Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates

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The adoptive transfer of antigen-specific T cells that have been expanded ex vivo is being actively pursued to treat infections and malignancy in humans. The T cell populations that are available for adoptive immunotherapy include both effector memory and central memory cells, and these differ in phenotype, function, and homing. The efficacy of adoptive immunotherapy requires that transferred T cells persist in vivo, but identifying T cells that can reproducibly survive in vivo after they have been numerically expanded by in vitro culture has proven difficult. Here we show that in macaques, antigen-specific CD8+ T cell clones derived from central memory T cells, but not effector memory T cells, persisted long-term in vivo, reacquired phenotypic and functional properties of memory T cells, and occupied memory T cell niches. These results demonstrate that clonally derived CD8+ T cells isolated from central memory T cells are distinct from those derived from effector memory T cells and retain an intrinsic capacity that enables them to survive after adoptive transfer and revert to the memory cell pool. These results could have significant implications for the selection of T cells to expand or to engineer for adoptive immunotherapy of human infections or malignancy.

Introduction

Studies in rodents have demonstrated that adoptive immunotherapy with antigen-specific CD8+ cytotoxic T cells is effective for cancer and infections, and there is evidence that this approach has therapeutic activity in humans (1–8). For clinical applications, T cells of a desired antigen specificity are isolated or engineered to express receptors that target infected or transformed cells and are then expanded in culture (9–14). In some settings the transfer of cloned T cells has been used to provide precise control of specificity and avoid toxicity. For example, in allogeneic stem cell transplantation, the administration of donor-derived T cell clones that target pathogens or malignant cells in the recipient can avoid graft-versus-host disease, which occurs with the infusion of unselected polyclonal donor T cells (3, 4, 15). However, the efficacy of adoptive immunotherapy in humans is often limited by the failure of cultured T cells, particularly cloned CD8+ T cells, to persist in vivo (16, 17), and insight into the basis for the poor survival of the transferred cells is lacking.

The pool of lymphocytes from which CD8+ T cells for adoptive immunotherapy can be derived includes naive T cells (Tn) and antigen-experienced memory T cells (Tm), which can be divided into central memory (Tcm) and effector memory (Tem) subsets that differ in phenotype, homing, and function (18). CD8+ Tcm express CD62L and CCR7, which promote migration into LNs and proliferate rapidly if reexposed to antigen (19). CD8+ Tem lack CD62L, enabling migration to peripheral tissues, and exhibit immediate effector function (19). In response to antigen stimulation, both CD8+ Tcm and Tem proliferate and differentiate into CD62L+ cytolytic effector T cells (T e) that express high levels of granzymes and perforin but are short lived (20). Thus acquisition of an effector phenotype during culture has been suggested as a major reason for the poor survival of transferred T cells (9).

In the normal host, T cell memory persists for life, indicating that some Tm cells may have the ability to self-renew or revert to the memory pool after differentiating to Tn in response to repeated antigen exposure (21). Tcm and Tem have distinct phenotypic and functional properties, but it is unknown whether T e cells derived from each of these Tm subsets retain any intrinsic properties of the parental cell. Using a nonhuman primate model relevant to human translation, we sought to determine whether T e clones derived from purified Tcm or Tem differed in their ability to persist in vivo or establish T cell memory after adoptive transfer. Here we show that antigen-specific CD8+ T e clones derived from the Tem subset of Tm survive in the blood for only a short duration after adoptive transfer, fail to home to LNs or BM, and do not reacquire phenotypic markers of Tm. By contrast, T e clones derived from Tcm persist long term after adoptive transfer, migrate to Tm niches, reacquire phenotypic properties of Tm, and respond to antigen challenge.

Results

Characterization of CMV-specific CD8+ T cell clones from CD62L+ Tcm and CD62L- Tem subsets. Immunocompetent Macaca nemestrina with latent CMV infection were used in this study. We identified CMV epitopes recognized by CD8+ T cells in individual macaques by stimulating aliquots of PBMCs with CMV immediate early 1 (IE-1)
Isolation and gene marking of T_CM- and T_EM-derived CMV-specific CD8\(^+\) T cell clones for adoptive transfer. (A) CMV IE-specific T cells are present in T_CM and T_EM subsets of CD8\(^+\) peripheral blood lymphocytes. PBMCs from macaque 02258 were stained with mAbs to CD28, Fas, and CD8 to identify CD28\(^+\)/CD8\(^+\)/Fas\(^+\) T_N, CD28\(^+\)/CD8\(^+\)/Fas\(^−\) T_CM, and CD28\(^−\)/CD8\(^+\)/Fas\(^+\) T_EM fractions (left panel) and assayed by cytokine flow cytometry after stimulation with a CMV peptide (right panels). (B) Sorted CD62L\(^+\)/CD8\(^+\)/CD28\(^+\)/Fas\(^+\) T_N (top panel), CD62L\(^−\)/CD8\(^+\)/CD28\(^+\)/Fas\(^−\) T_CM (middle panel), and CD62L\(^−\)/CD8\(^+\)/CD28\(^−\)/Fas\(^+\) T_EM (lower panel) T cells were stimulated with autologous CMV IE peptide-pulsed monocytes and assayed for cytolytic activity against peptide-pulsed (filled triangles) or unpulsed target cells (open circles). (C) Isolation of T_CM- and T_EM-derived CMV-specific CD8\(^+\) T cell clones. CD8\(^+\)/CD28\(^−\) and CD8\(^+\)/CD62L\(^+\) T cell subsets were cultured with peptide-pulsed monocytes. At day 7, T cell clones were isolated by limiting dilution, transduced to express ΔCD19 or CD20, and expanded for adoptive transfer. (D) Design of the retroviral vector constructs. MPSV-LTR, myeloproliferative sarcoma virus retroviral long terminal repeat; PRE, woodchuck hepatitis virus posttranscriptional regulatory element; ΔCD19, truncated macaque CD19 cDNA; CD20, full-length macaque CD20 cDNA. (E) Selection of ΔCD19- and CD20-modified CD8\(^+\) T cell clones. Transduced T cells were enriched for ΔCD19 or CD20 expression using immunomagnetic beads and analyzed by flow cytometry to assess purity (right panels). Unmodified T cells (left panels) served as control. The percentages of CD8\(^+\) T cells positive for CD19 or CD20 are indicated.
The TCM- and TEM-derived clones expressed a high level of CXCR4 and a low level of CD26, both of which may be involved in regulating cell migration to the BM (Supplemental Figure 1, B and C) (23, 24). The T cell clones of each pair recognized the same CMV peptide and had similar proliferation kinetics (Figure 2, B and C). Because telomere length was reported to...
correlate with in vivo persistence of transferred T cells in a prior study (25), we measured the telomere lengths of each clone using automated FISH and flow cytometry (flow-FISH), and although there was some variation, telomere length was not significantly different between T<sub>EM</sub>- and T<sub>CM</sub>-derived clones (Figure 2D).

In vivo persistence of CD8<sup>+</sup> T<sub>E</sub> clones derived from T<sub>CM</sub> or T<sub>EM</sub>. We administered the autologous gene-modified T cells intravenously and measured their frequency in the blood, LNs, and BM at intervals after infusion. The BM is known to be a niche for T<sub>CM</sub> and contains hematopoietic progenitor cells, which are a major site of latent CMV infection (26). A T<sub>EM</sub>-derived ΔCD19-modified T cell clone was transferred to macaque 02269 at a dose of 3 × 10<sup>8</sup> T cells/kg, which is approximately 5%–10% of the macaque total lymphocyte pool (27). CD19<sup>+</sup>CD8<sup>+</sup> T cells were detected in the blood 1 day after the infusion at a frequency of 1.2% of CD8<sup>+</sup> cells or 10 cells/μl of blood. The CD19<sup>+</sup>T cells peaked in the blood at 3.7% of CD8<sup>+</sup> T cells (40 cells/μl) on day 3 after infusion. However, transferred T cells were not detected in blood at day 5 or at multiple check points up to 42 days after infusion, or in BM or LN samples obtained 14 days after infusion (Figure 3A). PCR to amplify retroviral vec-

Figure 3
Persistence and migration of T<sub>EM</sub>- and T<sub>CM</sub>-derived CD8<sup>+</sup> T<sub>E</sub> clones in PBMCs, BM, and LNs following adoptive transfer. (A and B) ΔCD19-modified T<sub>EM</sub>-derived (A) and T<sub>CM</sub>-derived (B) clones were transferred to macaque 02269 in separate infusions at a cell dose of 3 × 10<sup>8</sup>/kg, and samples of PBMCs, BM, and LNs were collected before (pre) and at the indicated times following infusion. The frequency of transferred CD19<sup>+</sup> T cells was determined by flow cytometry and by PCR for vector sequences. Left: Percentage of CD19<sup>+</sup>CD8<sup>+</sup> T cells by flow cytometry in PBMCs, BM, and LNs before and after infusion of the T<sub>EM</sub>-derived (A) and T<sub>CM</sub>-derived (B) clones. Cells were gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells. Right: Absolute numbers of CD19<sup>+</sup>CD8<sup>+</sup> T cells/μl of blood determined by flow cytometry (gray bars; left y axis) and vector-positive T cells/10<sup>6</sup> PBMCs (filled diamonds; right y axis). (C and D) ΔCD19-modified T<sub>CM</sub>-derived and CD20-modified T<sub>EM</sub>-derived clones were transferred to macaque 02258 in separate infusions at a cell dose of 6 × 10<sup>8</sup>/kg. Aliquots of blood, BM, and LNs obtained at the indicated times were analyzed by flow cytometry and PCR to detect transferred CD19<sup>+</sup>CD8<sup>+</sup> (C) or CD20<sup>+</sup>CD8<sup>+</sup> (D) cells, respectively. Left: Percentage of CD8<sup>+</sup> T cells that expressed ΔCD19 (C) or CD20 (D) in PBMCs, BM, and LNs. Cells were gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells. Right: Absolute number of marked CD8<sup>+</sup> T cells/μl of blood (gray bars; left y axis) and vector-positive T cells/10<sup>6</sup> PBMCs (filled diamonds; right y axis).
tor sequences in DNA from peripheral blood, BM, and LNs confirmed the absence of the transferred T cells (Figure 3A and data not shown). We transferred the same dose of a ΔCD19-modified T cell clone derived from the TCM subset to this macaque. One day after infusion, the frequency of CD19+CD8+ T cells in the blood was 2.2% of CD8+ T cells (10 cells/μl), but in contrast to the TEM-derived clone, the TCM-derived cells persisted in the blood for longer than 56 days at approximately 0.2% of CD8+ T cells (3–6 cells/μl) and comprised 1.2% and 0.6% of CD8+ T cells in BM and LN samples, respectively, obtained 14 days after the infusion (Figure 3B).

In the second macaque (macaque 02258), we infused a higher cell dose (6 × 10^8/kg) and gave a ΔCD19-modified TCM-derived clone first. With this dose, the transferred cells were detected in the blood at a frequency of 26.3% of CD8+ T cells (228 cells/μl) on day 1 and 46.3% of CD8+ T cells (734 cells/μl) on day 3. The transferred TCM-derived clone was also detected in the BM and LN samples obtained on day 14 at 4.7% and 0.7% of CD8+ T cells, respectively (Figure 3C). The frequency of the transferred cells in the blood declined gradually over 28 days to 7–10 cells/μl but remained detectable by flow cytometry and PCR for more than 11 months after infusion (Figure 3C). The second infusion in this animal consisted of the same dose of a TEM-derived clone transduced with the CD20 retrovirus to enable the cells to be distinguished from the previously transferred ΔCD19-modified clone. The frequency of CD20+CD8+ T cells in the blood 1 day after the infusion was 16.3% of CD8+ T cells (103 cells/μl), but the transferred cells were not detected in the blood at day 5 or in BM and LN samples obtained on day 14 (Figure 3D). The disappearance of CD20-modified T cells was confirmed by PCR for vector sequences on DNA from samples of PBMCs, BM, and LNs (Figure 3D and data not shown).

CD8+ TEM-derived clones exhibit improved survival in IL-15 in vitro and decreased apoptosis in vivo. (A) Aliquots of TEM- and TCM-derived clones used for adoptive transfer were plated at the end of a 14-day stimulation cycle in medium alone (open triangles), IL-2 (1 ng/ml) (filled diamonds), or IL-15 (1 ng/ml) (filled squares), and viability was determined using Trypan blue dye exclusion. Data are representative of 13 TCM- and 11 TEM-derived clones from 3 macaques. (B) Expression of IL-15Rα, IL-2Rβ, and IL-2Rγ on TEM-derived (bold lines) and TCM-derived (black lines) clones transferred to macaque 02269 was measured by flow cytometry on days 13–14 after stimulation (dotted lines, isotype control mAb). Data are representative of clones administered to macaques 02258 and A99171. (C) Bcl-xl and Bcl-2 expression on 3 TEM-derived (white bars) and TCM-derived (gray bars) clones transferred to macaques 02269, 02258, and A99171 analyzed 14 days after stimulation. Mean ± SD of the MFI is shown on the y axis. (D) Apoptosis of TEM- and TCM-derived clones in vivo. The percent of CD19−CD8+ T cells in PBMCs that were Annexin V− and/or PI− 1 day after infusion of ΔCD19− TEM-derived or TCM-derived T cell clones to macaque A99171 was determined directly and after 24-hour culture. Samples were gated on CD8−CD19+ (white bars) and CD8−CD19+ T cells (black bars). Hatched bars show the T cells used for infusion. (E) Persistence and migration of TEM-derived and TCM-derived Tc clones in macaque A99171. PBMC, BM, and LN cells obtained at indicated times were analyzed by flow cytometry to detect transferred CD19−CD8+ Tc in samples gated on CD3+CD8+.

Figure 4

CD62L was not expressed by CD8-EM- and TEm-derived and TEm+EM- derived cells. On day 1, greater than 5% of CD8+ T cells were CD19+ and less than 20% were PI and/or Annexin V positive, both on direct analysis of PBMC and after 24 hours of culture (Figure 4D). As observed in prior animals, the TCM-derived clone migrated to the BM and LNs and persisted in the blood for more than 4 months (Figure 4E and data not shown).

The difference in persistence of TCM-derived and TEm-derived clones in the 3 macaques was significant (P < 0.03).

To address the possibility that a small proportion of transferred TEm might migrate to and persist only in tissue sites, we performed a necropsy on 1 macaque 10 days after the infusion of a TEm-derived clone. In this experiment, the intracellular truncated low-affinity nerve growth factor receptor (ΔLNGFR) was used as a marker to allow the cells to be distinguished from previously infused ΔCD19- and ΔCD20-marked T cells. As observed with prior infusions, the TEm-derived T cells were detected in the blood on day 1 at 7,376 ΔLNGFR+/10⁶ PBMCs (27 cells/μl) but declined rapidly and were not detected after day 5 (Figure 5A). DNA was prepared from samples of lung, liver, kidney, stomach, large intestine, skin, spleen, LNs, and BM obtained on day 10 and analyzed for ΔLNGFR vector sequences and for β-actin by real-time PCR. We did not detect ΔLNGFR-marked T cells in any tissue from this animal (Figure 5B). Together with the high levels of apoptosis in vivo after the infusion of TEm-derived clones, this data suggests that the lack of persistence of TEm-derived T cells in the blood reflects cell death rather than sequestration in tissues.

Adaptively transferred CD8+ TEm clones derived from TCM acquire a memory phenotype in vivo. CD262L was not expressed by CD8+ TCM-derived TEm clones at the time of adoptive transfer by flow cytometry, and we did not observe reexpression of CD262L when the TCM-derived clones were cultured in vitro with IL-2 or IL-15 (Figure 2A). However, the observation that the transferred TCM-derived cells migrated to LNs in all 3 animals suggested that CD262L might be reexpressed in vivo. CD262L was not expressed during the brief period the TEm-derived clones persisted in the blood in any of the macaques, but as early as 3 days after each infusion of TCM-derived TEm, a subset of the transferred cells in PBMC were CD262L+ (Figure 6A). CD262L+CD19+ T cells were also present in BM and LNs obtained 14 and 56 days after the infusions of TCM-derived clones (Figure 6, A and B). The proportion of transferred cells that were CD262L+ in the LNs was greater than in blood or BM (Figure 6B).
The expression of CD62L on transferred T cells after infusion prompted us to examine whether other memory markers were acquired that were absent on the infused T\textsubscript{E}. A subset of the CD19\textsuperscript{+}CD8\textsuperscript{+} T cells in PBMC and LNs obtained 14 days after transfer expressed CCR7, CD28, and CD127 (Figure 6, C–E). This analysis was repeated on samples obtained 56 days after cell transfer in macaque 02258 at days 14 and 56 after infusion of the ΔCD19-modified T\textsubscript{CM}-derived clone. The expression of phenotypic markers of T\textsubscript{CM}, including CD62L (B), CCR7 (C), CD28 (D), and CD127 (E) on the subset of transferred CD19\textsuperscript{+} T cells was determined by flow cytometry after gating on CD3\textsuperscript{+}CD8\textsuperscript{+} cells.

The expression of CD62L\textsuperscript{−} on transferred T cells after infusion prompted us to examine whether other memory markers were acquired that were absent on the infused T\textsubscript{E}. A subset of the CD19\textsuperscript{+}CD8\textsuperscript{+} T cells in PBMC and LNs obtained 14 days after transfer expressed CCR7, CD28, and CD127 (Figure 6, C–E). This analysis was repeated on samples obtained 56 days after cell transfer in macaque 02258. At this later time, the transferred CD19\textsuperscript{+}CD8\textsuperscript{+} T cells now comprised 1.4% of all CD8\textsuperscript{+} T cells in the LNs, and these cells were uniformly positive for markers of T\textsubscript{CM} including CD62L, CCR7, CD28, and CD127 (Figure 6, B–E). Transferred T cells were present at lower levels in PBMC and BM than in LNs and contained both CD62L\textsuperscript{−} and CD62L\textsuperscript{+} fractions. Thus despite expansion and differentiation of a single T\textsubscript{CM} cell to more than 5 \times 10\textsuperscript{9} T\textsubscript{E} cells, a significant fraction of the cells were able to persist long term after adoptive transfer, revert their phenotype to that of T\textsubscript{CM}, and compete with endogenous T cells to occupy anatomical niches of T\textsubscript{CM}.

T cells established by adoptive transfer exhibit functional properties of both T\textsubscript{CM} and T\textsubscript{EM}. We next examined whether the CD62L\textsuperscript{−} and CD62L\textsuperscript{+} CMV-specific T cells established by adoptive transfer were functional and had properties of T\textsubscript{CM} and T\textsubscript{EM}. Stimula-
Adoptively transferred CD8+ T cells exhibit functional properties of T\(_{\text{EM}}\) (A) IFN-\(\gamma\) production. PBMCs obtained from macaque 02269 before and 14 days following infusion of a ΔCD19+ CMV-specific T cell clone were stimulated with medium, PMA/ionomycin, or peptide antigen and examined by cytokine flow cytometry. Data are gated on CD3+CD8+ cells and are representative of results from macaque 02258. (B) Transferred T cells that reexpress CD62L lack direct cytotoxicity but acquire cytotoxic function after stimulation. Left: PBMCs obtained 14–70 days after infusion of a \(T_{\text{CM}}\)-derived ΔCD19+CD8+ clone to macaque 02258 were pooled, sorted into CD19+CD62L−CD8+ and CD19+CD62L+CD8+ fractions (purity >80%), and examined for lysis of autologous unpulsed (white bars) or peptide-pulsed target cells (black bars) (E/T ratio, 5:1). The cultured \(T_{\text{CM}}\)-derived ΔCD19+CD8+ clone served as positive control for lysis. Right: Sorted CD19+CD62L−CD8+ and CD19+CD62L+CD8+ T cells were stimulated using anti-CD3 and anti-CD28 mAbs for 14 days and then assayed for lysis of peptide-pulsed target cells (E/T ratio, 5:1). (C) Granzyme B expression. PBMCs and the transferred \(T_{\text{CM}}\)-derived clone were stained with mAbs to CD62L, CD8, and CD19 as well as intracellular granzyme B. Cells were analyzed by flow cytometry after gating on CD62L+CD8+, CD62L−CD8+, CD19+CD62L+CD8+, and CD19+CD62L−CD8+ cells. (D) Proliferation. PBMCs obtained from macaque 02269 on days 14–70 after infusion were sorted into CD19+CD62L+CD8+ (left panel) and CD19+CD62L−CD8+ subsets (right panel), labeled with CFSE, and stimulated with peptide-pulsed APCs as described in Methods. After 5 days, CFSE dilution was assessed by flow cytometry after gating on CD19+CD3+CD8+ cells. M3 gate, proportion of cells that have undergone more than 5 divisions.

CMV is latent in macaques, and reactivation is rare in immunocompetent hosts. Thus to determine whether the \(T_{\text{EM}}\) established by adoptive transfer could respond to antigen stimulation in vivo, we infused autologous T cells pulsed with CMV IE peptide (forming antigen-presenting T cells [T-APC]). We chose to use T-APCs because these cells could be easily prepared and prior human studies have shown that T cells expressing a foreign antigen can boost antigen-specific \(T_{\text{EM}}\) responses in vitro and in vivo (32, 33). Peptide-pulsed T-APCs were lysed by the CMV-specific T cell clone in vitro (Figure 8A) and were infused to macaque A99171 nine weeks after transfer of the CD19+ CD62L−ΔCD19+CD8+ clone. Seven days after the T-APC infusion, there was a 4- to 5-fold increase in the absolute numbers of both ΔCD19-modified T cells and
The persistence and antitumor efficacy of cultured T cells used for immunotherapy for infections and cancer and has been suggested to result from differentiation during in vitro culture (9, 16, 17). The inability of adoptively transferred T cells to persist in vivo may be an additional determinant of their fate in vivo. Here we improve the persistence of transferred T cells, suggesting that competition for cytokines and by the administration of IL-2 after lymphocytes before cell transfer to eliminate regulatory cells and therapy of cancer can sometimes be improved by depletion of host FAS+ cells. The data show the fold increase in absolute number of a previously transferred ΔCD19

cloned T cell from purified TCM and TEM and transferred these cells back into normal macaques without prior lymphodepletion or cytokine administration after cell transfer. In contrast to clinical trials of adoptive T cell therapy, where it is not possible to conclusively distinguish the transferred T cells from endogenous cells of the identical antigen and TCR specificity from which they were derived, we used gene marking to enable precise detection of transferred T cells. Although similar in function, phenotype, and proliferation before infusion, T cell clones derived from TCM or TEM exhibited remarkably different fates in vivo. TEM-derived clones persisted long term in the blood, migrated to LNs and BM, and underwent phenotypic conversion to both TCM and TEM after adoptive transfer. TEM-derived clones consistently failed to persist in the blood longer than 5 days and were not detected in LNs or BM. The demise of TEM-derived clones correlated with higher levels of apoptosis immediately after cell transfer, and a necropsy at day 10 after transferring a TEM-derived clone failed to detect these cells in any tissue site. Additional experiments analyzing T cell migration to tissues at earlier time points might provide further insight into the fate of TEM-derived clones. We chose to use culture conditions that are highly efficient and used commonly for propagating human T cells for clinical immunotherapy. Both TCM and TEM-derived clones proliferated equivalently well in vitro, but we cannot exclude the possibility that alternative culture conditions might yield cells with improved engraftment properties or that alterations to the host environment before or after transfer might enable TEM clones to persist.

A notable difference between TCM- and TEM-derived clones was the ability of IL-15, a cytokine required for maintenance of CD8+ T cells, to prevent apoptotic cell death after cytokine withdrawal. IL-15, which can directly bind to IL-15Rα on T cells or be transpresented by IL-15Rα-bearing cells (38), may be the initial stimulus that enables survival of TCM-derived cells in vivo, although the later acquisition of IL-7Rα on transferred T cells is consistent with a role for IL-7 in the reversion of T cell to long-lived TCM (39). Gene expression and epigenetic profiling may provide further insight into the basis for the marked differences in the survival of TEM clones derived from TCM and TEM and the ability of TCM-derived clones to reacquire phenotypic and functional properties of TCM (40, 41).

A prior study attempted to recapitulate in vivo programming of TCM and TEM by a single in vitro stimulation of naive tumor-specific CD8+ T cells from TCR-transgenic mice with antigen and either IL-2 to generate TEM or IL-15 to generate TCM (42). T cells cultured in IL-15 retained C6D6L, migrated to LNs, and were more effective in therapy in a murine model of melanoma. It remains to be determined whether the improved survival reflected incomplete differentiation of the T cells during the short-term in vitro culture in IL-15 or true programming of TCM in vitro. It is also unclear whether these properties would be retained through T cell cloning or the multiple cycles of stimulation that are necessary to generate polyclonal T cells for clinical immunotherapy from rare human tumor-specific or virus-specific TCR precursors.
Our discovery that Tc8 cells derived as clonal populations from Tcm are capable of reexpressing molecules required for homing to lymphoid organs and establishing reservoirs of functional Tcm and Tdm in vivo has implications for the selection of Tc cells for adoptive immunotherapy of viral diseases and cancer (1–8). For example, the results suggest that the durability of immune reconstitution achieved by adoptive transfer of donor virus-specific Tc cells to allogeneic stem cell transplant recipients would be improved by ensuring that Tc cells are expanded for therapy from the CD62L+ Tcm subset of the donor. Because virus-specific CD62L+ Tcm are typically present in lower frequency than Tdm in the blood, selection of Tcm cells prior to in vitro expansion would ensure that the cell product consisted of Tc cells that were able to persist and revert to the memory pool. The isolation of tumor-reactive Tc cells from cancer patients is difficult due to their low frequency and/or functional impairments in the tumor-bearing host (43, 44). Thus alternative approaches for generating autologous tumor-reactive Tc cells for cancer therapy have been developed, including the introduction of genes encoding TCRs specific for tumor antigens or chimeric immunoreceptors that target tumor cell surface molecules (11–14, 45). The results presented here suggest that isolating Tcm for insertion of genes that encode receptors for molecules on tumor cells may provide a tumor-reactive T cell population with an improved capacity to persist after adoptive transfer.

Methods

Animals and T cell transfer. Adult M. nemestrina were housed at the University of Washington National Primate Research Center under American Association for Accreditation of Laboratory Animal Care approved conditions. The Institutional Animal Care and Use Committee of the University of Washington approved the experimental protocols. Healthy macaques with a CD8+ T cell response to CMV IE-1 or IE-2 peptides were selected for the study (22). CMV-specific CD8+ T cell clones were infused intravenously at cell doses of 3 × 106 to 6 × 107/kg. Accredited clinical laboratories performed complete blood counts and serum chemistry. All macaques were followed at least 8 weeks after each T cell infusion. The persistence of transferred Tdm and Tdm-derived clones in the blood was measured by flow cytometry and PCR and compared using the log-rank test.

Retroviral vectors. A truncated macaque CD19 gene encoding for the extracellular and transmembrane domain (ACD19) and 4 aa of the cytoplasmic tail to abrogate signaling and the full-length CD20 gene were amplified by RT-PCR and cloned into the retroviral plasmid pMP71prox (a gift from W. Uckert, Max-Delbrück-Center, Berlin, Germany) (46). Retrovirus supernatant was produced in Phoenix Galv packaging cells (a gift from G. Nolan, Stanford University School of Medicine, Stanford, California, USA). A retroviral vector (LVV) containing ALNGFR as a cell surface marker has been described previously (47).

Epitope mapping. Cytokine flow cytometry was used to detect CD8+ T cells in PBMCs that expressed intracellular IFN-γ after stimulation with 24 pools of 15-mer overlapping peptides (NMI) arranged in an analytical grid and spanning the 558 aa rhesus CMV IE-1 protein sequence (GenBank accession no. M93360), or with an IE-2 peptide (a gift of L. Picker, Oregon Health & Science University, Beaverton, Oregon, USA) (22, 32). Immunogenic CMV peptides were identified by the responses of CD8+ T cells to intersecting peptides in the grid, and assays were repeated with each individual peptide and derivative 9-mer peptides to map specificity.

Flow cytometry and cell sorting. T cell clones and PBMCs were analyzed by flow cytometry after staining with fluorochrome-conjugated mAbs to CD3 (SP34), CD4, CD8, CD28, CD62L, CCR7, CD95, CD122 (IL-2Rβ), CD132 (IL-2Rγ), CXCR4, CD26 (BD), CD19, CD8β, and CD127 (Immunotech Coulter), IL-15Rα (R&D), and a secondary mouse IgG2b mAb (BD). For intracellular staining, cells were permeabilized using Cytofix/Cytoperm and stained with mAbs to granzyme B (BD), perforin (Kamiya Biomedical), Bcl-2 (BD), and Bcl-xL (Southern Biotech). Isotype-matched mAbs served as controls. In some experiments, samples were stained with Annexin V–phycocerythrin and PI according to the manufacturer’s instructions (BD). Samples were analyzed on a FACSCalibur using CellQuest Software (BD).

For sorting T cell subsets, PBMCs were stained with fluorochrome-conjugated anti-CD8, anti-CD28, and anti-Fas mAbs or with anti-CD8 and anti-CD26 mAbs and sorted using a Vantage BD cell sorter.

Culture and gene marking of CMV-specific T cell clones. Sorted-purified CD8+CD62L+ and CD8+CD62L− T cells containing Tcm and Tdm, respectively, were cocultured with autologous monocytes pulsed with CMVIE-1 or IE-2 peptides (1 μg/ml) in RPMI 1640 supplemented with 25 mM HEPES, 1-glutamine, 25 μM 2-mercaptoethanol (all from Invitrogen), and 10% human serum (Gemini, T cell media). IL-2 (10 U/ml; Chiron) was added on day 3. On day 7, T cells were replated at 0.3 cells/well in 96-well round-bottom plates with 1 × 105 γ-irradiated autologous peptide-pulsed PBMCs and 1 × 106 γ-irradiated B-lymphoblastoid lymphocytes in T cell media with 50 U/ml IL-2. After 12–14 days, aliquots from wells with visible growth were tested for recognition of 3H-Chromium-labeled peptide-pulsed or unpulsed target cells. CMV-specific T cell clones were stimulated with anti-CD3 and anti-CD28 mAbs, human γ-irradiated PBMCs and B-lymphoblastoid lymphocytes, and IL-2 (50 U/ml), as previously described (48, 49). On day 2, cells were exposed to ACD19 or CD20 retrovirus supernatant with IL-2 (50 U/ml) and polybrene (5 μg/ml; Chemicon), centrifuged at 1,000 g for 1 hour at 32°C, and incubated overnight. The cells were then washed and cultured in T cell media containing IL-2 and on day 8 selected for ACD19 or CD20 expression by immunomagnetic selection (Miltenyi Biotec). Cells were cryopreserved in aliquots and thawed subsequently for in vitro expansion for adoptive transfer as previously described (48, 49). The clonality of each infused T cell clone was confirmed by TCR-Vβ gene rearrangement analysis.

Cell viability assays. Aliquots of T cell clones at the end of the 14-day stimulation were plated at 2 × 104 to 4 × 105 cells/well in T cell media alone or with IL-2 (1 ng/ml or 16 U/ml; Chiron), IL-15 (1 ng/ml; R&D Systems), or IL-7 (10 ng/ml; R&D). Viability was assessed every 3–4 days by Trypan blue dye exclusion.

Cytotoxicity assays. Cytotoxic responses of CMV-specific T cells were examined as described (47–49). Briefly, autologous 3H-Chromium-labeled T cells were pulsed overnight with peptide antigen at various concentrations or with medium alone and used as targets for CD8+ T cell clones in a chromium-release assay.

Telomere length analysis. The average length of telomere repeats in individual lymphocytes was measured by automated flow-FISH (50, 51). Cryopreserved cells were thawed, hybridized with or without 0.3 μg/ml telomere-specific fluorescein isothiocyanate-conjugated (CCCTAA) PNA probe, washed, and counterstained with 0.01 μg/ml L6D5 751 (Exciton Chemical). To convert the fluorescence measured in sample cells hybridized with the telomere PNA probe into kilobytes of telomere repeats, fixed bovine thymocytes with known telomere length were used as internal control (51).

CFSE labeling of T cells. Sorted CD19+CD62L+ and CD19+CD62L− T cells were purified from PBMCs obtained after adoptive transfer and labeled with 0.625 μM CFSE (Invitrogen) in PBS for 10 minutes at 37°C and blocked with 20% FCS medium. CFSE-labeled T cells were plated at 1 × 106 cells/well in a 96-well plate with autologous CD40L-activated B cells (2.5 × 104 cells/well) pulsed with 200 ng/ml cognate peptide. CFSE dilution was analyzed by flow cytometry after 5 days.

PCR. PCR amplifications were performed using a quantitative PCR assay (49). DNA (0.3–1 μg) was amplified in duplicate using PCR primers and TaqMan probes (Applied Biosystems) designed to detect a unique sequence.
encompassing the junction of either the CD20 or CD19 gene and the retroviral vector pMP71pco. Standards consisted of DNA derived from the CD19- or CD20-modified T cell clones. Aliquots of peinuinfusion PBMCs served as a negative control. PCR to detect the ΔLNGFR vector was performed as described previously (47).

The M. nemestrina CD62L was cloned and sequenced, and a quantitative RT-PCR assay was used to determine the expression of CD62L in T cells. Briefly, total RNA was isolated from PBMCs and T cell clones using RNeasy Minikit (Qiagen) and used as template to prepare cDNA by reverse transcriptase (Superscript II; Invitrogen) following the manufacturer’s instructions. PCR was carried out in quadruplicate using a SYBR Green kit (Applied Biosystems) for 40 cycles in an ABI Prism 7700 Sequence Detector using SDS software 2.2.2 (Applied Biosystems). The GAPDH gene was used as a standard to correct for RNA quantity and quality. The expression level of CD62L was normalized to that of GAPDH and compared between PBMCs and T cell clones (ABI).

Preparation of T-APCs. Autologous T-APCs were expanded by stimulation with anti-CD3 and anti-CD28 mAbs and IL-2 (50 U/ml). After 14 days, T cells were harvested, pulsed for 90 min with CMV peptide (1 μg/ml), washed twice, and administered intravenously at a cell dose of 1 × 10^6 to 1 × 10^7/kg.

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