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(54) Title: ADOPTIVE TRANSFER OF CD8⁺ T CELL CLONES DERIVED FROM CENTRAL MEMORY CELLS

(57) **Abrégé/Abstract:**

The present invention provides a method of carrying out adoptive immunotherapy in a primate subject in need thereof by administering the subject a cytotoxic T lymphocytes (CTL) preparation in a treatment-effective amount. The method comprises administering as the CTL preparation a preparation consisting essentially of an in vitro expanded primate CTL population, the CTL population enriched prior to expansion for central memory T lymphocytes, and depleted prior to expansion of effector memory T lymphocytes. In some embodiments, the method may further comprise concurrently administering Interleukin-15 to the subject in an amount effective to increase the proliferation of the central memory T cells in the subject. Pharmaceutical formulations produced by the method, and methods of using the same, are also described.

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(57) Abstract: The present invention provides a method of carrying out adoptive immunotherapy in a primate subject in need thereof by administering the subject a cytotoxic T lymphocytes (CTL) preparation in a treatment-effective amount. The method comprises administering as the CTL preparation a preparation consisting essentially of an in vitro expanded primate CTL population, the CTL population enriched prior to expansion for central memory T lymphocytes, and depleted prior to expansion of effector memory T lymphocytes. In some embodiments, the method may further comprise concurrently administering Interleukin-15 to the subject in an amount effective to increase the proliferation of the central memory T cells in the subject. Pharmaceutical formulations produced by the method, and methods of using the same, are also described.



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**ADOPTIVE TRANSFER OF CD8⁺ T CELL CLONES DERIVED
FROM CENTRAL MEMORY CELLS**

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Field of the Invention

The present invention concerns methods and compositions for carrying out adoptive immunotherapy.

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Background of the Invention

Studies in rodents have demonstrated that adoptive immunotherapy with antigen specific T cells is effective for cancer and infections, and there is evidence this modality has therapeutic activity in humans¹⁻⁸. For clinical applications, it is necessary to isolate T cells of a desired antigen specificity or to engineer T cells to express receptors that target infected or transformed cells, and then expand these cells in culture⁹⁻¹⁴. The transfer of T cell clones is appealing because it enables control of specificity and function, and facilitates evaluation of in vivo persistence, toxicity and efficacy. Additionally, in the setting of allogeneic stem cell transplantation, the administration to recipients of T cell clones from the donor that target pathogens or malignant cells can avoid graft-versus-host disease that occurs with infusion of unselected donor T cells^{3,4,15}. However, it is apparent from clinical studies that the

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efficacy of cultured T cells, particularly cloned CD8⁺ T cells, is frequently limited by their failure to persist after adoptive transfer^{16,17}.

The pool of lymphocytes from which CD8⁺ T cells for adoptive immunotherapy can be derived contains naïve and long-lived, antigen experienced memory T cells (T_M). T_M can be divided further into subsets of central memory (T_{CM}) and effector memory (T_{EM}) cells that differ in phenotype, homing properties and function¹⁸. CD8⁺ T_{CM} express CD62L and CCR7, which promote migration into lymph nodes, and proliferate rapidly if re-exposed to antigen. CD8⁺ T_{EM} lack CD62L enabling migration to peripheral tissues, and exhibit immediate effector function¹⁹.

In response to antigen stimulation, CD8⁺ T_{CM} and T_{EM} both differentiate into cytolytic effector T cells (T_E) that express a high level of granzymes and perforin, but are short-lived²⁰. Thus, the poor survival of T cells in clinical immunotherapy trials may simply result from their differentiation during in vitro culture to T_E that are destined to die^{17, 21, 22}.

Summary of the Invention

A first aspect of the present invention is a method of carrying out adoptive immunotherapy in a primate subject in need thereof by administering the subject a cytotoxic T lymphocytes (CTL) preparation in a treatment-effective amount. The method comprises administering as the CTL preparation a preparation consisting essentially of an *in vitro* expanded (*e.g.*, grown *in vitro* for 1, 2, or 3 days up to 3 or 4 weeks or more) primate CTL population, the CTL population enriched prior to expansion for central memory T lymphocytes, and depleted prior to expansion of effector memory T lymphocytes. In some embodiments, the method may further comprise concurrently administering Interleukin-15 to the subject in an amount effective to increase the proliferation of the central memory T cells in the subject.

A further aspect of the invention is a pharmaceutical formulation comprising, consisting essentially of or consisting of an *in vitro* expanded primate cytotoxic T lymphocyte (CTL) population, the CTL population enriched prior to expansion for central memory T lymphocytes, and depleted prior to expansion of effector memory T lymphocytes.

A further aspect of the invention is the use of a formulation as described herein for the preparation of a medicament for carrying out a method as described herein (*e.g.*, treating cancer or an infectious disease in a human, or primate, subject).

In some embodiments the CTL preparation (or population) is produced by the process of: (a) collecting a first CTL population from a donor; (b) separating a CTL subpopulation enriched for CD62L⁺ central memory T lymphocytes and depleted of CD62L⁻ effector memory T lymphocytes to produce a central memory-enriched CTL subpopulation; (c) expanding the central memory-enriched CTL subpopulation *in vitro* in a culture medium (*e.g.*, for 1, 2, or 3 days up to 3 or 4 weeks or more); and then (d) collecting cells from the culture medium to produce the CTL preparation. The separating step "b" can in some embodiments be carried out by: (i) contacting the first CTL population to anti-CD62L antibody, wherein the antibody is immobilized on a solid support, so the central memory cells bind to the support; then (ii) separating the support from the CTL population with central memory cells bound thereto; (iii) and then separating the central memory cells from the solid support to produce the central memory enriched CTL subpopulation. In some embodiments, the expanding step "c" further comprises administering Interleukin-15 to the central memory subpopulation *in vitro*.

In some embodiments, the central memory-enriched T cells are modified *in vitro* with at least one gene that targets (*e.g.*, specifically binds to) cancer cells or other pathogenic cells in the subject.

In another aspect, there is provided a CD8⁺ cytotoxic T lymphocyte (CTL) preparation, wherein the CTL preparation is a primate CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells, wherein the CD8⁺ T_E cells of the CTL preparation comprise an introduced polynucleotide encoding a receptor that specifically binds to a target on a cell, and wherein the 60% or more CD8⁺ T_E cells have:

- (a) decreased expression of CD62L as compared to CD8⁺ central memory T (T_{CM}) cells, and
- (b) improved survival and/or expansion for at least 10 days when cultured *in vitro* in the presence of IL-15 in the absence of antigen stimulation as compared to CD8⁺ effector memory T (T_{EM}) cells.

In another aspect, there is provided a use of the CD8⁺ CTL preparation as described herein for the preparation of a medicament for use in adoptive immunotherapy in a human subject.

In another aspect, there is provided a use of the CD8⁺ CTL preparation as described herein for use in adoptive immunotherapy in a human subject.

In another aspect, there is provided a method for producing a CD8⁺ cytotoxic T lymphocyte (CTL) preparation useful for adoptive immunotherapy, comprising the steps of:

- (a) enriching a subpopulation of CD62L⁺ CD8⁺ central memory T (T_{CM}) lymphocytes from a primate subject's T lymphocyte population;
- (b) introducing, into cells of the T_{CM}-enriched subpopulation, a polynucleotide encoding a receptor that specifically binds to a target on a cell; and
- (c) expanding the T_{CM}-enriched subpopulation *in vitro* to produce a CD8⁺ CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells that have decreased expression of CD62L as compared to CD8⁺ T_{CM} cells.

In another aspect, there is provided a CD8⁺ cytotoxic T lymphocyte (CTL) preparation produced by the method described herein, wherein the CTL preparation is a primate CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells, wherein the CD8⁺ T_E cells of the CTL preparation comprise an introduced polynucleotide encoding a receptor that specifically binds to a target on a cell, and wherein the 60% or more CD8⁺ T_E cells have:

- (a) decreased expression of CD62L as compared to CD8⁺ central memory T (T_{CM}) cells, and
- (b) improved survival and/or expansion for at least 10 days when cultured *in vitro* in the presence of IL-15 in the absence of antigen stimulation as compared to CD8⁺ effector memory T (T_{EM}) cells.

Brief Description of the Drawings

Figure 1. Isolation and genetic modification of CMV-specific CD8⁺ T cell clones from CD62L⁻ and CD62L⁺ T cell subsets for adoptive transfer.

Figure 1a. CMV IE-specific memory T cells are present in both CD62L⁺ and CD62L⁻ subsets of CD8⁺ peripheral blood lymphocytes. PBMC were sorted into CD62L⁻CD8⁺ and CD62L⁺CD8⁺ fractions after staining with anti-CD8 and antiCD62L antibodies (upper panels). The CD62L⁻ and CD62L⁺ fractions were stimulated for 6 hours with autologous CD40L-activated B cells pulsed with medium alone or with CMV-IE peptide. Brefeldin A was added for the final 4 hours and CD8⁺ T cells that produced IFN- γ were detected by staining for intracellular IFN- γ . The lower left panels show IFN- γ production by the CD62L⁻CD8⁺ subset and the lower right panels

show IFN- γ production by the CD62L⁺CD8⁺ subset. Data from macaque 02269 is shown and is representative of data obtained in four consecutive macaques.

Figure 1b. Strategy for the isolation of CMV-specific CD8⁺ T cell clones from CD62L⁺T_{EM} and CD62L⁺T_{CM} subsets. Aliquots of PBMC were obtained from each macaque and sorted into CD62L⁺CD8⁺ and CD62L⁺CD8⁺ fractions after staining with anti-CD8 and anti-CD62L antibodies. The sorted T cells were cultured with autologous monocytes pulsed with the CMV IE peptide and after 1 week of stimulation, T cells from the cultures were cloned by limiting dilution. T cell clones were screened to identify those that lysed autologous peptide-pulsed target cells, and then transduced with a retroviral vector that encoded a cell surface Δ CD19 or CD20 molecule. Individual T cell clones were expanded in vitro to $>5 \times 10^9$ cells by stimulation with anti-CD3 and anti-CD28 antibodies in cultures supplemented with γ -irradiated human PBMC and EBV-LCL prior to adoptive transfer.

Figure 1c. Retroviral vector constructs encoding for macaque cell surface marker genes. Abbreviations: MPSV-LTR, myeloproliferative sarcoma virus retroviral long terminal repeat; Ψ +, extended packaging signal; PRE, woodchuck hepatitis virus posttranscriptional regulatory element; Δ CD19, truncated macaque CD19 cDNA encoding the extracellular and transmembrane domains, and 4 aa of the cytosolic tail; CD20, full-length macaque CD20 cDNA.

(d) Immunomagnetic bead selection of Δ CD19 and CD20-modified CD8⁺ T cell clones. Transduced T cells were enriched for Δ CD19 or CD20 expressing cells on day 8 after transduction using a two-step immunomagnetic selection with anti-CD19 or anti-CD20 antibodies and rat anti-mouse IgG coupled microbeads. Aliquots of unmodified (left panels) and Δ CD19 or CD20-modified T cells (right panels) were removed after selection, co-stained with anti-CD8 and anti-CD19 or anti-CD20 antibodies, and analyzed by flow cytometry to assess purity. The percentages of CD8⁺ T cells positive for the transgene are indicated.

Figure 2. CMV-specific CD8⁺ T cell clones derived from T_{CM} and T_{EM} subsets exhibit an effector phenotype, and comparable avidity and proliferation in vitro.

Figure 2a. Individual T_{CM} and T_{EM}-derived macaque CMV-specific CD8⁺ T cell clones were examined by flow cytometry for expression of CD62L, CCR7,

CD28, CD127, granzyme B and perforin (bold line) and stained with isotype control antibodies (dotted line). Inset values represent the mean fluorescence intensity (MFI). The data is shown for T cell clones that were adoptively transferred to macaque 02269 and is representative of the data for all of the T cell clones used in adoptive transfer experiments in the three macaques.

Figure 2b. Cytotoxic activity of each pair of T_{EM} (filled triangle) and T_{CM} (filled square) -derived CMV-specific CD8⁺ T cell clones used in adoptive transfer was examined in a 4-hour chromium release assay at an effector to target ratio of 20:1 using autologous target cells pulsed with the CMV IE peptide at various concentrations. The sequences of the IE-1 peptides were KKGDIDRV (02269) and EEHVKLFFK (A99171), the sequence of the IE-2 peptide was ATTRSLEYK (02258).

Figure 2c. In vitro growth of CMV-specific CD8⁺ T cell clones. The T_{EM} (filled triangle) and T_{CM} (filled square) derived T cell clones used for adoptive transfer were stimulated with anti-CD3 and anti-CD28 in the presence of γ -irradiated feeder cells and IL-2 (50 U/ml). Cell growth over 14 days of culture was measured by counting viable cells using trypan blue exclusion.

Figure 2d. Telomere length in CMV-specific T cell clones derived from CD62L⁺ and CD62L⁻ subsets. The median telomere length of duplicate samples was measured by automated flow-FISH in peripheral blood T lymphocytes (shaded squares), in the infused T cell clones (iT_{EM} filled squares, iT_{CM} open squares), and in each of two additional randomly selected T_{EM} (left hatched squares) and T_{CM}-derived (right hatched squares) T cell clones from each macaque.

Figure 3. Persistence and migration of T_{EM} and T_{CM}-derived CD8⁺ T_E clones in peripheral blood, bone marrow, and lymph nodes following adoptive transfer.

Figures 3a-b. In vivo persistence and migration of Δ CD19-modified T_{EM}-derived (a) and T_{CM}-derived (b) T cell clones in macaque 02269. The T cell clones were transferred in separate infusions given intravenously more than 10 weeks apart at a cell dose of 3×10^8 /kg. Samples of PBMC were collected at intervals for 6-8 weeks after each infusion. Bone marrow and lymph node samples were collected before infusion and fourteen days after each T cell infusion. Samples were stained

with fluorochrome-conjugated anti-CD3, CD8, and CD19 antibodies, respectively. The frequency of transferred CD19⁺ T cells was determined by flow cytometry with gating on CD3⁺CD8⁺ cells. Left panels show the percentage of CD8⁺ T cells that expressed CD19 in blood, bone marrow and lymph nodes prior to and at intervals
 5 after infusion of the T_{EM}-derived (a) and T_{CM}-derived (b) T cell clones. Right panels show the absolute numbers of CD19⁺CD3⁺CD8⁺ T cells per μ l of blood. This was determined by calculating the absolute number of CD3⁺CD8⁺ T cells per μ l of blood at the indicated days (% of CD3⁺CD8⁺ T cells in an aliquot of mononuclear cells x mononuclear cell count per μ l blood/100). Subsequently, the number of
 10 CD19⁺CD3⁺CD8⁺ T cells was derived (% CD19⁺CD3⁺CD8⁺ T cells x absolute CD3⁺CD8⁺ count per μ l blood/100).

Figures 3c-d. In vivo persistence and migration of adoptively transferred Δ CD19-modified T_{CM} derived and CD20-modified T_{EM}-derived T cell clones in macaque 02258. CMV-specific CD8⁺ T cell clones were adoptively transferred in
 15 separate intravenous infusions at a cell dose of 6×10^8 /kg. Aliquots of blood, bone marrow, and lymph nodes were obtained before and at the indicated times after each T cell infusion and analyzed by flow cytometry using specific antibodies to detect the transferred CD19⁺CD8⁺ or CD20⁺CD8⁺ T cells, respectively. Left panels show the percentage of CD8⁺ T cells that expressed Δ CD19 (c) or CD20 (d) in blood, bone
 20 marrow and lymph nodes at intervals after each infusion. Right panels show the absolute number of CD3⁺CD8⁺ T cells per μ l of blood that expressed Δ CD19 (c) or CD20 (d) at intervals after infusion.

**Figure 4. T_{CM}-derived T_E clones undergo less apoptosis after adoptive transfer compared with T_{EM}-derived T_E clones, and are rescued from cell death
 25 in vitro by IL-15.**

Figure 4a. Expression of bcl-xl and bcl-2 by T_{EM}-derived (open squares) and T_{CM}-derived (filled squares) CD8⁺ T cell clones adoptively transferred to macaques 02269, 02258, and A99171. The T cells were analyzed at the end of a 14-day stimulation cycle by intracellular staining with bcl-xl and bcl-2 specific antibodies.
 30 The mean fluorescence intensity of staining is shown on the y axis.

Figure 4b. Apoptosis of T_{EM}-derived and T_{CM}-derived CD8⁺ T_E clones in vivo. A Δ CD19-modified T cell clone derived from the CD62L T_{EM} fraction was adoptively

transferred at a dose of 6×10^8 /kg to macaque A99171, followed 5 weeks later by the infusion of the same cell dose of a Δ CD19-modified T cell clone derived from the CD62L⁺ T_{CM} fraction. Samples of PBMC were obtained on day 1 after transfer and the proportion of Δ CD19-expressing CD8⁺ T cells that bound Annexin V and/or stained positive for PI was determined by flow cytometry after staining with anti-CD8 and CD19 antibodies, and with Annexin V and PI. PBMC were analyzed directly and after 24 hours of culture in CTL media. The analysis was performed after gating on CD8⁺CD19⁻ T cells (open squares) and on CD8⁺CD19⁺ T cells (filled squares), respectively. The hatched bars show the proportion of T cells in each of the cell products prior to infusion that bound Annexin V or stained positive for PI.

Figure 4c. Persistence and migration of T_{EM}-derived and T_{CM}-derived T_E clones in macaque A99171. Peripheral blood, bone marrow, and lymph node cells obtained at intervals after each T cell infusion were analyzed by flow cytometry to detect transferred CD19⁺CD8⁺ T cells. The samples were analyzed after gating on CD3⁺CD8⁺ cells.

Figure 4d. IL-15 supports the in vitro survival of T_{CM}-derived but not T_{EM}-derived CD8⁺ T_E clones. Aliquots of the CMV-specific T_{EM}-derived and T_{CM}-derived CD8⁺ T cell clones that were used for adoptive transfer to macaques 02269, 02258, and A99171 respectively, were plated in wells containing medium alone (open triangles) or medium supplemented with IL-2 (16.6 U/ml; 1 ng/ml) (filled diamonds) or IL-15 (1 ng/ml) (filled squares), respectively. The number of viable cells was determined at the indicated days (up to 34 days) by counting cells using trypan dye exclusion.

Figure 4e. CD8⁺ T_{CM}-derived clones express higher levels of IL-15 receptor (IL-15R) chains. Expression of IL-15R α , IL-2R β , and IL-2R γ on aliquots of CMV-specific T_{CM}-derived (bold line) and T_{EM}-derived (black line) T cell clones was measured by flow cytometry on day 13-14 after stimulation. Staining with isotope control antibody is shown by the dotted line. The data is shown for the T cell clones used for the adoptive transfer experiments in macaque 02269, and is representative of the data obtained for the T cell clones administered to macaques 02258 and A99171, respectively.

Figure 5. Adoptively transferred T_{CM} -derived T_E cells reacquire markers of T_{CM} in vivo and persist in memory cell niches.

Figure 5a. Expression of CD62L on T_{EM} -derived (upper panels) or T_{CM} -derived (lower panels) T_E clones after adoptive transfer. Samples of PBMC were obtained before and on day 3 after the infusion of T_{CM} and T_{EM} -derived T cell clones, and analyzed by flow cytometry after staining with fluorochrome-conjugated anti-CD3, CD8, CD62L, and either CD19 or CD20 antibodies. Cells were gated to identify CD3⁺CD8⁺ T cells and the percentage of T cells that expressed the CD19 or CD20 marker gene and CD62L is shown in the upper right quadrant of each panel.

Figures 5b-e. A major fraction of CD8⁺ T cells that persist after adoptive transfer acquire phenotypic markers of T_{CM} and reside in lymph nodes. Aliquots of PBMC, lymph nodes (LN), and bone marrow (BM) were obtained from macaque 02258 at day 14 and day 56 after the infusion of the Δ CD19-modified T_{CM} -derived CMV-specific T cell clone. The expression of phenotypic markers of T_{CM} including CD62L (b), CCR7 (c), CD28 (d), and CD127 (e) on the subset of transferred CD19⁺ T cells was determined by flow cytometry after gating on CD3⁺CD8⁺ cells.

Figure 6. Adoptively transferred CD8⁺ T cells that persist in vivo exhibit functional properties of memory T cells.

Figure 6a. Adoptively transferred CD8⁺ T cells that persist in vivo produce IFN- γ after antigen stimulation. PBMC were obtained before the T cell infusion (Pre) and 21 days after (Post) the infusion of a Δ CD19-modified T_{CM} -derived CMV-specific CD8⁺ T cell clone. Aliquots of PBMC were stimulated with medium alone, PMA/ionomycin, or the CMV IE peptide recognized by the transferred T cell clone. IFN- γ production by CD8⁺ T cells was examined by cytokine flow cytometry after staining with anti-CD3, CD8, CD19, and IFN- γ antibodies. The analysis was performed after gating on CD3⁺CD8⁺ T cells. The data is shown for macaque 02269 and is representative of a separate experiment in macaque 02258.

Figure 6b. Adoptively transferred T cells that re-express CD62L lack direct cytotoxicity but differentiate into cytotoxic cells after TCR stimulation. Left panel: PBMC obtained 14-70 days after the infusion of a Δ CD19-modified T_{CM} -derived CD8⁺ T cell clone were pooled and sorted into CD19⁺CD62L⁻CD8⁺ and CD19⁺CD62L⁺CD8⁺ fractions that were at least 80% pure. The sorted cells were

examined for recognition of autologous target cells alone (open squares) or pulsed with CMV IE peptide (closed squares) in a 4-hour chromium release assay at an E/T ratio of 5:1. The lysis of target cells by the in vitro cultured T_{CM}-derived CD19-modified CD8⁺ T cell clone served as a positive control. Right panel: Aliquots of the sorted CD62L⁻ΔCD19⁺CD8⁺ and CD62L⁺ΔCD19⁺CD8⁺ T cells were stimulated in vitro using anti-CD3 and CD28 antibodies, in the presence of γ-irradiated feeder cells and IL-2 (50 U/ml). After 14 days of culture, the cultures were assayed for recognition of peptide pulsed target cells in a 4-hour chromium release assay (E/T ratio of 5:1). The data is shown for macaque 02258 and is representative of that observed with macaque 02269.

Figure 6c. Adoptively transferred T cells that reacquire CD62L in vivo exhibit more rapid proliferation than those that remain CD62L⁻. Aliquots of the sorted ΔCD19⁺CD62L⁺CD8⁺ and ΔCD19⁺CD62L⁻CD8⁺ T cells from macaque 02269 were labeled with CFSE (upper panels) and stimulated in vitro with either CMV IE peptide-pulsed autologous CD40L activated B cells or medium alone. After 5 days, dilution of CFSE was assessed by flow cytometry after gating on ΔCD19⁺CD3⁺CD8⁺ cells. The M3 gate identifies cells that have undergone more than five divisions.

Figure 6d. Adoptively transferred T cells can be driven to expand in vivo by the infusion of TAPC. Autologous T cells obtained and cryopreserved from macaque A99171 prior to any T cell infusions were thawed and expanded by stimulation with anti-CD3 and anti-CD28 antibodies, and IL-2. After expansion, the T cells were pulsed with the CMV IE peptide recognized by the adoptively transferred ΔCD19-modified T_{CM} clone. Eight weeks after the T cell infusion when a stable level of CD19⁺ T cells was established in vivo, a dose of 1 x 10⁷ T-APC/kg was administered intravenously. The absolute number of CD3⁺CD8⁺ T cells and CD19⁺CD8⁺ T cells/μl of blood was measured on samples obtained prior to administration of T-APC, and on day +3, +5 and +7 after the infusion of T-APC. The number of endogenous IE-specific CD8⁺ T cells was measured at the same time points by cytokine flow cytometry after gating on CD19⁻CD3⁺CD8⁺ cells. The data shows the fold-increase in the absolute numbers of CD3⁺CD8⁺ T cells (open squares), CD19⁺CD8⁺ T cells (shaded squares), and CD19⁻IE-specific CD8⁺ T cells (filled squares) at day +3, +5 and +7 after the infusion of T-APC.

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Figure 6e. T-APC pulsed with IE peptide are lysed by IE-specific CD8⁺ T cells. T-APC generated from macaque A99171 either pulsed with IE peptide (filled squares) or with media alone (open squares) were labeled with ⁵¹Cr and used as targets for an aliquot of the autologous IE-specific CD8⁺ T cell clone that was
 5 adoptively transferred to macaque A99171.

The present invention is explained in greater detail below.

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Detailed Description of the Preferred Embodiments

"T cells" or "T lymphocytes" as used herein may be from any mammalian, preferably primate, species, including monkeys, dogs, and humans. In some embodiments the T cells are allogenic (from the same species but different donor) as the recipient subject; in some embodiments the T cells are autologous (the donor and
 15 the recipient are the same); in some embodiments the T cells are syngeneic (the donor and the recipients are different but are identical twins).

Cytotoxic T lymphocyte (CTL) as used herein refers to a T lymphocyte that expresses CD8 on the surface thereof (*i.e.*, a CD8⁺ T cell). In some embodiments such cells are preferably "memory" T cells (T_M cells) that are antigen-experienced.

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"Central memory" T cell (or "T_{CM}") as used herein refers to a CTL that expresses CD62L on the surface thereof (*i.e.*, CD62L⁺CD8⁺ cells).

"Effector memory" T cell (or "T_{EM}") as used herein refers to a CTL that does not express CD62L on the surface thereof (*i.e.*, CD62L⁻CD8⁺ cells).

"Enriched" and "depleted" as used herein to describe amounts of cell types in a
 25 mixture refers to the subjecting of the mixture of the cells to a process or step which results in an increase in the number of the "enriched" type and a decrease in the number of the "depleted" cells. Thus, depending upon the source of the original population of cells subjected to the enriching process, a mixture or composition may contain 60, 70, 80, 90, 85, or 99 percent or more (in number or count) of the
 30 "enriched" cells and 40, 30, 20, 10, 5 or 1 percent or less (in number or count) of the "depleted" cells.

Interleukin-15 is a known and described in, for example, US Patent No. 6,344,192.

I. In vitro expansion.

T lymphocytes can be collected in accordance with known techniques and enriched or depleted by known techniques such as affinity binding to antibodies such as flow cytometry and/or affinity binding. After enrichment and/or depletion steps, in vitro expansion of the desired T lymphocytes can be carried out in accordance with known techniques (including but not limited to those described in US Patent No. 6,040,177 to Riddell et al.), or variations thereof that will be apparent to those skilled in the art.

For example, the desired T cell population or subpopulation may be expanded by adding an initial T lymphocyte population to a culture medium in vitro, and then adding to the culture medium feeder cells, such as non-dividing peripheral blood mononuclear cells (PBMC), (*e.g.*, such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (*e.g.* for a time sufficient to expand the numbers of T cells). The order of addition of the T cells and feeder cells to the culture media can be reversed if desired. The culture can typically be incubated under conditions of temperature and the like that are suitable for the growth of T lymphocytes. For the growth of human T lymphocytes, for example, the temperature will generally be at least about 25 degrees Celsius, preferably at least about 30 degrees, more preferably about 37 degrees.

The T lymphocytes expanded are typically cytotoxic T lymphocytes (CTL) that are specific for an antigen present on a human tumor or a pathogen.

The non-dividing feeder cells can comprise gamma-irradiated PBMC feeder cells. In some embodiments, the PBMC are irradiated with gamma rays in the range of about 3000 to 3600 rads.

Optionally, the expansion method may further comprise the step of adding non-dividing EBV-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of about 6000 to 10,000 rads. The LCL feeder cells may be provided in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least about 10:1.

Optionally, the expansion method may further comprise the step of adding anti-CD3 monoclonal antibody to the culture medium (*e.g.*, at a concentration of at

least about 0.5 ng/ml). Optionally, the expansion method may further comprise the step of adding IL-2 and/or IL-15 to the culture medium (*e.g.*, wherein the concentration of IL-2 is at least about 10 units/ml).

In some embodiments it may be desired to introduce functional genes into the
5 T cells to be used in immunotherapy in accordance with the present invention. For example, the introduced gene or genes may improve the efficacy of therapy by promoting the viability and/or function of transferred T cells; or they may provide a genetic marker to permit selection and/or evaluation of *in vivo* survival or migration; or they may incorporate functions that improve the safety of immunotherapy, for
10 example, by making the cell susceptible to negative selection *in vivo* as described by Lupton S. D. et al., *Mol. and Cell Biol.*, **11:6** (1991); and Riddell et al., *Human Gene Therapy* 3:319-338 (1992); see also the publications of PCT/US91/08442 and PCT/US94/05601 by Lupton et al., describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable marker with a negative
15 selectable marker. This can be carried out in accordance with known techniques (*see, e.g.*, US Patent No. 6,040,177 to Riddell et al. at columns 14-17) or variations thereof that will be apparent to those skilled in the art based upon the present disclosure.

Various infection techniques have been developed which utilize recombinant infectious virus particles for gene delivery. This represents a currently preferred
20 approach to the transduction of T lymphocytes of the present invention. The viral vectors which have been used in this way include virus vectors derived from simian virus 40, adenoviruses, adeno-associated virus (AAV), and retroviruses. Thus, gene transfer and expression methods are numerous but essentially function to introduce and express genetic material in mammalian cells. Several of the above techniques
25 have been used to transduce hematopoietic or lymphoid cells, including calcium phosphate transfection, protoplast fusion, electroporation, and infection with recombinant adenovirus, adeno-associated virus and retrovirus vectors. Primary T lymphocytes have been successfully transduced by electroporation and by retroviral infection

30 Retroviral vectors provide a highly efficient method for gene transfer into eukaryotic cells. Moreover, retroviral integration takes place in a controlled fashion and results in the stable integration of one or a few copies of the new genetic information per cell.

It is contemplated that overexpression of a stimulatory factor (for example, a lymphokine or a cytokine) may be toxic to the treated individual. Therefore, it is within the scope of the invention to include gene segments that cause the T cells of the invention to be susceptible to negative selection in vivo. By "negative selection" is meant that the infused cell can be eliminated as a result of a change in the in vivo condition of the individual. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to an administered agent, for example, a compound. Negative selectable genes are known in the art, and include, inter alia the following: the Herpes simplex virus type I thymidine kinase (HSV-I TK) gene (Wigler et al., Cell 11:223, 1977) which confers ganciclovir sensitivity; the cellular hypoxanthine phosphoribosyltransferase (HPRT) gene, the cellular adenine phosphoribosyltransferase (APRT) gene, bacterial cytosine deaminase, (Mullen et al., Proc. Natl. Acad. Sci. USA. 89:33 (1992)).

In some embodiments it may be useful to include in the T cells a positive marker that enables the selection of cells of the negative selectable phenotype in vitro. The positive selectable marker may be a gene which, upon being introduced into the host cell expresses a dominant phenotype permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, inter alia, hygromycin-B phosphotransferase gene (hph) which confers resistance to hygromycin B, the aminoglycoside phosphotransferase gene (neo or aph) from Tn5 which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and the multi-drug resistance (MDR) gene.

Preferably, the positive selectable marker and the negative selectable element are linked such that loss of the negative selectable element necessarily also is accompanied by loss of the positive selectable marker. Even more preferably, the positive and negative selectable markers are fused so that loss of one obligatorily leads to loss of the other. An example of a fused polynucleotide that yields as an expression product a polypeptide that confers both the desired positive and negative selection features described above is a hygromycin phosphotransferase thymidine kinase fusion gene (HyTK). Expression of this gene yields a polypeptide that confers hygromycin B resistance for positive selection in vitro, and ganciclovir sensitivity for negative selection in vivo. See Lupton S. D., et al, Mol. and Cell. Biology 11:3374-3378, 1991. In addition, in preferred embodiments, the polynucleotides of the

invention encoding the chimeric receptors are in retroviral vectors containing the fused gene, particularly those that confer hygromycin B resistance for positive selection in vitro, and ganciclovir sensitivity for negative selection in vivo, for example the HyTK retroviral vector described in Lupton, S. D. et al. (1991), supra.

- 5 See also the publications of PCT/US91/08442 and PCT/US94/05601, by S. D. Lupton, describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable markers with negative selectable markers.

Preferred positive selectable markers are derived from genes selected from the group consisting of hph, neo, and gpt, and preferred negative selectable markers are
10 derived from genes selected from the group consisting of cytosine deaminase, HSV-I TK, VZV TK, HPRT, APRT and gpt. Especially preferred markers are bifunctional selectable fusion genes wherein the positive selectable marker is derived from hph or neo, and the negative selectable marker is derived from cytosine deaminase or a TK gene.

- 15 A variety of methods can be employed for transducing T lymphocytes, as is well known in the art. For example, retroviral transductions can be carried out as follows: on day 1 after stimulation using REM as described herein, provide the cells with 20-30 units/ml IL-2; on day 3, replace one half of the medium with retroviral supernatant prepared according to standard methods and then supplement the cultures
20 with 5 ug/ml polybrene and 20-30 units/ml IL-2; on day 4, wash the cells and place them in fresh culture medium supplemented with 20-30 units/ml IL-2; on day 5, repeat the exposure to retrovirus; on day 6, place the cells in selective medium (containing, e.g., an antibiotic corresponding to an antiobiotic resistance gene provided in the retroviral vector) supplemented with 30 units/ml IL-2; on day 13,
25 separate viable cells from dead cells using Ficoll Hypaque density gradient separation and then subclone the viable cells.

II. Compositions and methods.

- Subjects that can be treated by the present invention are, in general, human
30 and other primate subjects, such as monkeys and apes for veterinary medicine purposes. The subjects can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects.

Subjects that can be treated include subjects afflicted with cancer, including but not limited to colon, lung, liver, breast, prostate, ovarian, skin (including melanoma), bone, and brain cancer, etc. In some embodiments the tumor associated antigens are known, such as melanoma, breast cancer, squamous cell carcinoma, colon cancer, leukemia, myeloma, prostate cancer, etc. (in these embodiments memory T cells can be isolated or engineered by introducing the T cell receptor genes). In other embodiments the tumor associated proteins can be targeted with genetically modified T cells expressing an engineered immunoreceptor. Examples include but are not limited to B cell lymphoma, breast cancer, prostate cancer, and leukemia.

Subjects that can be treated also include subjects afflicted with, or at risk of developing, an infectious disease, including but not limited to viral, retroviral, bacterial, and protozoal infections, etc.

Subjects that can be treated include immunodeficient patients afflicted with a viral infection, including but not limited to Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus infections in transplant patients, etc.

Cells prepared as described above can be utilized in methods and compositions for adoptive immunotherapy in accordance with known techniques, or variations thereof that will be apparent to those skilled in the art based on the instant disclosure. *See, e.g.*, US Patent Application Publication No. 2003/0170238 to Gruenberg et al; *see also* US Patent No. 4,690,915 to Rosenberg.

In some embodiments, the cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "pharmaceutically acceptable" carrier) in a treatment-effective amount. Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumen.

A treatment-effective amount of cells in the composition is at least 10^9 , typically greater than 10^9 , at least 10^{10} cells, and generally more than 10^{10} . The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For example, if cells that are specific for a particular antigen are desired, then the population will contain greater

than 70%, generally greater than 80%, 85% and 90-95% of such cells. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 mls or less, even 250 mls or 100 mls or less. Hence the density of the desired cells is typically greater than 10^6 cells/ml and generally is greater than 10^7 cells/ml, generally
5 10^8 cells/ml or greater. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^9 , 10^{10} or 10^{11} cells.

In some embodiments, the lymphocytes of the invention may be used to confer immunity to individuals. By "immunity" is meant a lessening of one or more physical
10 symptoms associated with a response to infection by a pathogen, or to a tumor, to which the lymphocyte response is directed. The amount of cells administered is usually in the range present in normal individuals with immunity to the pathogen. Thus, the cells are usually administered by infusion, with each infusion in a range of at least 10^6 to 10^{10} cells/ m^2 , preferably in the range of at least 10^7 to 10^9 cells/ m^2 . The
15 clones may be administered by a single infusion, or by multiple infusions over a range of time. However, since different individuals are expected to vary in responsiveness, the type and amount of cells infused, as well as the number of infusions and the time range over which multiple infusions are given are determined by the attending physician, and can be determined by routine examination. The generation of sufficient
20 levels of T lymphocytes (including cytotoxic T lymphocytes and/or helper T lymphocytes) is readily achievable using the rapid expansion method of the present invention, as exemplified herein. *See, e.g.,* US Patent No. 6,040,177 to Riddell et al. at column 17.

25 The present invention is illustrated further in the examples set forth below.

EXPERIMENTAL

In the normal host, T cell memory persists for life indicating the capacity for selfrenewal in addition to differentiation²³. This quality of T cell memory has been suggested to reside in the $CD62L^+$ T_{CM} subset, although a unique subset of T cells that
30 self-renew and differentiate has been identified in mice^{18, 23, 24}. We sought to determine if T_E clones differentiated in vitro from T_{CM} or T_{EM} subsets might differ intrinsically in their potential to persist after adoptive transfer. Here, we show in a non-human primate model relevant for human translation, that antigen-specific T_E

clones derived from T_{CM} but not T_{EM} precursors can persist, migrate to lymph nodes and bone marrow, reacquire phenotypic properties of T_{CM} and T_{EM}, and respond to antigen challenge.

5 METHODS

Animals and experimental design. Adult macaques (*Macaca nemestrina*) were housed at the University of Washington Regional Primate Research Center, under American Association for Accreditation of Laboratory Animal Care approved conditions. The Institutional Animal Care and Use Committee approved the
10 experimental protocols.

Healthy macaques were selected for this study if they had evidence of prior CMV infection as determined by a positive lymphoproliferative response to CMV antigen and a CD8⁺ T cell response to CMV IE-1 or IE-2 peptides²⁵. CMV-specific CD8⁺ T cell clones were isolated from CD62L⁺CD8⁺ and CD62L⁻CD8⁺ T cells
15 obtained by cell sorting of PBMC from each macaque, modified by retroviral gene transfer, expanded in vitro and infused intravenously at cell doses of 3-6 x 10⁸/kg. Blood samples were obtained by venipuncture at intervals after each infusion and bone marrow from the posterior iliac crest and a lymph node from the inguinal region were obtained under anaesthesia.

20 Accredited clinical laboratories performed CBC and serum chemistry on blood obtained before and at intervals after each T cell infusion. The macaques were followed for at least 7 weeks after each T cell infusion.

Cytokine flow cytometry for detection of CMV-specific CD8⁺ T cells. PBMC were isolated from peripheral blood by Ficoll-Hypaque gradient separation.
25 Multiparameter flow cytometry was used to detect CD8⁺ T cells in PBMC that expressed intracellular IFN-γ after stimulation with pools of 15-mer synthetic peptides with an 11 amino acid (aa) overlap that spanned the 558 aa sequence of the rhCMV IE1 protein (GenBank accession number: M93360), or with an IE-2 peptide kindly provided by Dr. L. Picker (Oregon Health Sciences University)²⁵. The peptides that
30 comprised the panel were synthesized using standard Fmoc chemistry (NMI) and arranged in an analytic grid composed of 24 pools, each containing 11-12 peptides. Aliquots of PBMC were stimulated for 6 hours at 37 °C with the peptide pools (5

5 $\mu\text{g/ml}$) or medium alone in the presence of 1 $\mu\text{g/ml}$ monoclonal anti-CD28 and anti-CD49d antibodies. Stimulation with PMA (10 ng/ml ; Sigma) and ionomycin (1 $\mu\text{g/ml}$; Sigma) was performed as a positive control. After two hours, Brefeldin A (10 $\mu\text{g/ml}$; Sigma) was added. The cells were first stained with phycoerythrin (PE)-labeled anti-CD8 β (Immunotech Coulter) and peridinin chlorophyll protein (Percep)-Cy5.5-labeled anti-CD4, then permeabilized using Cytofix/Cytoperm Permeabilization Solution (BD Biosciences (BD)), and stained with a fluorescein isothiocyanate (FITC)-labeled anti-IFN- γ antibody (BD). All analyses were performed on a FACSCalibur and the data analyzed using CellQuest Software (BD).

10 Immunogenic CMV peptides were identified by the responses of CD8 $^+$ T cells to intersecting peptides in the grid and the assays were repeated with each individual peptide and derivative 9-mer peptides to confirm and map specificity.

To determine if CD8 $^+$ T cells for CMV IE peptides were present in the T_{CM} and T_{EM} subsets, PBMC were stained with fluorochrome conjugated anti-CD8 and anti-CD62L monoclonal antibodies, and CD62L $^+$ CD8 $^+$ T cells and CD62L $^-$ CD8 $^+$ T cells were sorted using a Vantage BD cell sorter. The sorted cells were assayed by cytokine flow cytometry after stimulation with autologous B cells that were activated by culture on NIH 3T3 cells modified to express human CD40L, and then pulsed with individual IE peptides or with media alone. The CD40L-activated B cells were

15 cells were sorted using a Vantage BD cell sorter. The sorted cells were assayed by cytokine flow cytometry after stimulation with autologous B cells that were activated by culture on NIH 3T3 cells modified to express human CD40L, and then pulsed with individual IE peptides or with media alone. The CD40L-activated B cells were

20 cocultured with the purified CD62L $^+$ CD8 $^+$ and CD62L $^-$ CD8 $^+$ T cells for 6 hours prior to the addition of Brefeldin A for an additional 4 hours, followed by permeabilization and staining for intracellular IFN- γ .

Retroviral vectors. A truncated macaque CD19 gene encoding for the extracellular and transmembrane domain (ΔCD19) and four aa of the cytoplasmic tail to abrogate signaling, and the full length CD20 gene were amplified by RT-PCR from

25 cDNA generated from macaque PBMC and cloned into pcDNA3.1 vector (Invitrogen). The ΔCD19 and CD20 genes were subcloned into the retroviral plasmid pMP71GFP_{pre} (W. Uckert, Max-Delbrück-Center, Berlin, Germany) after removing the GFP gene⁴³. Retrovirus supernatant was produced in the packaging cell line

30 Phoenix Galv (G. Nolan, Stanford University, USA) after transfection using Fugene G according to the manufacturer's instruction (Roche Diagnostics).

Culture and genetic modification of CMV-specific CD8⁺ T cell clones for adoptive transfer. CD62L⁺CD8⁺ and CD62L⁻CD8⁺ T cell fractions were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% human AB serum, 25 μ M 2-mercaptoethanol, and 4 mM L-glutamine (T-cell media), and co-cultured with autologous monocytes pulsed with CMV IE-1 or IE-2 peptides (1 μ g/ml). IL-2 (10 U/ml; Chiron Corporation) was added on day 3 of the culture. On day 7, T cell clones were generated by plating 0.3 T cells per well in 96-well round bottom plates with 1×10^5 γ -irradiated autologous PBMC pulsed with CMV IE-1 or IE-2 peptides (0.5 μ g/ml) and 1×10^4 γ -irradiated B-lymphoblastoid cells (LCL) as feeder cells in the presence of 50 U/ml IL-2.

After 14 days, an aliquot from cloning wells with visible growth was tested in a chromium release assay for recognition of autologous target cells either pulsed with CMV peptide or with medium alone. T cell clones that only lysed peptide pulsed target cells were expanded by stimulation with monoclonal anti-CD3 and anti-CD28 antibodies in the presence of human γ -irradiated PBMC and EBV transformed LCL, and IL-2 (50 U/ml) as described^{44, 45}. On day 2 after stimulation, the cells were pelleted, resuspended in Δ CD19 or CD20 retroviral supernatant with IL-2 (50 U/ml) and polybrene (5 μ g/ml), centrifuged at 1000 g for 1 hour at 32 °C, and incubated overnight. The cells were then washed and cultured in CTL medium containing IL-2. On day 6 after retroviral transduction, T cells that expressed the Δ CD19 or CD20 transgene were selected with immunomagnetic beads. Briefly, the transduced T cells were incubated with monoclonal anti-CD19 (Immunotech Coulter) or anti-CD20 antibody (BD), washed, incubated with rat anti-mouse IgG-coupled magnetic beads (Miltenyi Biotec), and selected using the MidiMACS device. The selected cells were then cultured for an additional 6 days and cryopreserved in aliquots that could be thawed subsequently for adoptive transfer experiments. The clonality of the infused T cell clones was confirmed by analysis of TCR V β gene rearrangements.

To assess cell viability in vitro, aliquots of the T cell clones at the end of the 14-day stimulation cycle and immediately prior to adoptive transfer were plated in 12-well tissue culture plates at $2-4 \times 10^6$ cells/well in T-cell media alone, media 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).

The viability of T cells was assessed every 3-4 days by trypan blue dye exclusion. In some experiments, aliquots of the T cell clones were cultured with media containing IL19 21 (30 ng/ml) (R&D) or cocultured with autologous PBMC and mature monocytederived dendritic cells⁴⁶.

5 **Generation of T-APC.** To generate T-APC, we expanded aliquots of autologous polyclonal T cells by stimulation with anti-CD3 and anti-CD28 monoclonal antibodies and IL-2 (50 U/ml). At the end of a 14 day stimulation cycle, the T cells were harvested, pulsed for 30 min with 1 µg/ml of the cognate CMV IE peptide, washed twice in media, suspended in normal saline and administered to the
10 animal by intravenous infusion at a cell dose of 1×10^7 /kg. An aliquot of the autologous T cells before and after pulsing with the CMV IE peptide were labeled with ⁵¹Cr and tested as targets for the CMV-specific T cell clone.

Cytotoxicity assays. Cytotoxic responses of CMV-specific T cells were examined as described^{44,45,47}. Briefly, autologous ⁵¹Cr-labeled T cells were pulsed
15 overnight with peptide antigen at various concentrations or medium alone and used in a chromium release assay for recognition by CD8⁺ T cell clones.

Flow cytometry. PBMC and T cell clones were analyzed by flow cytometry after staining with fluorochrome-conjugated monoclonal anti-CD3 (SP34), CD4, CD8, CD27, CD28, CD45RA, CD62L, CCR7, CD122 (IL2-Rβ), CD132 (IL2-Rγ)
20 (BD), CD19, CD8β, CD45RO, and CD127 (Immunotech Coulter), and IL15Rα (R&D) antibodies. For intracellular staining, cells were suspended in CYTOFIX/CYTOPERM™ Permeabilization Solution (BD) according to the manufacturer's instruction and stained with monoclonal antibodies to granzyme B (BD), perforin (Kamiya Biomedical), bcl-2 (BD), and bcl-xl (Southern Biotech).
25 Isotype-matched irrelevant control monoclonal antibodies served as controls (BD). In some experiments, samples of PBMC were labeled with anti-CD8-FITC and anti-CD19 allophycocyanin (APC) and stained with Annexin V-PE and PI according to the manufacturer's instruction. All analyses were performed on a FACSCalibur and the data analyzed using CellQuest Software (BD).

30 **CFSE-labeling of T cells.** CD19⁺CD62L⁺CD8⁺ and CD19⁺CD62L⁺CD8⁺ T cells were flow sorted from pooled PBMC samples obtained after adoptive transfer and labeled with 0.625 µM CFSE (Molecular Probes) in PBS for 10 minutes at 37 °C

under constant agitation, followed by blocking with 20% FCS medium. CFSE-labeled T cells were plated at 1×10^5 cells/well in a 96-well plate with autologous CD40L activated B cells (2.5×10^4 cells/well) pulsed with 200 ng/ml CMV IE peptide. CFSE dilution was analyzed by flow cytometry after five days.

5 **Fluorescent Probe PCR.** PCR amplifications and analyses were performed using a quantitative real-time PCR assay (Perkin-Elmer Applied Biosystems)^{45, 48}. DNA (0.3-1 μ g) was amplified in duplicate using PCR primers and TaqMan probes (Synthegen) designed to detect a unique MP71CD20_{pre} sequence using a fluorescent-tagged probe encompassing the junction of the CD20 gene and the retroviral vector
10 pMP71_{pre}.

Standards consisted of DNA derived from the infused CD20+ T cells. Aliquots of preinfusion PBMC served as negative control.

Telomere length analysis. The average length of telomere repeats in individual lymphocytes was measured by automated flow-FISH as described^{49, 50}.
15 Cells, which were previously frozen in 10% (v/v) DMSO 20% (v/v) human serum, were thawed and processed in microtiter plates for automated flow-FISH. Cells were hybridized with or without 0.3 μ g/ml telomere specific FITC-conjugated (CCCTAA)₃ PNA probe, washed and counterstained with 0.01 μ g/ml LDS 751 (Exciton Chemical). To convert the fluorescence measured in sample cells hybridized with the
20 FITC-labeled telomere PNA probe into kilobases (kb) of telomere repeats, fixed bovine thymocytes with known telomere length as an internal control were processed simultaneously with each sample⁵⁰. FITC-labeled fluorescent beads were used to correct for daily shifts in the linearity of the flow cytometer and fluctuations in the laser intensity and alignment. Flow cytometric data collection was performed on a
25 FACSCaliburTM apparatus and the data analysis was performed using a CellQuestProTM data analysis system (BD).

RESULTS

Characterization of Cytomegalovirus (CMV)-specific CD8⁺ T cell clones
30 **derived from CD62L⁺ T_{CM} and CD62L⁻ T_{EM} subsets.** Immunocompetent *Macaca nemestrina* with latent CMV infection were used in this study. To identify CMV-specific CD8⁺ T cells, we stimulated aliquots of peripheral blood mononuclear cells

(PBMC) from four macaques with rhesus CMV immediate early (IE)-1 or IE-2 peptides and analyzed interferon-gamma (IFN- γ) production by flow cytometry²⁵. After identification of an immunogenic CMV epitope for each macaque, we evaluated whether specific CD8⁺ T cells were present in CD62L⁺ and CD62L⁻ T cell fractions, purified from PBMC and containing T_{CM} and T_{EM} respectively. Stimulation of CD62L⁺ and CD62L⁻ T cells with CMV peptides identified CD8⁺ T cells that produced IFN- γ in both subsets (**Figure 1a**). The majority of CD62L⁺ T cells were positive for CCR7 and CD28, and expressed absent or low levels of granzyme B. The CD62L⁻ T cells were mostly negative for CD28 and CCR7, and positive for granzyme B (not shown).

We next generated CD8⁺ CMV-specific T cell clones for adoptive transfer from purified CD62L⁺CD8⁺ (T_{CM}) and CD62L⁻CD8⁺ (T_{EM}) cells of three macaques (**Figure 1b**). Cytolytic CD8⁺ CMV-specific T cell clones were obtained from both T_{CM} subsets in all macaques. The cloning efficiency was 13.6-23.2% for cultures initiated from T_{CM}, and 0.4-10.4% for cultures initiated from T_{EM}. Individual T cell clones were transduced with a retroviral vector encoding either a truncated cell surface CD19 (Δ CD19) or full-length CD20 molecule to permit tracking the cells in vivo, and transduced T cells were selected with immunomagnetic beads (**Figures 1c, d**). A pair of T_{CM} and T_{EM}-derived clones was randomly selected from each of the three macaques, and expanded in culture to $\geq 5 \times 10^9$ cells over a total culture duration of 49 days before adoptive transfer. Independent of their derivation from CD62L⁺ or CD62L⁻ cells, all of the CMV-specific T cell clones had differentiated to T_E and were negative for CD62L, CCR7, CD28, and CD127, and positive for granzyme B and perforin (**Figure 2a**). The T cell clones of each pair recognized the same CMV peptide, had comparable avidity, displayed nearly identical growth after T cell receptor (TCR) stimulation, and had similar telomere lengths (**Figures 2b-d**).

In vivo persistence of CD8⁺ T_E clones derived from CD62L⁺ T_{CM} or CD62L⁻ T_{EM}. We administered the autologous gene-modified T cells intravenously and measured their frequency in the blood, lymph node, and bone marrow at intervals after infusion. A Δ CD19-modified T cell clone derived from T_{EM} was transferred to macaque 02269 at a dose of 3×10^8 T cells/kg, which is approximately 5-10% of the macaque total body lymphocyte pool²⁶. CD19⁺CD8⁺ T cells were easily detected in

the blood one day after the infusion at a frequency of 1.2% of CD8⁺ cells or 10 cells/ μ l of blood. The CD19⁺ T cells in the blood peaked at 3.7% of CD8⁺ T cells (40 cells/ μ l) on day three after the infusion. However, the T cells were not detected in blood obtained at day five or multiple times up to forty-two days after infusion, and were not present in bone marrow or lymph node samples obtained fourteen days after the infusion (**Figure 3a**). We transferred the same dose of a Δ CD19-modified CMV-specific T cell clone derived from the T_{CM} subset to this macaque. On day one post infusion, the frequency of CD19⁺CD8⁺ T cells in the blood was 2.2% of CD8⁺ T cells (10 cells/ μ l). In contrast to the T_{EM}-derived clone, the T_{CM}-derived cells persisted in the blood for greater than fifty-six days after infusion at ~0.2% of CD8⁺ T cells (3-6 cells/ μ l), and comprised 1.2% and 0.6% of CD8⁺ T cells in bone marrow and lymph node samples respectively, obtained fourteen days after the infusion (**Figure 3b**). In a second macaque, we infused a Δ CD19-modified T_{CM}-derived clone first at a higher cell dose of 6×10^8 /kg. With this dose, transferred cells were detected in the blood at a frequency of 26.3% of CD8⁺ T cells (228 cells/ μ l) on day one and 46.3% of CD8⁺ T cells (734 cells/ μ l) on day three. The transferred T_{CM}-derived clone was present in the bone marrow and lymph node obtained on day fourteen 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 twenty-eight days to a stable level of 7-10 cells/ μ l that persisted for greater than eleven months after infusion (**Figure 3c**). We administered the same dose of a T_{EM}-derived clone transduced to express CD20 to enable the cells to be tracked and distinguished from the previously transferred Δ CD19-modified T cells. The frequency of CD20⁺CD8⁺ T cells in the blood one day after the infusion was 16.3% of CD8⁺ T cells (103 cells/ μ l), but the transferred cells disappeared from the blood by day five and were not detected in the bone marrow and lymph node obtained fourteen days after the infusion (**Figure 3d**). The disappearance of CD20-modified T cells was confirmed by PCR for vector sequences on DNA isolated from peripheral blood, bone marrow and lymph node mononuclear cells obtained on day 14 (data not shown).

CD8⁺ T_E cells derived from T_{EM} undergo rapid apoptosis after adoptive transfer and respond poorly to IL-15. The failure of T_E clones derived from T_{EM} to persist in blood, marrow or lymph nodes after adoptive transfer could be due to cell death in vivo and/or migration to other tissue sites. T_{EM}-derived clones expressed

lower levels of the anti-apoptotic proteins bclxl and bcl-2 than T_{CM}-derived clones, suggesting they may be more susceptible to apoptosis (**Figure 4a**). Therefore, we infused a ΔCD19-modified T_{EM}-derived T cell clone to the third macaque and measured the proportion of transferred cells in PBMC that were positive for propidium iodide (PI) and Annexin V binding²⁷. Approximately 40% of the CD19⁺CD8⁺ T cells in PBMC were PI and/or Annexin V positive when analyzed directly on day one, and 45% were positive after culturing the PBMC for 24 hours (**Figure 4b**).

Consistent with the prior animals, the T_{EM}-derived clone was only detected in the blood for three days, and was not present in the day fourteen bone marrow or lymph node samples (**Figure 4c**). We then infused a ΔCD19-modified T_{CM}-derived T cell clone into the same animal and analyzed the proportion of apoptotic cells. Less than 20% of the transferred T_{CM} cells were PI and/or Annexin V positive in PBMC analyzed directly, and this fraction decreased to 12% after 24 hours of culture (**Figure 4b**). The T_{CM}-derived clone migrated to the bone marrow and lymph nodes (**Figure 4c**), and persisted in the blood for greater than seventy-seven days as observed previously (data not shown). We reasoned that the selective survival of T_{CM}-derived T cells in vivo might be due to responsiveness to homeostatic cytokines such as IL-15 that maintain endogenous CD8⁺ memory T cells²⁸⁻³⁰, and cultured aliquots of the T_{CM} and T_{EM}-derived clones in media containing low doses of IL-15, IL-2, or IL-7. Both T_{EM} and T_{CM}-derived T_{EE} clones lacked expression of IL-7Rα (**Figure 2a**) and died rapidly in vitro when cultured in IL-7 or in media alone (**Figure 4d**). Culture in IL-15 or IL-2 also did not improve the viability of T_{EM}-derived clones (**Figure 4d**). By contrast, T_{CM}-derived clones were rescued from cell death for over 30 days in IL-15 and exhibited improved survival in IL-2 (**Figure 4d**). The responsiveness of T_{CM}-derived clones to IL-15 correlated with higher levels of cell surface IL-15Rα, IL-2Rβ®, and IL-2Rγ compared with T_{EM}-derived clones (**Figure 4e**).

Adoptively transferred CD8⁺ T_E clones derived from T_{CM} acquire a memory phenotype in vivo. The migration of T_{CM}-derived CD8⁺ T_E clones to lymph nodes suggested that CD62L might be re-expressed on transferred cells in vivo. CD62L was not detected on the T cell clones at the time of adoptive transfer (**Figure 2a**), or after culture in the absence of antigen stimulation with cytokines (IL-2, IL-7, IL-15, IL-21),

autologous PBMC, or monocyte-derived dendritic cells (data not shown). However, in all three macaques, CD19⁺CD62L⁺CD8⁺ cells were observed in the blood as early as three days after the infusion of the T_{CM}-derived clones and were detected in the blood, bone marrow and lymph node samples obtained on day 14 (**Figure 5a, b**). The proportion of transferred T cells that were CD62L⁺ in the lymph node was greater than in blood or marrow (**Figure 5b**).

CD62L was not expressed during the brief period the T_{EM}-derived clone persisted in the blood in any of the three macaques (**Figure 5a**). We next examined whether the T cells that persisted in vivo after adoptive transfer acquired other phenotypic markers of memory T cells that were absent on the infused T_E. Fourteen days after cell transfer, a subset of the CD19⁺CD8⁺ T cells in blood and lymph node expressed CCR7, CD28, and CD127 (**Figures 5c-e**). We repeated this analysis on blood, bone marrow, and lymph node samples obtained two months after the infusion of the T_{CM}-derived clone in macaque 02258. At this later time, the infused CD19⁺ T cells comprised 1.4% of the CD8⁺ T cells in the lymph node and these T cells were uniformly positive for T_{CM} markers including CD62L, CCR7, CD28, and CD127 (**Figures 5b-e**). Transferred T cells were present at lower levels in the blood and bone marrow, and contained both CD62L⁺ and CD62L⁻ fractions. The transferred CD19⁺ T cells that persisted long-term in the blood and expressed CD62L, also expressed CCR7, CD28 and CD127 (**not shown**). Thus, despite differentiation to T_E during expansion of a single T_{CM} cell to more than 5 x 10⁹ cells, a significant fraction of the transferred T cell clones were able to persist long-term, compete with endogenous T cells for anatomical niches of T_{CM}, and re-express phenotypic markers of T_{CM}.

Memory T cells established by adoptive transfer exhibit functional properties of both T_{CM} and T_{EM}. An objective of T cell immunotherapy is to establish a stable pool of memory T cells that can respond to antigen by producing cytokines and differentiating into cytolytic effector cells. The transferred CD19⁺ T_{CM}-derived cells that persisted in the blood produced IFN-γ after peptide stimulation and their frequency was comparable to endogenous CD19⁻ CMV-specific CD8⁺ T cells (**Figure 6a**). The cytolytic function of these T cells was examined by sorting T cells from the blood into CD19⁺CD62L⁺CD8⁺ and CD19⁺CD62L⁻CD8⁺ fractions. For these experiments, it was necessary to pool PBMC samples from multiple time points to

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obtain sufficient cells for the assays. The CD19⁺CD62L⁻T cells demonstrated cytolytic activity nearly equivalent to that of the T cell clone and expressed granzyme B, while the CD19⁺CD62L⁺ T cells lacked direct cytolytic activity and had low granzyme B expression (**Figure 6b**). In vitro stimulation of the CD19⁺CD62L⁺ T cells with anti-CD3 and anti-CD28 monoclonal antibodies generated T_E cells with cytolytic activity, demonstrating these cells could be re-induced to differentiate to cytolytic T_E (**Figure 6b**). We also labeled the sorted CD19⁺CD62L⁺ and CD19⁺CD62L⁻ T cell fractions with CFSE and monitored proliferation five days after antigen stimulation in vitro. Seventy-four percent of CD62L⁺ cells underwent 5 or more cell divisions, but only 13% of CD62L⁻ cells underwent 5 or more divisions (**Figure 6c**). Thus, the adoptive transfer of a T_E clone derived from the T_{CM} pool established distinct populations of cells in vivo with the functional properties of T_{CM} and T_{EM}.

We have previously shown in human studies that the infusion of T cells that express a foreign antigen can prime and boost endogenous antigen-specific memory T cell responses³¹. Thus, to determine if T_M established by adoptive transfer could respond to antigen stimulation in vivo, we infused a small dose (1 x 10⁷/kg) of autologous T cells pulsed with CMV IE peptide (T-APC) into macaque A99171 two months after administering the ΔCD19-modified T_{CM}-derived T cells. The peptide pulsed T-APC were efficiently lysed by the CMV-specific T cell clone in vitro (**Figure 6e**). Within 7 days of the infusion of T-APC, there was a 4 to 5-fold increase in the absolute numbers of both ΔCD19-modified and endogenous IE-specific T cells in the blood (**Figure 6d**). Thus, the memory T cells established by adoptive transfer were as capable as endogenous memory T cells of expanding in response to antigen in vivo.

DISCUSSION

Poor survival of adoptively transferred T cells that target infected or cancerous cells has correlated with lack of therapeutic efficacy in clinical trials^{14,16,16}. The persistence and efficacy of cultured T cells can sometimes be improved by depletion of host lymphocytes prior to cell transfer to eliminate regulatory cells and competition for pro-survival cytokines, and by the administration of IL-2 after cell transfer^{16,17}. However, these interventions are not uniformly successful in improving cell persistence suggesting that intrinsic properties of T cells isolated for adoptive therapy

may be critical for establishing durable immunity. Here, we used gene marking to show that antigen-specific CD8⁺ T_E clones derived in vitro from the T_{CM} but not from the T_{EM} subset, persist longterm in vivo, occupy memory T cell niches, and reacquire phenotypic and functional properties of T_{CM} after adoptive transfer.

5 After encountering antigen in vivo, T cells undergo proliferation and programmed differentiation evoked by signals from the TCR, co-stimulatory and adhesion molecules, and cytokine receptors^{18,20,32}. These events result in the generation of large numbers of T_E that die as antigen is cleared, and a smaller pool of phenotypically distinct T_{CM} and T_{EM} that persist for life and respond to antigen re-
10 exposure by differentiating into T_E. Data from both murine and human studies support a linear differentiation model in which T_{CM} give rise to both T_{EM} and T_E, although it remains possible that T_{CM} and T_{EM} represent separate lineages^{18,33-35}. The capacity of T cell memory to be maintained for life suggests that at least some T_M must be capable of both self-renewal and differentiation²³.

15 The results presented here suggest that isolating T_{CM} for gene insertion may provide a tumor-reactive T cell population with an improved capacity to persist after adoptive transfer.

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The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents

10 of the claims to be included therein.

That which is claimed is:

1. A CD8⁺ cytotoxic T lymphocyte (CTL) preparation, wherein the CTL preparation is a primate CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells, wherein the CD8⁺ T_E cells of the CTL preparation comprise an introduced polynucleotide encoding a receptor that specifically binds to a target on a cell, and wherein the 60% or more CD8⁺ T_E cells have:
 - (a) decreased expression of CD62L as compared to CD8⁺ central memory T (T_{CM}) cells, and
 - (b) improved survival and/or expansion for at least 10 days when cultured *in vitro* in the presence of IL-15 in the absence of antigen stimulation as compared to CD8⁺ effector memory T (T_{EM}) cells.
2. The CD8⁺ CTL preparation according to claim 1, wherein the CD8⁺ T_E cells of the CTL preparation are positive for granzyme B, perforin or both.
3. The CD8⁺ CTL preparation of claim 1 or 2, wherein the CTL preparation comprises 70%, 80%, 85% or 90% or more of the primate CD8⁺ T_E cells.
4. The CD8⁺ CTL preparation according to any one of claims 1-3, wherein the CTL preparation consists essentially of the primate CD8⁺ T_E cells.
5. The CD8⁺ CTL preparation according to any one of claims 1-4, wherein the CD8⁺ T_E cells of the CTL preparation have improved survival and/or expansion for at least 30 days when cultured *in vitro* in the presence of IL-15.

6. The CD8⁺ CTL preparation according to any one of claims 1-5, wherein the CD8⁺ effector T (T_E) cells persist *in vivo* upon adoptive transfer to a subject for at least 5 days, at least 7 days, at least 10 days, at least 14 days, at least 21 days, at least 28 days, at least 42 days, at least 56 days, at least 70 days, at least 77 days, at least 215 days, or at least 344 days.
7. The CD8⁺ CTL preparation according to any one of claims 1-6, wherein the receptor that specifically binds to the target on the cell is an engineered immunoreceptor.
8. The CD8⁺ CTL preparation according to any one of claims 1-7, wherein the receptor that specifically binds to the target on the cell specifically binds to pathogenic cells.
9. The CD8⁺ CTL preparation according to any one of claims 1-8, further comprising IL-15.
10. The CD8⁺ CTL preparation according to any one of claims 1-9, wherein the primate is a human.
11. Use of the CD8⁺ CTL preparation according to any one of claims 1-10 for the preparation of a medicament for use in adoptive immunotherapy in a human subject.
12. Use of the CD8⁺ CTL preparation according to any one of claims 1-10 for use in adoptive immunotherapy in a human subject.
13. The use according to claim 11 or 12, wherein the CD8⁺ CTL preparation is allogeneic to the subject, syngeneic to the subject, or autologous to the subject.
14. The use according to any one of claims 11-13, wherein the adoptive immunotherapy comprises concurrent administration of IL-15 to the subject in an amount effective to increase cell proliferation of the CD8⁺ CTL preparation in the subject.

15. The use according to any one of claims 11-14, wherein the adoptive immunotherapy is for treating cancer.
16. The use according to claim 15, wherein the cancer is selected from colon cancer, lung cancer, liver cancer, breast cancer, prostate cancer, ovarian cancer, skin cancer, bone cancer, brain cancer, breast cancer, squamous cell carcinoma, melanoma, leukemia, or myeloma.
17. The use according to any one of claims 11-14, wherein the adoptive immunotherapy is for treating an infectious disease.
18. The use according to claim 17, wherein the infectious disease is selected from a viral infection, bacterial infection, or protozoal infection.
19. The use according to any one of claims 11-18, wherein the subject is immunodeficient.
20. A method for producing a CD8⁺ cytotoxic T lymphocyte (CTL) preparation useful for adoptive immunotherapy, comprising the steps of:
 - (a) enriching a subpopulation of CD62L⁺ CD8⁺ central memory T (T_{CM}) lymphocytes from a primate subject's T lymphocyte population;
 - (b) introducing, into cells of the T_{CM}-enriched subpopulation, a polynucleotide encoding a receptor that specifically binds to a target on a cell; and
 - (c) expanding the T_{CM}-enriched subpopulation *in vitro* to produce a CD8⁺ CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells that have decreased expression of CD62L as compared to CD8⁺ T_{CM} cells.
21. The method according to claim 20, wherein the enriching step is carried out by contacting the primate subject's T lymphocyte population with an anti-CD62L antibody.

22. The method according to claim 21, wherein the anti-CD62L antibody is immobilized on a solid support.
23. The method according to any one of claims 20-22, wherein the T_{CM}-enriched subpopulation comprises at least 70% CD62L⁺ CD8⁺ cells.
24. The method according to any one of claims 20-23, wherein the 60% or more CD8⁺ effector T (T_E) cells are positive for granzyme B, perforin or both.
25. The method according to any one of claims 20-24, wherein the receptor that specifically binds to the target on the cell is an engineered immunoreceptor.
26. The method according to any one of claims 20-25, wherein the receptor that specifically binds to the target on the cell specifically binds to pathogenic cells.
27. The method according to any one of claims 20-26, wherein the expanding step comprises expanding the CD8⁺ T_{CM}-enriched subpopulation in the presence of an anti-CD3 antibody.
28. The method according to any one of claims 20-27, wherein the expanding step comprises expanding the CD8⁺ T_{CM}-enriched subpopulation in the presence of IL-2 and/or IL-15.
29. The method according to any one of claims 20-28, wherein the CD8⁺ CTL preparation is for allogeneic, syngeneic, or autologous use.
30. The method according to any one of claims 20-29, wherein the primate is a human.

31. A CD8⁺ cytotoxic T lymphocyte (CTL) preparation produced by the method of any one of claims 20-30, wherein the CTL preparation is a primate CTL preparation comprising 60% or more CD8⁺ effector T (T_E) cells, wherein the CD8⁺ T_E cells of the CTL preparation comprise an introduced polynucleotide encoding a receptor that specifically binds to a target on a cell, and wherein the 60% or more CD8⁺ T_E cells have:

- (a) decreased expression of CD62L as compared to CD8⁺ central memory T (T_{CM}) cells, and
- (b) improved survival and/or expansion for at least 10 days when cultured *in vitro* in the presence of IL-15 in the absence of antigen stimulation as compared to CD8⁺ effector memory T (T_{EM}) cells.

32. The CD8⁺ CTL preparation according to any one of claims 1-10 and 31, wherein the CTL preparation is formulated in a pharmaceutically acceptable carrier.

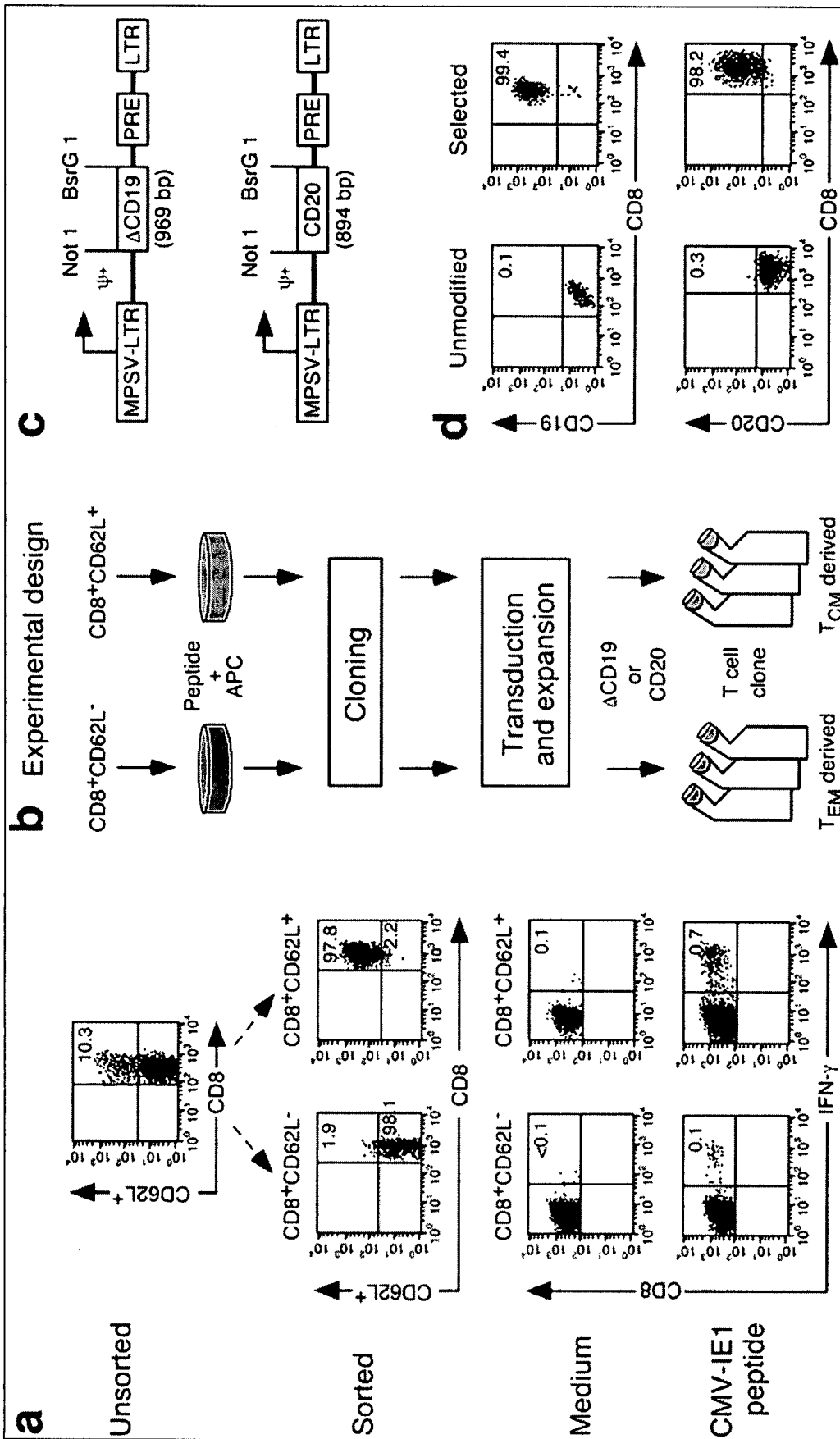


Figure 1

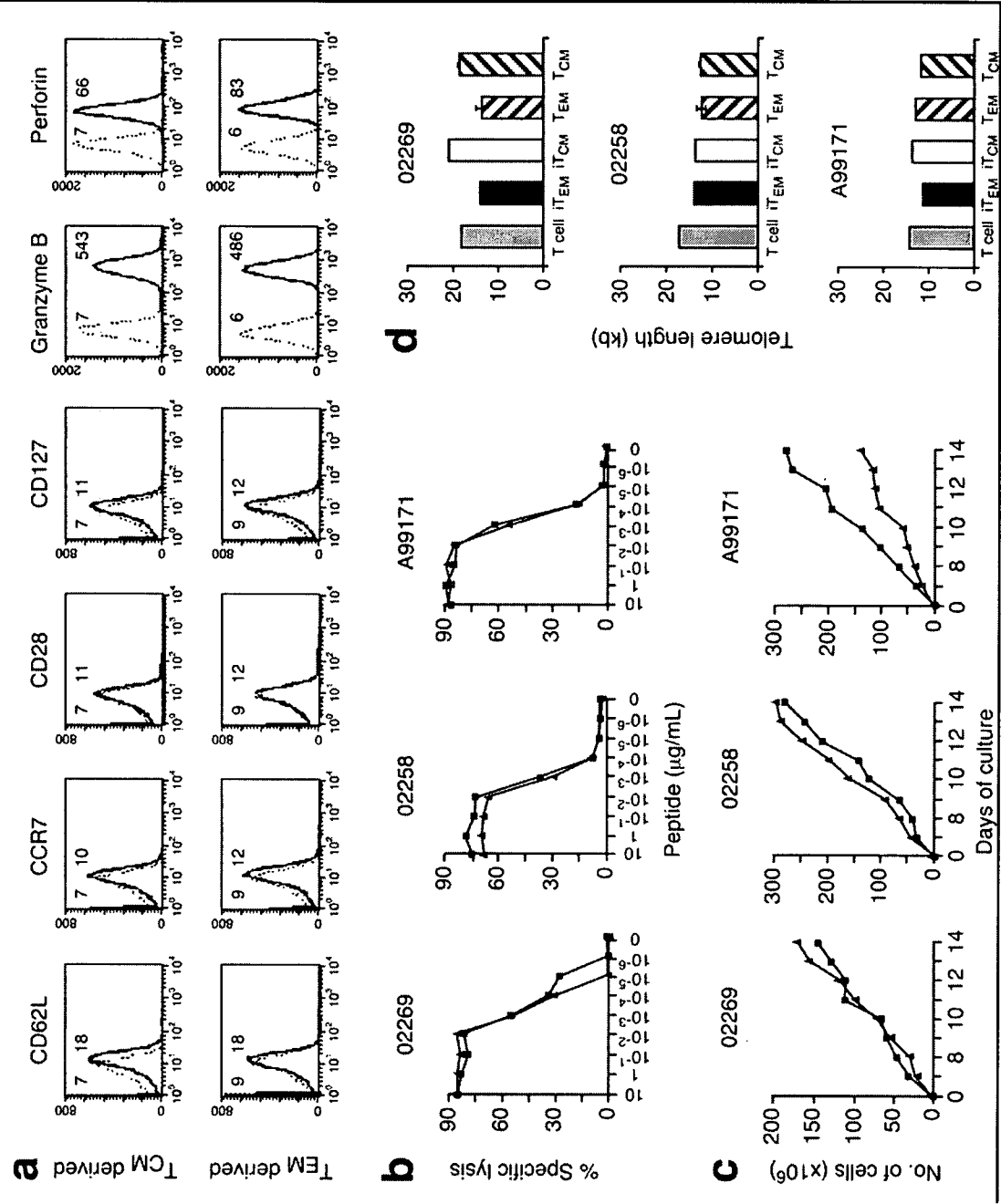


Figure 2

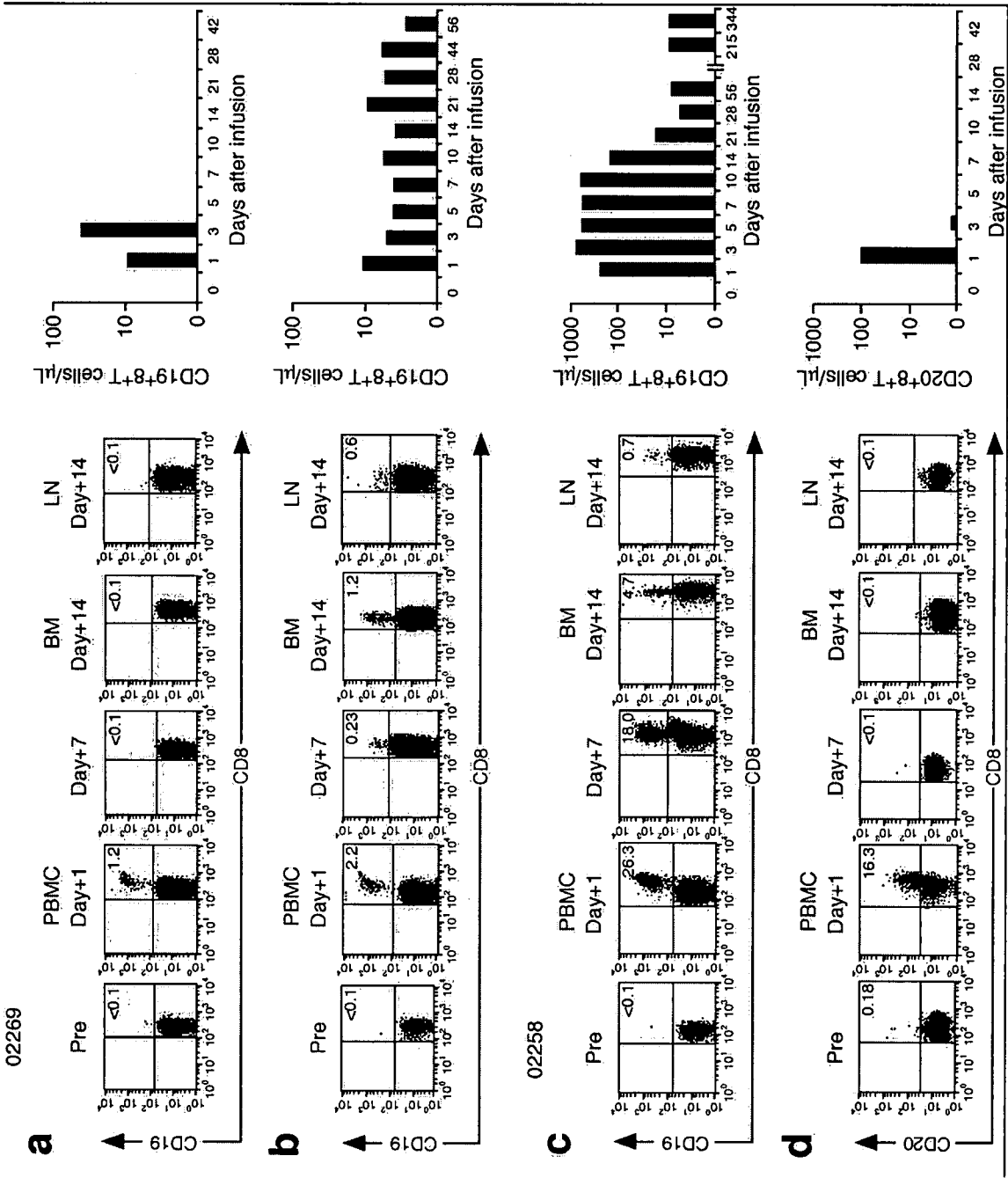
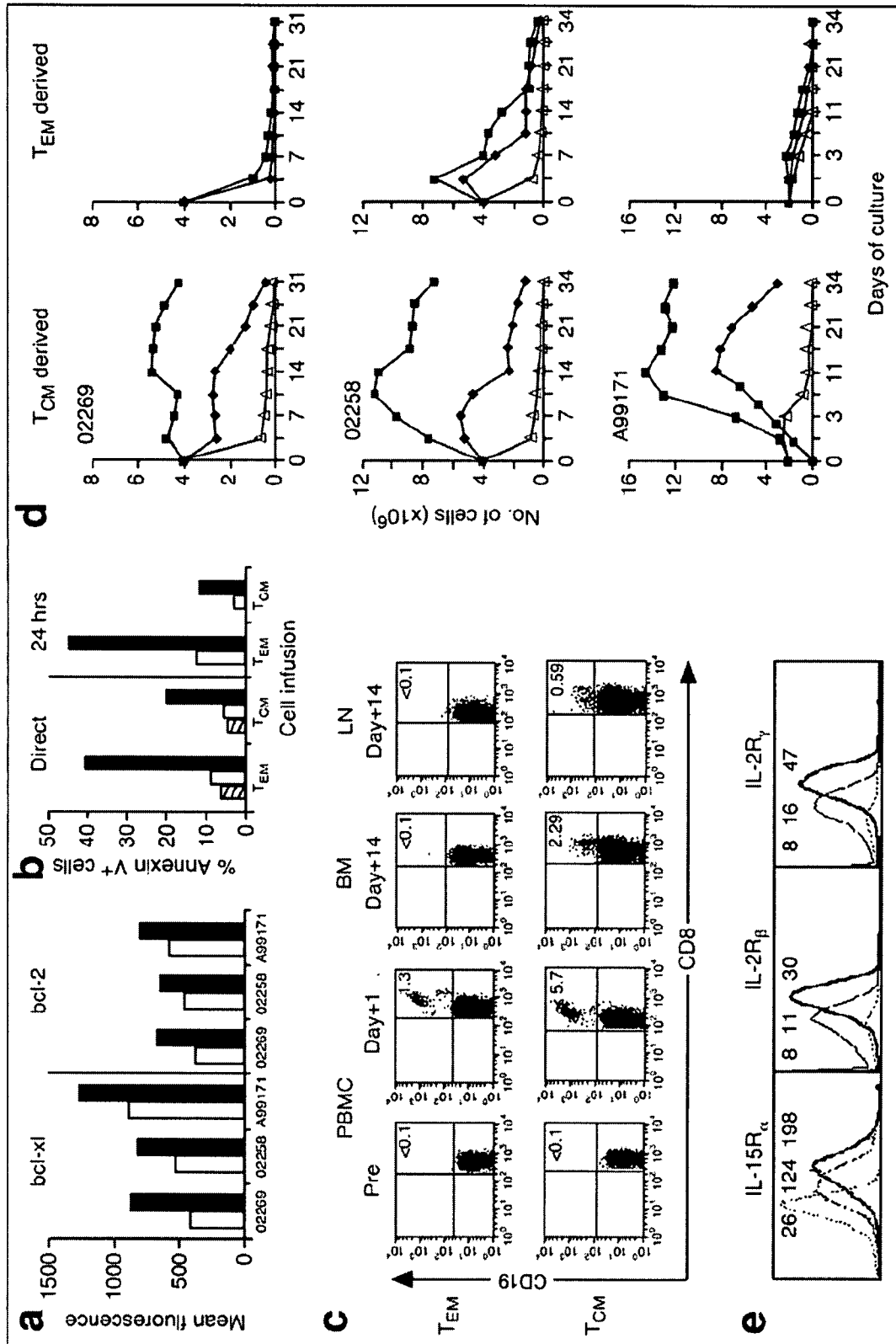
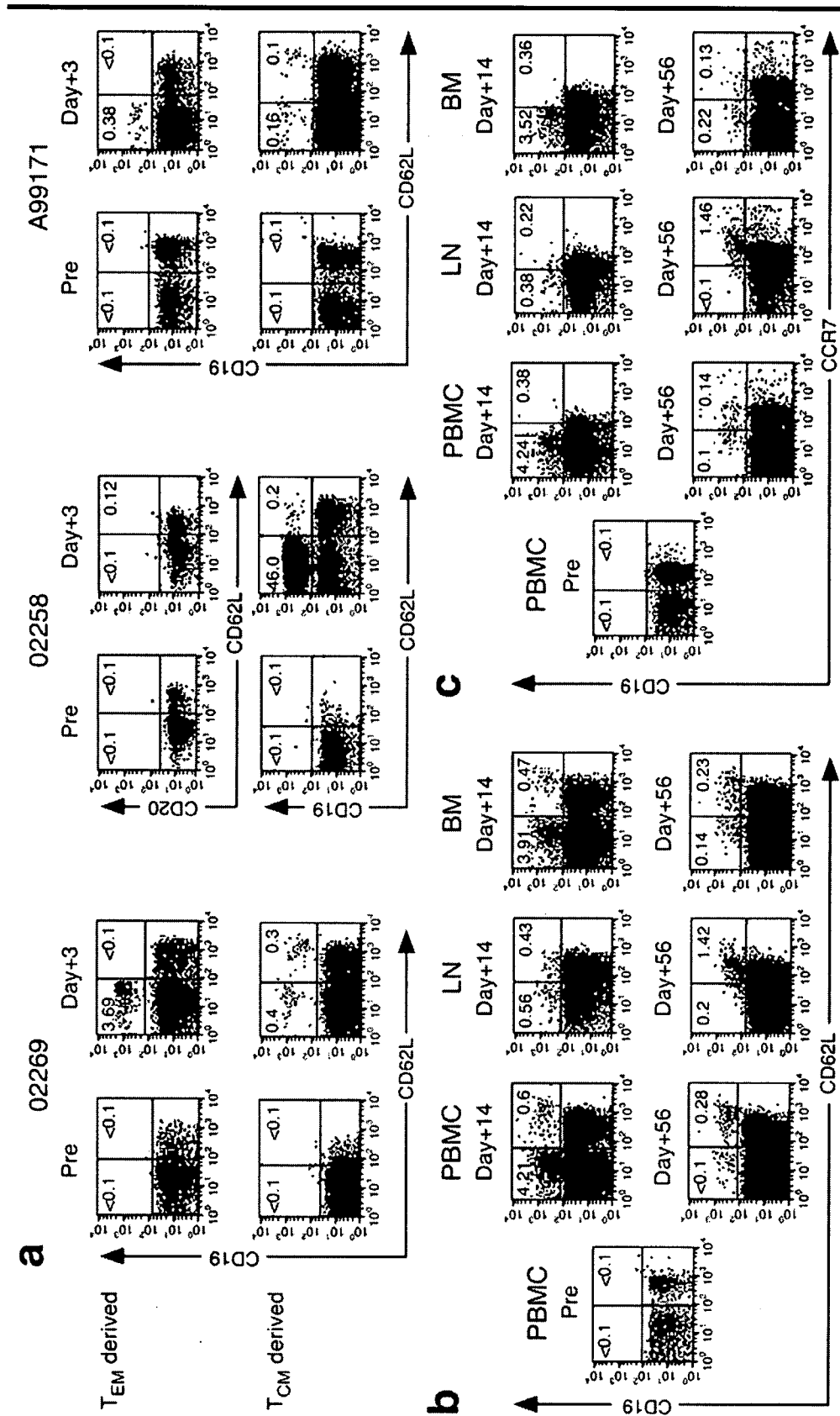


Figure 3





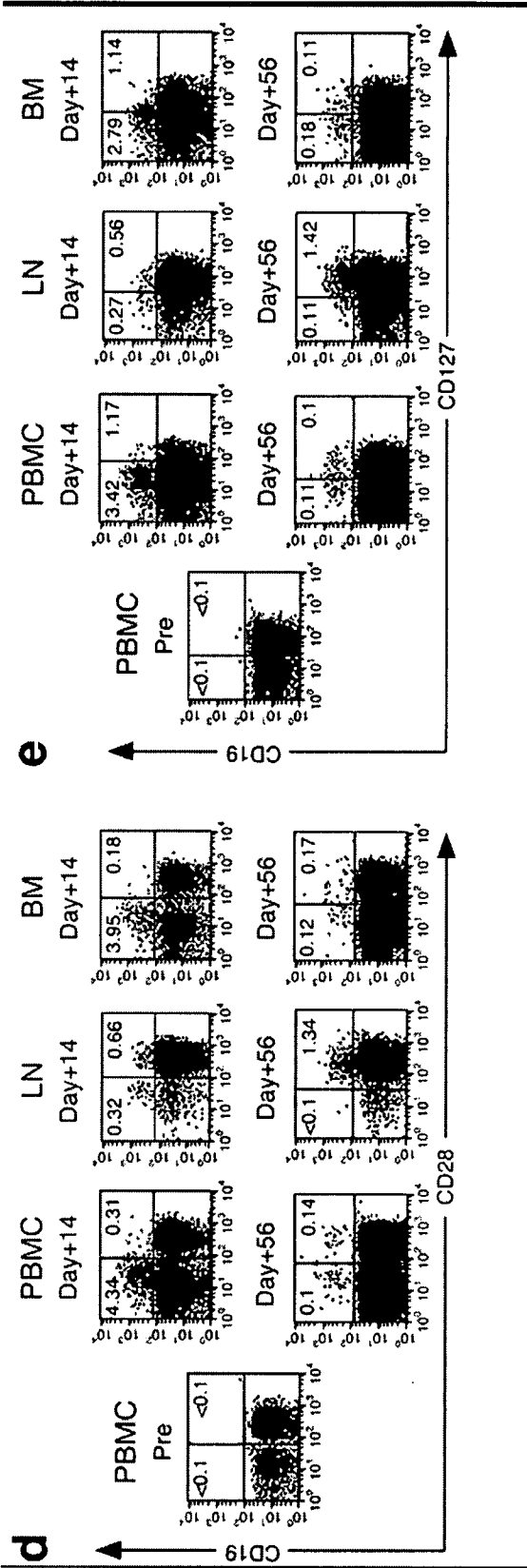


Figure 5 (2 of 2)

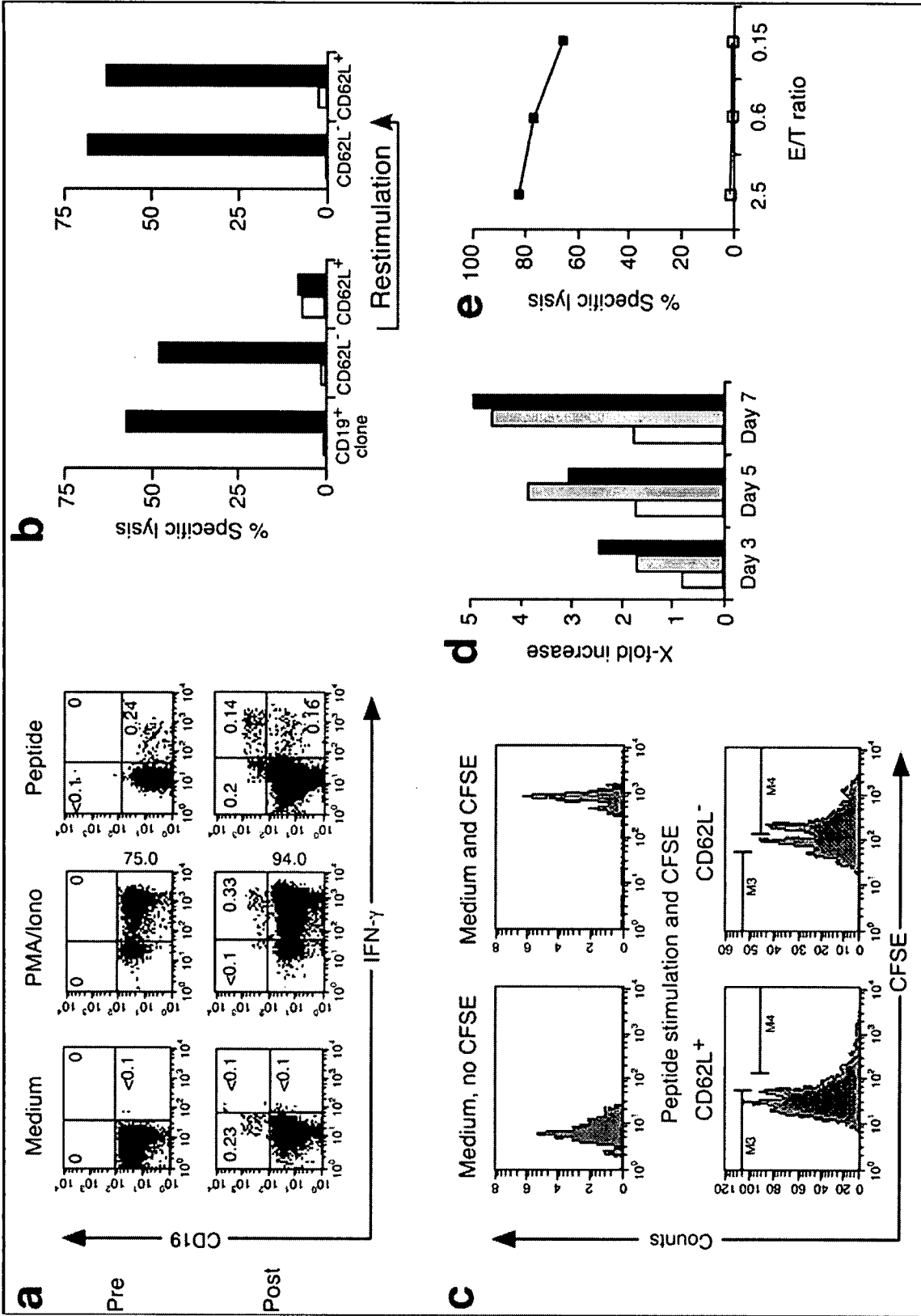


Figure 6