_{CM} origin. These findings suggest that the prospective isolation of T_{CM} for subsequent use in antiviral and antitumor adoptive therapy would yield cell products with superior engraftment fitness and therapeutic activity.

Methods

Flow cytometry

Human peripheral blood mononuclear cells (PBMCs) and T cells were analyzed by flow cytometry after staining with fluorochrome-conjugated monoclonal antibodies (mAbs) to CD4, CD8, CD62L, CD45RO, CD127, CD28, CD45, CD3, perforin, granzyme A, Ki-67, interferon-γ (IFN-γ), CD122 (IL-2Rβ), CD132 (IL-2Rγ; BD Biosciences), and CCR7, and IL-15Rα (R&D Systems). Phycoerythrin (PE)-conjugated CMV pp65 (NLVPMVATV)–HLA-A2*0201 iTAg MHC tetramer, PE-conjugated multiallele negative tetramer, and the IOTestBeta Mark TCR V β Repertoire Kit (representing ~ 70% of normal TCR Vβ repertoire) were obtained from Beckman Coulter. Isotype-matched mAbs served as controls. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen. CaspScreen Flow Cytometric Apoptosis Detection Kit with D_2R substrate was purchased from BioVision. All mAbs, tetramers, and CFSE were used according to the manufacturer's instructions. Data acquisition was performed on a FACSCalibur (BD Biosciences) using FCS Express, Version 3 software (De Novo Software).

DNA constructs and lentiviral vector

The HygroR-pp65_pEK plasmid contains the hygromycin resistance gene derived from pMG (Invivogen) and the full-length CMV *Pp65* gene (gift from Dr John Zaia, City of Hope National Medical Center), fused by polymerase chain reaction and ligated into the mammalian expression vector pEK, which originated from pcDNA3.1(+; Invitrogen), and in which the CMV promoter and the ampicillin gene were replaced by the human elongation factor 1α promoter derived from pMG. Pp65–2A-eGFP-ffluc_epHIV7 contains the full-length CMV *Pp65* gene, the 2A peptide sequence, an enhanced *Gfp* gene (Clontech), and the firefly luciferase gene (Invivogen) within the epHIV7 lentiviral vector, which was generated from the pHIV7 vector (gift from Jiing-Kuan Yee, COHNMC) that had its CMV promoter replaced with elongation factor 1α promoter.

The GFP-IMPDH2dm-2A-IL-15_pcDNA3.1(+) plasmid contains a fusion of the *Gfp* gene, which confers fluorescence, and the human inosine monophosphate dehydrogenase II (T3331, S351Y) gene, which confers resistance to mycophenolic acid, followed by the 2A self-cleaving peptide sequence[13] and a human recombinant IL-15 gene. OKT3–2A-Hygro_pEK contains the antihuman-CD3ϵ immunoglobulin gene (gift from Andrew Raubitschek, COHNMC), the 2A peptide sequence, and the hygromycin resistance gene within the pEK vector. All construct and construction-associated polymerase chain reaction primer sequences are available on request.

Generation of CMV-specific antigen-presenting cells and T cells

Human PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech) from heparinized peripheral blood obtained from healthy, HLA-$A2^+$ CMV-immune volunteer donors. HLA-A2 positivity was determined based on screening a PBMC sample with a PE-conjugated anti-HLA-A2 antibody BB7.2 (BioLegend). The protocol and consent forms were approved by the COHNMC Internal Review Board.

To generate antigen-presenting cells that express CMV pp65 (vAPCs), PBMCs were resuspended in nucleofection solution using the Human T cell Nucleofector kit (Amaxa), and 5×10^7 cells were aliquoted into 0.2-cm cuvettes containing 10 μg HygroR-pp65_pEK (or pmaxGFP, Amaxa; as a transfection control) in a final volume of 100 μL/cuvette. The cells were electroporated using the Amaxa Nucleofector I, program U-14, after which cells were allowed to recover for 6 hours at 37°C, and then γ-irradiated to a dose of 1200 cGy. For separation of T_{CM} and T_{EM} subsets, PBMCs were washed, labeled with fluorochrome-conjugated anti-CD45RO and anti-CD62L antibodies, and $CD62L^+CD45RO^+$ T_{CM} and $CD62L^-CD45RO^+$ T_{EM} cells were sort-purified using a MoFlo MLS (Dako Cytomation). To generate CMV-specific T cells, the sort-purified T_{CM} or T_{EM} were then stimulated with autologous irradiated vAPCs in the presence of 5 U/mL IL-2 (Chiron) at a 4:1 (responder/stimulator) ratio once a week for 3 weeks, in RPMI 1640 supplemented with 2mM L-glutamine, 25mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% human serum (CM). After enrichment by antigen stimulation, the CMV pp65-specific T cells were expanded using a rapid expansion method (REM) by which 10^6 T cells were stimulated with 30 ng/mL anti-CD3ϵ (OKT3; Ortho Biotech), 5×10^7 γ-irradiated PBMCs (3500 cGy), and 10^7 γ-irradiated lymphoblastoid cell lines (LCLs, 8000 cGy) in 50-mL culture media.[14] Cultures were then supplemented with 50 U/mL IL-2 and 10 ng/mL rhIL-15 (CellGenix) every 48 hours for 14 days, after which a second REM stimulation with IL-2/IL-15 was performed for another 14 days before in vitro analysis and adoptive transfer.

EBV-transformed LCLs were made from PBMCs as previously described.[15] LCLs that expressed OKT3 (LCL-OKT3) were derived by suspending allogeneic LCLs in nucleofection solution using the Amaxa Nucleofector kit T, adding the OKT3–2A-Hygromycin_pEK plasmid (5 μg plasmid/10^7 cells), and electroporating the LCLs using the Amaxa Nucleofector I, program T-20. Stable transfectants were obtained by growing the transfected LCLs in CM containing 0.4 mg/mL hygromycin. To generate LCL-pp65, autologous LCLs (auto-LCLs) were transduced with lentiviral vector pp65–2A-eGFP-ffluc_epHIV7 at a multiplicity of infection of 20 in the presence of 5 μg/mL polybrene in 500 μL CM. The initial transduction efficiency was 48.5% as assessed by GFP expression, and the LCL-pp65 were subsequently purified to more than 98% purity by sorting GFP^+ cells.

NS0-IL-15 cells were generated by transfecting the mouse myeloma line NS0 (gift from Andrew Raubitschek, COHNMC) with GFP-IMPDH2dm-2A-IL-15_pcDNA3.1. NS0 cells were resuspended in nucleofection solution using the Amaxa Nucleofector kit T, 5 μg of plasmid DNA was added to 5×10^6 cells, and the cells were electroporated using the Amaxa Nucleofector I, program T-27. The transfected cells were grown in Dulbecco modified Eagle medium (Irvine Scientific) supplemented with 10% fetal calf serum, 25mM HEPES, and 2mM L-glutamine in the presence of 6μM mycophenolic acid, and screened for human IL-15 transgene expression by cytometric bead array with a Bio-Plex assay (Bio-Rad Laboratories).

Cytokine production assays

T cells (10^5) were cocultured overnight in 96-well tissue culture plates with 10^5 of LCL-OKT3, auto-LCL-pp65, auto-LCLs, or auto-LCLs that had been pulsed (2 hours at 37°C in CM) with 10 μg/mL of either the HLA-A2-restricted pp65 peptide (NLVPMVATV) or an HLA-A2-restricted control peptide (VLQELNVTV[16]; City of Hope DNA/RNA Peptide Synthesis Facility). Supernatants were harvested 18 hours after stimulation and analyzed by cytometric bead array using the Bio-Plex Human Cytokine 17-Plex Panel (Bio-Rad Laboratories) according to the manufacturer's instructions.

Cytotoxicity assays

Four-hour ^{51}Cr release assays were performed as previously described[17] using the indicated effector cells and ^{51}Cr-labeled target cells.

Proliferation assays

Proliferation of $T_{CM/E}$ and $T_{EM/E}$ (5×10^4) was determined on incubation with different concentrations of rhIL-15 in 200 μL CM. After 48 hours,

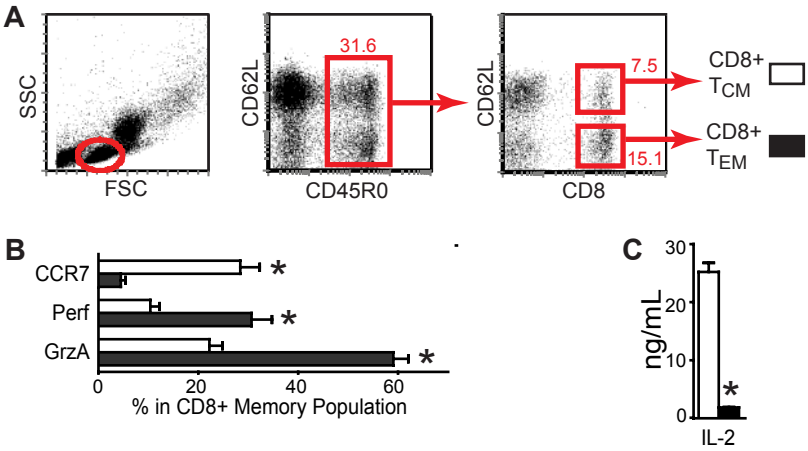

Figure 1. Frequency of CD8$^+$ T-cell memory subsets in human peripheral blood. (A) Flow cytometric analysis of PBMCs from 4 different human donors gated on the lymphoid population by forward and side scatter (left panel) and analyzed for CD45RO, CD62L, and CD8 expression. CD45RO$^+$ lymphocytes were then gated for CD8$^+$CD62L$^+$ T_{CM} and CD8$^+$CD62L$^-$ T_{EM} (middle and right panels) and analyzed by multicolor flow cytometry with anti-CCR7, anti-perforin, or anti-granzyme A mAbs (B). Percentage of cells in each gate (red) is indicated, and mean percentage of the CD8$^+$ T_{CM} or T_{EM} cells that were CCR7, perforin, or granzyme A positive (± SE; n = 3 donors) is indicated. *$P < .05$, CD8$^+$ T_{CM} versus T_{EM} cells (unpaired Student *t* test). (C) Cytokine production profiles of the freshly isolated CD8$^+$ T_{CM} and T_{EM}. Supernatants were collected after overnight coincubation with LCL-OKT3, and cytokine levels (mean ± SE of triplicate wells) were determined as described in "Cytokine production assays." *$P < .0001$, cytokine levels of CD8$^+$ T_{CM} versus T_{EM} cells (unpaired Student *t* test).

50 μCi/mL [^{3}H]-thymidine was added, and then T cells were harvested the next day (PHD Harvester, Brandel). [^{3}H]-Thymidine incorporation was measured with a liquid scintillation LS 6500 counter (Beckman Coulter) using a standard [^{3}H]-thymidine incorporation assay.

Xenograft models

All mouse experiments were approved by the COH Institute Animal Care and Use Committee. Six- to 10-week old NOD/*Scid* IL-2Rγc ^{null} (NOG) mice were injected intravenously on day 0 with 10^7 $T_{CM/E}$ or $T_{EM/E}$ cells. Irradiated (8000 cGy) NS0-IL-15 cells (1.5×10^7) were administered intraperitoneally 3 times a week starting on day 0 to provide a systemic supply of human IL-15 in vivo. Peripheral blood was harvested by retro-orbital bleeding, and leukocytes were analyzed by flow cytometry to monitor human T-cell engraftment. Where indicated, 10^7 irradiated (8000 cGy) or 2.5×10^6 nonirradiated LCL-pp65 cells were injected intravenously, and luciferase activity was measured by Xenogen imaging as previously described.[18]

D_2R assay

Caspase activity was determined with CaspSCREEN Flow Cytometric Apoptosis detection Kit according to the manufacturer's instructions. Briefly, aliquots of harvested cells from recipient mice were washed with phosphate-buffered saline twice and resuspended in 0.3 mL D_2R incubation buffer; 3 μL of 1M dithiothreitol, 1 μL of D_2R reagent, and 5 μL peridinin chlorophyll protein-conjugated anti-huCD45 mAb and then incubated in the dark for 20 minutes at 37°C. Flow cytometric analysis was performed on CD45$^+$-gated cells using the FL-1 channel for D_2R cleavage-associated fluorescence.

Results

Frequency of CD8$^+$ T_{CM} and T_{EM} in healthy donor peripheral blood

Human CD45RO$^+$ memory T cells can be segregated into T_{CM} and T_{EM} based on differential expression of CD62L.[8] Using multiparameter flow cytometry, we analyzed blood samples from 11 healthy donors to determine the proportions of CD45RO$^+$ CD8$^+$ T_{CM} and T_{EM} cells (Figure 1A; Table 1). Using forward and side scatter profiles to gate on lymphocytes, we found that CD45RO$^+$ CD8$^+$ cells in the blood have a predominantly CD62L$^-$ T_{EM} phenotype, with CD8$^+$ T_{CM} accounting, on average, for only 5.1% of total lymphocytes. Consistent with previous reports on human memory T-cell heterogeneity, we observed that CD8$^+$CD45RO$^+$CD62L$^+$ T_{CM} more frequently, but not uniformally, coexpressed CCR7 compared with their CD8$^+$ T_{EM} counterparts ($P < .05$).[7,19,20] Conversely, CD8$^+$CD45RO$^+$CD62L$^-$ T_{EM} more frequently expressed perforin and granzyme A ($P < .05$) than CD8$^+$CD45RO$^+$CD62L$^+$ T_{CM} (Figure 1B).

We then fluorescence-activated cell sorter-purified CD8$^+$ cells from the T_{CM} and T_{EM} fractions to greater than 90% purity (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) and assayed their direct effector function in vitro using OKT3 to provide a T-cell receptor (TCR) signal. Direct lysis of target cells expressing OKT3 was similar in CD8$^+$ T_{CM} and CD8$^+$ T_{EM} (36% lysis; data not shown), but OKT3 stimulation elicited significantly greater IL-2 production by CD8$^+$ T_{CM} cells compared with their T_{EM} counterparts ($P < .0001$; Figure 1C).

Differentiated T_E cells derived from T_{CM} and T_{EM} are similar in phenotype and function in vitro

We chose CMV pp65 as a model antigen based in part on clinical experience with adoptive therapy targeting this antigen. We generated CMV pp65-specific T_E lines from sort-purified T_{CM} ($T_{CM/E}$) or T_{EM} ($T_{EM/E}$) by stimulating the T cells weekly for 3 weeks with autologous irradiated PBMCs that were transfected with a full-length pp65 transgene as antigen-presenting cells (vAPCs; Figure 2A-B; supplemental Figure 2). The resulting polyclonal T-cell lines were enriched for pp65-specific T cells as determined by staining with an HLA A2/pp65 tetramer (Figure 2C) and were then expanded using anti-CD3 mAb and irradiated feeder cells. After

Table 1. Percentage of lymphocytes from human PBMCs that are CD8$^+$ T_{CM}

Donor	CD8$^+$ T_{CM},* %	CD8$^+$ T_{CM}/T_{EM}
1	3.1	0.4
2	2.8	0.4
3	7.9	0.4
4	8.7	0.6
5	2.0	0.5
6	4.3	0.5
7	3.1	0.4
8	5.7	2.1
9	7.7	0.8
10	5.6	1.1
11	5.6	0.8
Mean (SE)	5.1 (0.6)	0.7 (0.2)

*Values are percentages in lymphocyte gate based on forward scatter versus side scatter.

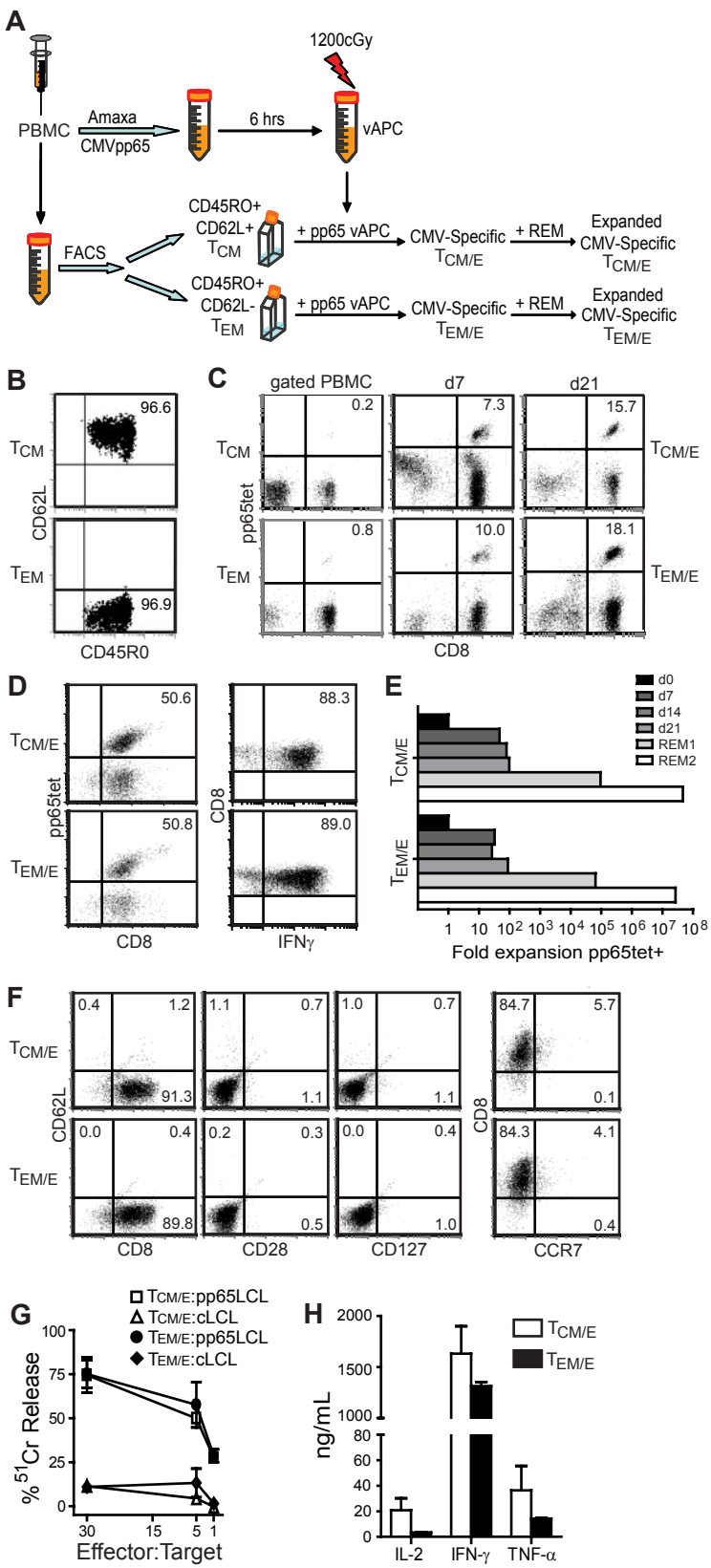

Figure 2. T_E cells derived from T_{CM} and T_{EM} in vitro are similar in phenotype and function. (A) Schematic of methods for deriving CMV-specific $T_{CM/E}$ and $T_{EM/E}$. Purified T_{CM}, T_{EM}, and pp65-expressing vAPCs were generated from the same CMV-seropositive donor's PBMCs. (B) CD45RO and CD62L staining of T_{CM} (top) and T_{EM} (bottom) after sorting from PBMCs. (C) CD8 and pp65-tetramer staining of gated PBMCs (left panels) and of CMV-specific $T_{CM/E}$ and $T_{EM/E}$ at day 7 (middle panels) and 21 (right panels) after stimulation with vAPCs. Histogram quadrants are based on staining with isotype and negative tetramer controls, and percentage of double-positive cells is indicated. (D) pp65 tetramer and intracellular IFN-γ staining of $T_{CM/E}$ and $T_{EM/E}$ before infusion, after overnight coincubation with LCL-pp65. (E) Fold expansion of pp65-tetramer$^+$ cells was determined by multiplying the total number of cells by the percentage pp65tet$^+$ (determined as shown in panel C) found at days 0, 7, 14, and 21 of vAPC stimulation and 14 days after the first and second anti-CD3 (REM) stimulations; these values were then normalized to the input cell number (day 0). (F) Expression of CD62L, CD127, CD28, CCR7, and CD8 on the $T_{CM/E}$ and $T_{EM/E}$ cell products. (G) Cytotoxic activity of $T_{CM/E}$ and $T_{EM/E}$ cell products against auto-LCLs loaded with either an HLA-A2-restricted control peptide (cLCL) or CMV pp65 peptide (pp65LCL). Mean percentage of ^{51}Cr release (± SD) of triplicate wells is depicted. (H) Cytokine production by $T_{CM/E}$ and $T_{EM/E}$. Supernatants were collected after coincubating T cells overnight with CMV pp65 peptide-loaded auto-LCLs, and mean (± SD of triplicate wells) cytokine levels were determined using cytometric bead array.

2 rounds of expansion, the majority (~ 90%) of the CD8$^+$ T cells secrete IFN-γ in response to overnight stimulation with antigen, with 50% of the CD8$^+$ T cells staining positive for the HLA-A2/pp65 tetramer, which represents specificity to a single epitope of pp65 (Figure 2D). The expansion of pp65-specific T cells after stimulation with vAPCs and with anti-CD3 mAbs and feeders was equivalent for cells derived from T_{CM} or T_{EM}, with each culture expanding to cell numbers that approximated or exceeded clinical cell doses ($> 10^9$ cells; Figure 2E). Cultures derived from CD45RO$^+$CD62L$^+$ T_{CM} or CD45RO$^+$CD62L$^-$ T_{EM} precursors

exhibited an identical $CD8^+$ T_E phenotype ($CD62L^-$, $CD127^-$, $CCR7^-$, and $CD28^-$; Figure 2F) and equivalent cytolytic activity (Figure 2G). Cytokine secretion after engaging pp65-expressing target cells tended to be higher from $T_{CM/E}$ than $T_{EM/E}$, but this was not statistically significant (Figure 2H).

Engraftment of CMV-specific $T_{CM/E}$ in huIL-15 NOG mice is greater than that of $T_{EM/E}$

We next examined the ability of human pp65-specific $CD8^+$ $T_{CM/E}$ and $T_{EM/E}$ cells to persist after intravenous injection into NOG mice (Figure 3A). Because IL-15 is required for maintenance of memory T cells, we generated a murine NS0 cell line that constitutively secretes human IL-15 and developed an intraperitoneal dosing regimen of irradiated NS0-IL-15 cells that provided steady-state serum levels of human IL-15 in the range of 6 to 8 pg/mL (Figure 3B inset). To determine whether IL-15 was required for T-cell engraftment, we inoculated NOG mice intravenously with human $T_{CM/E}$ or $T_{EM/E}$ alone or with IL-15-producing NS0 cells. Mice inoculated with $T_{CM/E}$ or $T_{EM/E}$ alone did not exhibit sustained engraftment of human T cells. However, both $T_{CM/E}$ and $T_{EM/E}$ engrafted in mice that received NS0 cells that produced human IL-15, although the engraftment of $T_{CM/E}$ was markedly superior to that of $T_{EM/E}$ (20% vs 3% of circulating cells 21 days after adoptive transfer, $P < .05$; Figure 3B). The engraftment of $T_{CM/E}$ in spleen and bone marrow was also significantly higher than $T_{EM/E}$ in mice that received NS0-IL-15 cells ($P < .05$; Figure 3C).

To examine the possibility that the superior engraftment of $T_{CM/E}$ reflected the preferential expansion or survival of one or a few cells in the polyclonal culture, or that the failure of $T_{EM/E}$ to engraft was the result of limited diversity in the infused population, we performed TCR Vβ analysis on an aliquot of the input $T_{CM/E}$ and $T_{EM/E}$ and on aliquots of human T cells present in mice engrafted 21 days previously with $T_{CM/E}$ (Figure 3D). The in vivo analysis could not be performed in mice that received $T_{EM/E}$ because there were too few cells for analysis. The TCR repertoire was diverse in both $T_{CM/E}$ and $T_{EM/E}$ cultures with similar expansions of individual Vβ clonotypes, a result that excluded limited diversity of $T_{EM/E}$ as an explanation for poor engraftment. Moreover, the TCR Vβ diversity of the T cells that persisted in mice that received $T_{CM/E}$ was remarkably similar to that of the input population, indicating that engraftment fitness is a general feature of $CD8^+$ $T_{CM/E}$. To ensure that our results are not unique to CMV-specific cells, we also generated EBV-specific $T_{CM/E}$ and $T_{EM/E}$ to examine their engraftment potential (supplemental Figure 3A). After 3 stimulations using an EBV-transformed autologous lymphoblastoid cell line (auto-LCL), 73% and 75%, respectively, of viral-specific $T_{CM/E}$ and $T_{EM/E}$ are $CD8^+$ (data not shown). Both EBV-specific $T_{CM/E}$ and $T_{EM/E}$ showed 70% cytotoxic activity against autologous, but not allogeneic, LCLs at a 30:1 effector/target ratio (supplemental Figure 3B). On adoptive transfer, these $CD8^+$ $T_{CM/E}$ again showed enhanced engraftment fitness compared with their $T_{EM/E}$ counterparts ($P < .05$; supplemental Figure 3C).

Human $CD8^+$ $T_{CM/E}$ persist long-term in huIL-15 NOG mice and reacquire attributes of their T_{CM} precursors

We followed a cohort of NOG mice that continued to receive injections of NS0-IL-15 cells for 100 days to determine the duration that $CD8^+$ pp65-specific $T_{CM/E}$ and $T_{EM/E}$ would persist after adoptive transfer in response to IL-15. The engraftment of the $CD8^+$ $T_{CM/E}$ in the blood remained steady at approximately 2% of mononuclear cells, whereas $T_{EM/E}$ remained at or below the level of detection (Figure 4A). Mice engrafted with $T_{CM/E}$ also had significant populations of human T cells in the spleen and bone marrow, whereas $T_{EM/E}$ were only detected in the bone marrow.

To enable analysis of diversity and phenotype of the long-term engrafted populations, we pooled bone marrow harvested from 5 mice in each cohort, sorted human T cells based on expression of CD45 using an AutoMACS (Milteny Biotec), and expanded the sort-purified T cells in vitro for analysis. Similar to that observed in short-term engraftment experiments, T cells present in the bone marrow of mice engrafted with $T_{CM/E}$ displayed a broad TCR Vβ usage, whereas mice engrafted with $T_{EM/E}$ had only low levels of T cells that consisted of a single TCR clonotype (Vβ 13.1; Figure 4B). Although the phenotype and function of $T_{CM/E}$ and $T_{EM/E}$ were similar before infusion (Figure 2D-H), the T cells that persisted long-term in mice given $T_{CM/E}$ were 40% $CD28^+$ compared with 4% $CD28^+$ in mice given $T_{EM/E}$ (Figure 4C). On pp65 antigen stimulation, the $T_{CM/E}$ that persisted in vivo secreted 1.3 ng/mL IL-2, whereas $T_{EM/E}$ secreted only 0.05 ng/mL ($P < .0001$; Figure 4D). The persistent $T_{CM/E}$ displayed lower antigen-specific cytolytic activity and IFN-γ production compared with $T_{EM/E}$ (Figure 4E-F). Together, these data show that T_E cells derived from T_{CM} have a uniform capacity to persist long-term after infusion and to reacquire attributes of their T_{CM} precursors, whereas the engraftment of $T_{EM/E}$ is poor and limited to a small subset of cells that retain immediate effector function.

Adoptively transferred $CD8^+$ $T_{EM/E}$ exhibit both greater proliferation and cell death than $CD8^+$ $T_{CM/E}$ in huIL-15 NOG mice

A potential mechanism by which the in vivo engraftment of $T_{CM/E}$ in NOG mice given irradiated NS0-IL-15 may be superior to $T_{EM/E}$ was that $T_{CM/E}$ may be more responsive to IL-15. Thus, we examined the cell surface expression of IL-15Rα, IL-2Rβ, and IL-2Rγ on CMV-specific $T_{CM/E}$ and $T_{EM/E}$ before infusion. Similar to the results we observed with T-cell clones derived from each memory subset in nonhuman primates,[5] it was found that $T_{CM/E}$ expressed higher levels of surface IL-15Rα, IL-2Rβ, and IL-2Rγ (Figure 5A). This also conferred on $T_{CM/E}$ a slightly enhanced proliferative response when cultured in soluble IL-15 compared with $T_{EM/E}$ (Figure 5B). These results suggested that $T_{CM/E}$ might proliferate more vigorously in response to IL-15 in vivo. However, when we inoculated subsequent cohorts of huIL-15 NOG mice with $T_{CM/E}$ and $T_{EM/E}$ that were labeled with CFSE before in vivo administration, we observed greater proliferation of $T_{EM/E}$ in the first 9 days after adoptive transfer as indicated by the percentage of cells that diluted CFSE (ie, 81% for $T_{EM/E}$ and 25% for $T_{CM/E}$, respectively; Figure 5C). Yet as in previous experiments, $T_{CM/E}$ again exhibited superior persistence in the blood, spleen, and bone marrow compared with $T_{EM/E}$ (Figure 5D).

The finding that $T_{EM/E}$ proliferated more vigorously than $T_{CM/E}$ in vivo, but were present in lower numbers (ie, at day 12), suggested that these cells may undergo more rapid cell death. To evaluate this possibility, D_2R cleavage was used as a measure of caspase activity and a surrogate for apoptosis. We found that 31.6% of $T_{EM/E}$ but only 5.8% of $T_{CM/E}$ were positive for activated caspase activity at day 9 after infusion (Figure 5E). We also found that the percentage of $CD28^+$ cells markedly increased within the in vivo engrafted $T_{CM/E}$ population compared with the $T_{CM/E}$ before infusion (16% ± 2% vs 1%), whereas the percentage of $CD28^+$ $T_{EM/E}$ remained the same (1%). These results are consistent with superior survival of $CD8^+$ $T_{CM/E}$ in huIL-15 NOG mice resulting from less cell death rather than increased proliferation.

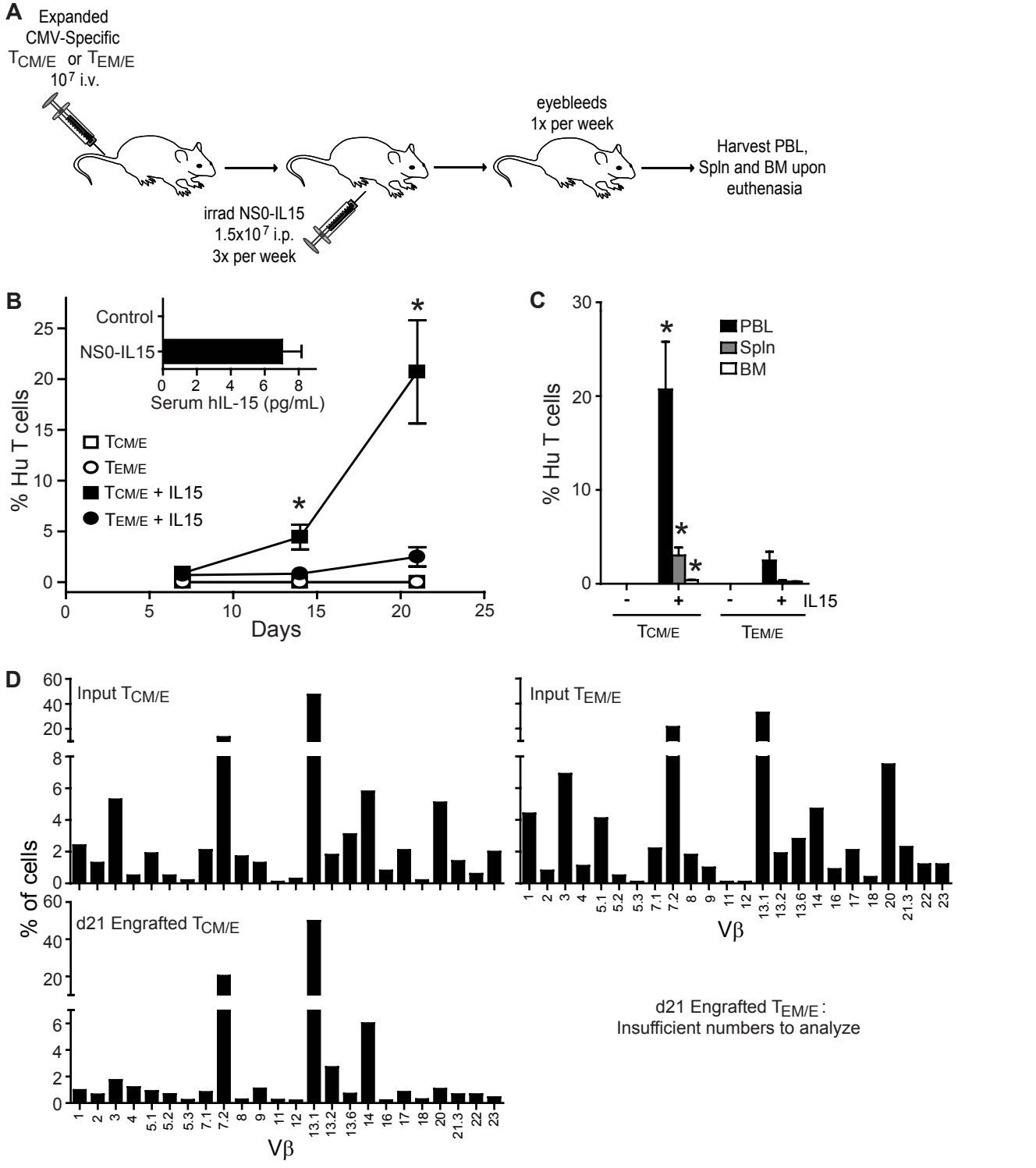

Figure 3. IL-15-dependent engraftment of CMV-specific $T_{CM/E}$ cells in NOG mice is greater than that of $T_{EM/E}$. (A) Schematic of the experiment. (B) Mean percentage (± SE) of human T cells ($CD45^+$ $CD8^+$) in peripheral blood lymphocytes (PBLs) of mice engrafted with $T_{CM/E}$ (squares) or $T_{EM/E}$ (circles) was determined by flow cytometry (n = 5). $^*P < .05$, $T_{CM/E}$ versus $T_{EM/E}$ cell engraftment in the presence of NS0-IL-15 cells (unpaired Student *t* test). (Inset) Mean levels of human IL-15 (± SE) in day 7 serum of NOG mice that had received 3 intraperitoneal injections of 1.5×10^7 irradiated NS0-IL-15 cells (n = 6) or in control mice (n = 10). (C) Mean percentage of human T cells ($CD45^+$ $CD8^+$) plus or minus SE in mouse PBL, bone marrow, and spleen at day 21. $^*P < .05$, $T_{CM/E}$ cell engraftment in each organ versus that of $T_{EM/E}$ in the presence of NS0-IL-15 cells. (D) TCR Vβ repertoire of the CMV-specific $T_{CM/E}$ and $T_{EM/E}$ before (Input) and after (d21) engraftment. Percentage of $CD3^+$ cells (Input) or $CD45^+$ $CD3^+$ cells (d21) that were positive for the indicated TCR Vβ genes was determined by flow cytometry.

Adoptively transferred $CD8^+$ $T_{CM/E}$ proliferate and exhibit superior protective immunity after in vivo antigen challenge

We next evaluated the ability of CMV-specific T cells engrafted in the huIL-15 NOG mice to respond to antigen challenge. $CD8^+$ $T_{CM/E}$ and $T_{EM/E}$ were transferred into cohorts of 12 mice each and then irradiated auto-LCLs that were transduced to express CMV-pp65 were administered to half of the mice in each cohort on days 3, 10, and 17 after T-cell transfer (Figure 6A). Mice that received

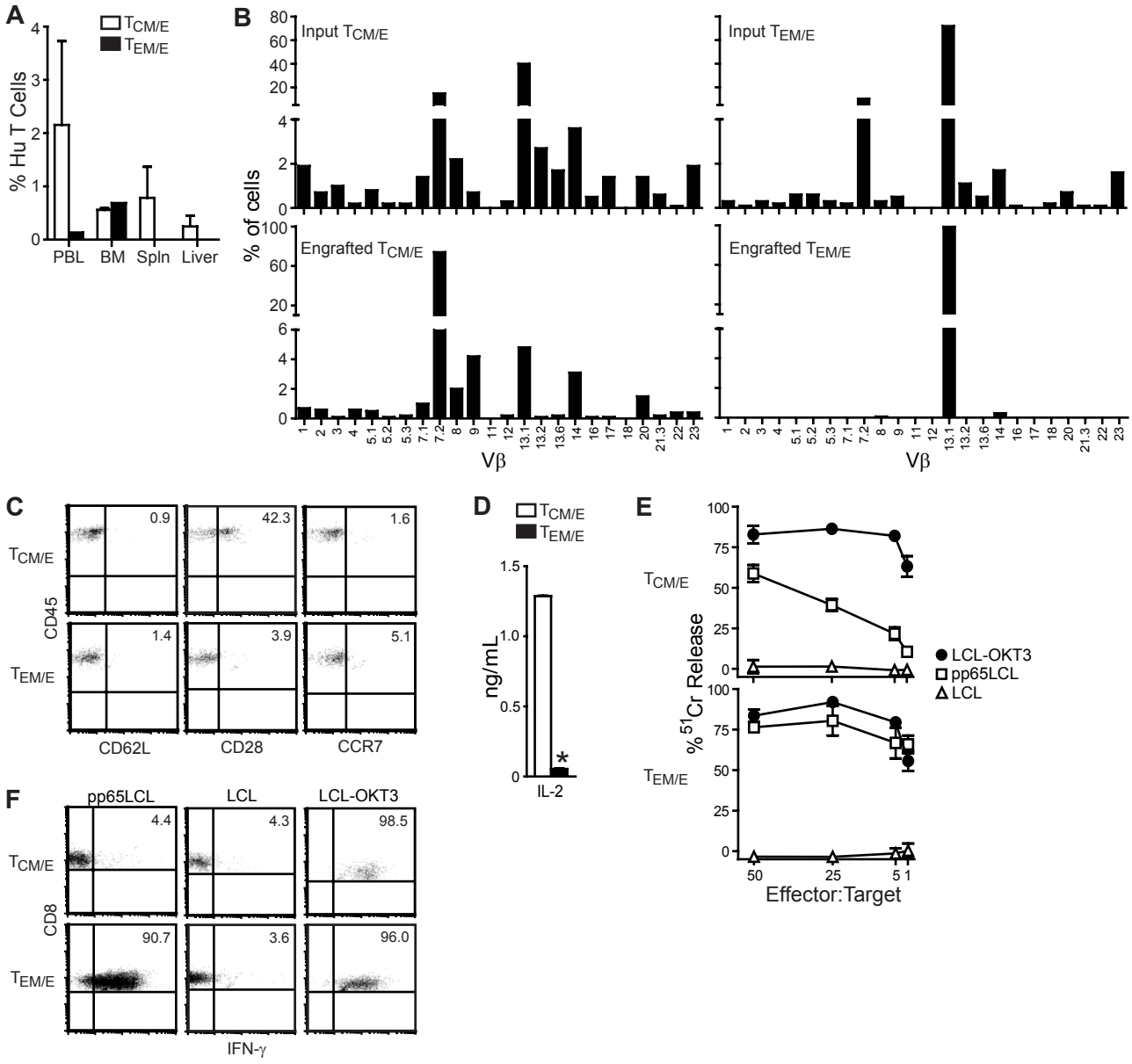

Figure 4. Human CD8$^+$ $T_{CM/E}$ persist long-term (100 days) in huIL-15 NOG mice and remain functional. $T_{CM/E}$ and $T_{EM/E}$ (10^7) were injected intravenously at day 0, and irradiated NS0-IL-15 cells (1.5×10^7) were administered 3 times a week starting at day 0, until mice were killed at day 100. (A) Mean percentage of human CD45$^+$CD8$^+$ cells (± SE) in mouse PBL, bone marrow, and spleen at day 100 was determined by flow cytometry (n = 5). (B) TCR Vβ repertoire of the input and long-term engrafted $T_{CM/E}$ and $T_{EM/E}$. Bone marrow was pooled from mice, and human CD45$^+$ cells were sorted and expanded by stimulation with anti-CD3. The percentage of CD3$^+$ cells positive for the indicated TCR Vβ genes was determined by flow cytometry. (C) Bone marrow harvested at day 100 from mice engrafted with $T_{CM/E}$ and $T_{EM/E}$ was analyzed by flow cytometry for expression of human CD45, CD62L, CCR7, and CD28. Gating was based on staining with isotype control mAb, and the percentage of double-positive cells is indicated. (D) IL-2 production from CD45$^+$ T cells derived from day 100 bone marrow of mice engrafted with $T_{CM/E}$ and $T_{EM/E}$. Supernatants were collected after T cells were coincubated overnight with LCL-pp65, and IL-2 levels were determined using cytometric bead array. (E) Cytotoxic activity of human T cells derived from day 100 bone marrow of mice engrafted with $T_{CM/E}$ and $T_{EM/E}$, and stimulated with anti-CD3 mAb. Target cells included OKT3-expressing LCLs, auto-LCLs or LCL-pp65. Mean percentage of ^{51}Cr release (± SD) of triplicate wells. (F) Intracellular IFN-γ staining of human T cells derived from day 100 bone marrow of mice engrafted with $T_{CM/E}$ and $T_{EM/E}$ and coincubated overnight with LCL-pp65, LCL-OKT3, or auto-LCLs.

CD8$^+$ $T_{CM/E}$ and antigen challenge exhibited significantly higher levels of human T-cell engraftment in the blood at days 14 and 21 than mice that received CD8$^+$ $T_{CM/E}$ alone ($P < .05$). As before, the engraftment of CD8 $T_{EM/E}$ was significantly lower than that of $T_{CM/E}$ and was not augmented by antigen challenge ($P > .05$; Figure 6B). In this experiment, we assessed proliferation by staining human T cells harvested from mice for Ki-67 expression and found that nearly equivalent fractions (9%) of $T_{CM/E}$ and $T_{EM/E}$ were Ki-67 positive at this time point (Figure 6C). However, similar to the results in mice that did not receive antigen stimulation, 94% of the engrafted $T_{EM/E}$ were positive for activated caspase activity compared with 25% of the engrafted $T_{CM/E}$, consistent with a higher propensity of the $T_{EM/E}$ to undergo apoptosis.

The higher levels of engraftment and superior response to antigen stimulation in vivo achieved with infusions of $T_{CM/E}$ suggested that this subset would have superior therapeutic activity. To evaluate this, we designed an experiment to assess the ability of transferred CMV-specific T_E cells derived from either T_{CM} or T_{EM} to protect against the lethal outgrowth of auto-LCLs transduced to express both CMV-pp65 and firefly luciferase (ffLuc, a reporter for biophotonic imaging of tumor growth). In this experiment, pp65$^+$ffLuc$^+$ LCLs were inoculated intravenously into NOG mice

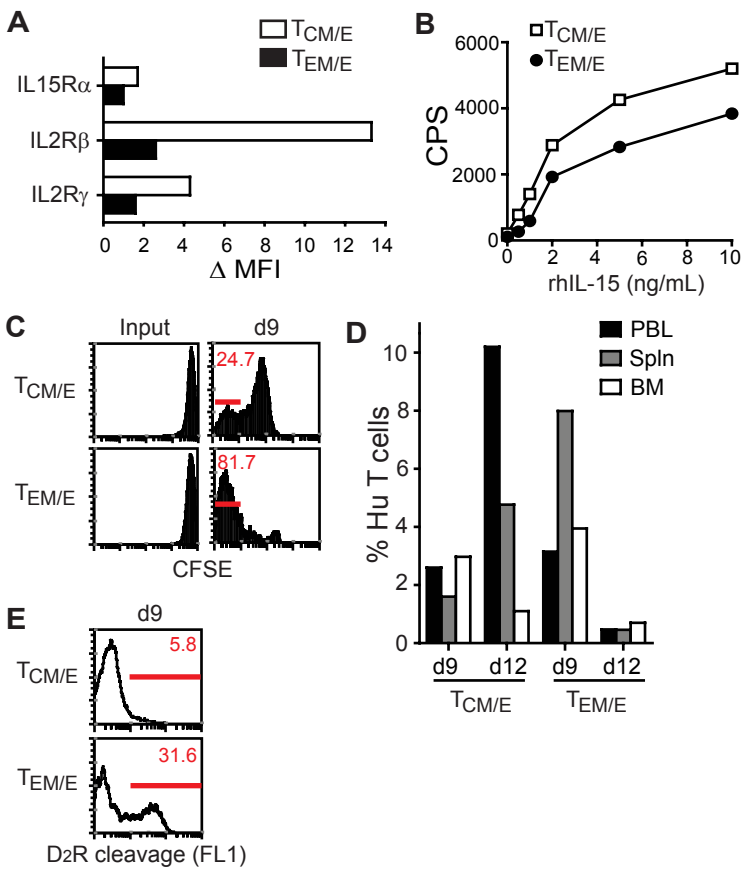

Figure 5. Differential cytokine receptor expression, IL-15–mediated proliferation, and caspase activity of $T_{CM/E}$ and $T_{EM/E}$. (A) IL-15Rα, IL-2Rβ, or IL-2Rγ expression by $T_{CM/E}$ and $T_{EM/E}$. Mean fluorescence intensity was normalized to that of isotype control staining in each case to determine ΔMFI. (B) Proliferation of $T_{CM/E}$ and $T_{EM/E}$ was determined after 48-hour incubation with different concentrations of rhIL-15 using a standard [3H]-thymidine incorporation assay. (C-D) CFSE-labeled $T_{CM/E}$ and $T_{EM/E}$ (10^7) were injected intravenously into mice at day 0, and irradiated NS0-IL-15 cells (1.5×10^7) were administered 3 times a week starting at day 0, until mice were killed at either day 9 or day 12. (C) CFSE profiles of the input and engrafted $T_{CM/E}$ and $T_{EM/E}$ in day 9 PBL was assessed by flow cytometry. Percentage of CFSE-diluted cells that fall within the first log are indicated. (D) Engraftment of the $CD45^+$ human T cells in the PBL, bone marrow, and spleen was assessed on days 9 and 12 by flow cytometry. (E) FL-1 profiles of $CD45^+$ human T cells in the PBL were assessed on day 9 as a readout for cleavage of the caspase substrate D_2R. The percentage of cells with cleaved D_2R is depicted.

and allowed to engraft for one week, after which the mice were either untreated or treated with a single infusion of either CMV-specific $CD8^+$ $T_{CM/E}$ or $T_{EM/E}$ with irradiated NS0-IL-15 support (Figure 7A). Tumors progressed in mice receiving $T_{EM/E}$ at a rate similar to untreated control mice over a 10-day observation period, whereas administration of $T_{CM/E}$ caused a significant reduction in tumor growth ($P < .05$; Figure 7B). The superior antitumor response mediated by $T_{CM/E}$ correlated with significantly higher levels of engraftment (4.3% $T_{CM/E}$ vs 0.3% $T_{EM/E}$; $P < .05$; Figure 7C).

Discussion

We have developed a NOG mouse model for studying the ability of human effector T cells derived from distinct memory T-cell subsets to establish persistent engraftment and respond to subsequent antigen stimulation. A unique feature of this model was the introduction of irradiated NS0 cells transfected to express human IL-15, which provided serum levels of IL-15 similar to those reported in normal humans. IL-15 has been shown to be essential for maintenance of memory T cells and enabled us to establish durable engraftment of antigen-specific human T cells in NOG mice after the infusion of differentiated T_E cells.

A key finding of our study was that human antigen-specific $CD8^+$ T_E cells derived from T_{CM} exhibit superior engraftment in hu-IL-15 NOG mice compared with T_E cells derived from T_{EM}. This result is in agreement with a study in nonhuman primates,[5] in which we found that antigen-specific $CD8^+$ T-cell clones derived from T_{CM}, but not T_{EM}, were capable of engrafting long-term and reverting to the memory T-cell pool. Our experiments in huIL-15 NOG mice extend the primate experiment in several ways. First, we used polyclonal T cells and included a comparison of the TCR Vβ repertoire of the input cells with that of persistently engrafted cells. This analysis demonstrated retention of a broad TCR repertoire in the human $T_{CM/E}$ cells that persisted long-term, suggesting that engraftment fitness is a general trait common to all T_E cells derived from T_{CM}, and not attributable to only a subset of these cells. The rare T cells that persisted from $T_{EM/E}$ were derived from a very restricted clonotype, consistent with the lack of engraftment being a general trait of T_E cells derived from T_{EM}. Second, we show that transfer of $T_{CM/E}$ provides superior protection from a tumor challenge, which was not possible in the nonhuman primate model. Finally, our study is the first, to our knowledge, to show that providing human IL-15 is sufficient to support the long-term persistence (> 3 months) of functional human virus-specific T cells in NOG mice.

In the studies in which $T_{CM/E}$ clones were transferred to nonhuman primates, a subset of the T cells that persisted long-term reexpressed surface markers of T_{CM}, including CD62L and CCR7, and occupied memory cell niches in the lymph nodes and bone marrow.[5] The human T cells that persisted long-term in huIL-15 NOG mice after the infusion of $T_{CM/E}$ did not reacquire expression of CD62L and CCR7. However, we did observe that a major fraction of the $T_{CM/E}$ that persisted in huIL-15 NOG mice highly expressed CD28 in vivo and were capable of autocrine IL-2 secretion in response to antigen stimulation. The persisting $T_{CM/E}$ were also capable of expanding in vivo more robustly in response to antigen reexposure and mediating superior protection from a lethal challenge with CMV-$pp65^+$ tumor cells. This suggests that the huIL-15 NOG mouse model does not provide human T cells with all of the signals or niches required for the acquisition of

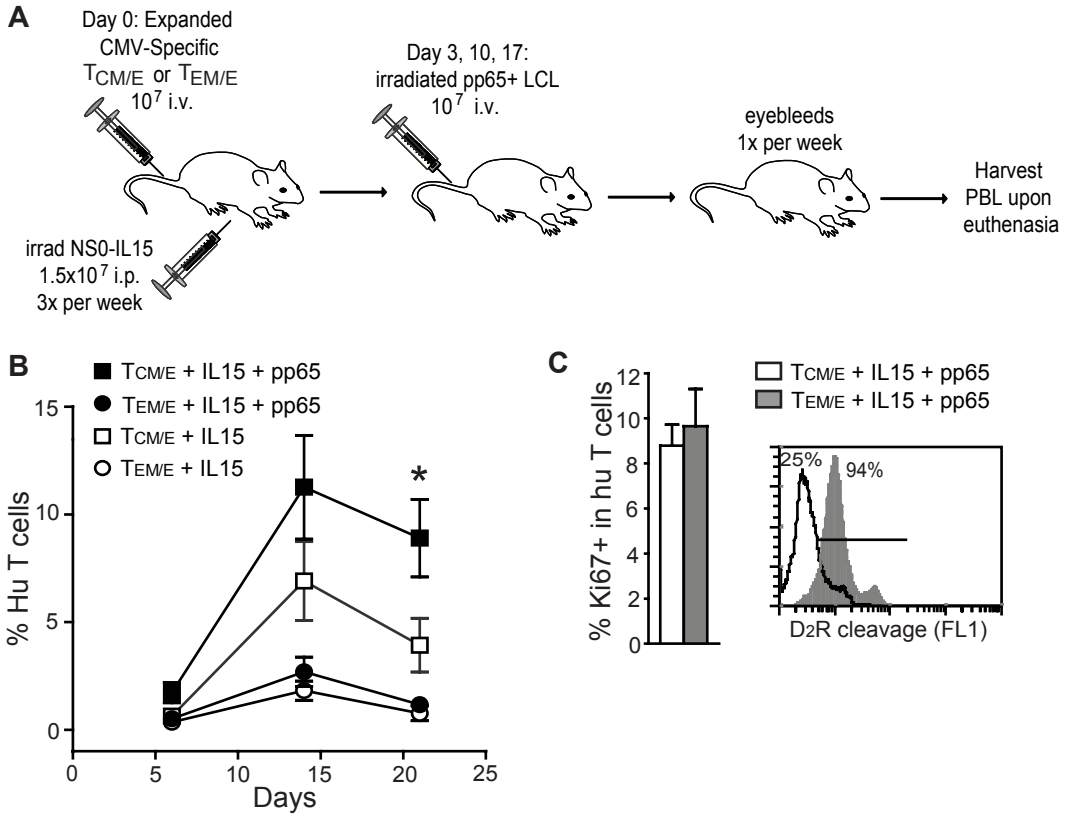

Figure 6. Adoptively transferred CMV-specific CD8$^+$ $T_{CM/E}$ exhibit a better response to antigen challenge in vivo than $T_{EM/E}$. (A) Schematic of in vivo antigenic stimulation of engrafted CMV-specific $T_{CM/E}$ and $T_{EM/E}$. (B) Engraftment of CMV-specific $T_{CM/E}$ (squares) or $T_{EM/E}$ (circles) was carried out with (black) or without (white) administration of irradiated CMV pp65-expressing LCLs at days 3, 10, and17; and mean percentage (± SE) of human T cells (CD45$^+$ CD8$^+$) in mouse PBL was determined by flow cytometry (n = 6). *$P < .05$, engraftment of $T_{CM/E}$ alone versus pp65-driven $T_{CM/E}$ engraftment (unpaired Student *t* test). (C) On euthanasia at day 28, PBLs were harvested and analyzed by flow cytometry for percentage of Ki-67$^+$ cells in the human T-cell population (left) and for the ability of CD45$^+$ human T cells to cleave the caspase substrate D_2R (right).

CD62L and CCR7 but does provide sufficient cues for $T_{CM/E}$ to revert to resting functional memory T cells.

The observation that $T_{CM/E}$ exhibit superior engraftment potential has obvious implications for human adoptive T-cell therapy, yet

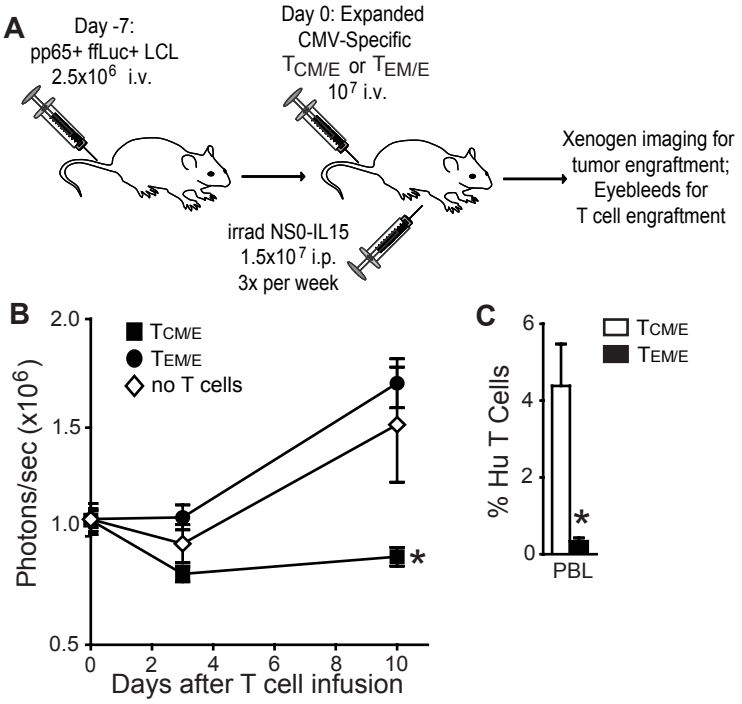

Figure 7. Adoptively transferred CMV-specific CD8$^+$ $T_{CM/E}$ exhibit superior protection from tumor challenge. (A) Schematic of the in vivo tumor challenge experiment. (B) Engraftment of pp65$^+$ ffLuc$^+$ LCL in animals treated with or without CMV-specific $T_{CM/E}$ or $T_{EM/E}$ was determined by Xenogen imaging; and mean (± SE) of total flux levels of luciferase activity are shown for each group (n = 5). *$P < .05$, animals treated with $T_{CM/E}$ versus either untreated or $T_{EM/E}$-treated animals (analysis of variance). (C) Mean percentage (± SE) of human CD45$^+$CD8$^+$ cells in day 10 mouse PBL was determined by flow cytometry. *$P < .05$, $T_{CM/E}$ versus $T_{EM/E}$ engraftment (unpaired Student *t* test).

the basis for the profound differences in the fate of T_E cells is not completely understood. In the nonhuman primate studies, $CD8^+$ $T_{CM/E}$ clones were found to express higher levels of IL-15 receptor chains and exhibited superior survival in vitro in response to IL-15 compared with $T_{EM/E}$ and less apoptosis in vivo. Our data comparing the fate of human $T_{CM/E}$ and $T_{EM/E}$ in vivo confirm a requirement for IL-15 for in vivo survival of transferred human T cells. Transferred $T_{EM/E}$ proliferate more in vivo than $T_{CM/E}$ in response to IL-15 but exhibit higher levels of caspase activation and cell death, consistent with a different cellular response to IL-15 as a potential mechanism that contributes to their poor survival. However, it is probable that additional cell-intrinsic properties dictate the capacity of T_E cells to revert to the memory pool. This model also provides an opportunity to study other human cytokines, including inhibitory cytokines such as IL-10 for their effect on T-cell persistence and function.

Much of our current understanding of memory T-cell lineage fate decisions is derived from studies in mice and has focused on the transition of T_E cells derived from the naive T-cell pool to the T_{CM} and T_{EM} subsets.[6,21,22] Our work with human and nonhuman primate T_E cells derived from the T_{CM} and T_{EM} subsets suggests that the initial lineage fate choice imparts cell-intrinsic programming that is retained through subsequent rounds of T_E differentiation and influences the differential engraftment fitness after adoptive transfer. Recently, Araki et al described histone methylation as a transcriptional regulatory mechanism that is differentially maintained in human $CD8^+$ central and effector memory subsets.[23] Our data are consistent with an epigenetic programming mechanism that is retained on memory cell differentiation ex vivo to effector cells. Ongoing studies in the laboratory are evaluating broader epigenetic mechanisms, including genome-wide analysis of DNA methylation and the evaluation of ex vivo epigenetic modification of T cells to program a broader repertoire of effector cells for engraftment fitness.

We have focused on memory cells as a reservoir of antigen-specific precursor cells for adoptive therapy, but the extent to which human naive cells can be programmed in vitro for downstream therapeutic applications remains to be determined. Studies in murine models have suggested that T_E cells derived from naive T cells may have a superior ability to persist in vivo compared with T_{CM}, providing the culture duration before adoptive transfer is very short.[24] However, these experiments were performed with T cells from a TCR transgenic mouse and would be difficult to easily translate to humans. Our studies in humans and nonhuman primates demonstrate that long-term culture of $T_{CM/E}$ cells is not an impediment to effective T-cell transfer, suggesting that the cell-intrinsic qualities that dictate engraftment are more permanently established in $T_{CM/E}$ than in T_E cells derived from naive T cells. Pharmacologic manipulation of WNT signaling during culturing of murine naive T cells or manipulation of mammalian target of rapamycin signaling during the induction of an effector response from naive T cells in vivo may promote the acquisition of T-cell memory.[25,26] It will be of interest to determine whether these findings can be extrapolated to human T cells. Achieving reproducible high level engraftment of human antigen-specific T cells in clinical trials of adoptive therapy for cancer has been a formidable obstacle; this is in contrast to the trials in which virus-specific T_E generated from healthy immune donors were transferred to recipients to reconstitute viral immunity after allogeneic hematopoietic stem cell transplantation.[1,27,28] To date, no cancer adoptive therapy clinical trial has used the transfer of effector cells from a defined population of precursor cells, whether they be naive, central memory, or effector memory in origin. Our studies and others show that $CD8^+$ T_{CM} are present in low frequency in the blood, constituting only 2% to 8% of peripheral blood T cells, and it is likely that this pool contains very rare T cells specific for tumor-associated antigens. Thus, adoptive T-cell therapy for cancer may need to use gene transfer to endow T_{CM} or other subsets with tumor reactivity, such as could be accomplished by the introduction of genes encoding tumor-specific T-cell receptors or chimeric antigen receptors.[29,30] Our group has therefore focused on developing methods to purify human T-cell subsets, including polyclonal and virus-specific T_{CM} for the generation of tumor reactive T_E via subsequent genetic modification using viral vectors. This will enable the incorporation of defined precursor cells into clinical trials designed to determine which subsets of T cells will provide superior in vivo persistence and therapeutic efficacy.

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Authorship

Contribution: X.W. designed and performed research, collected, analyzed, and interpreted data, and cowrote the manuscript; C.B. designed research, contributed analytic tools, analyzed and interpreted data, and cowrote the manuscript; C.W.W. performed research and collected data; S.J.F. analyzed and interpreted data; and S.R.R. and M.C.J. designed research, analyzed and interpreted data, and cowrote the manuscript.

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