The Road to Memory: An Early Rest for the Long Journey

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Central memory T lymphocytes were reported to develop after acute but not chronic infection, which prompted this feasibility study on generating long-term CD8 T cells ex vivo, by applying a culture condition that simulates an acute infection. During 35 d of culture, naïve T cells (CD45RA\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{+}\), CXCR3\(^{+}\)) first developed into effector T cells (CD45RA\(^{-}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{-}\), CXCR3\(^{-}\)), followed by three intermediate stages: intermediate T cells 1 (CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{-}\), CXCR3\(^{-}\)), intermediate T cells 2 (CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{+}\), CD62L\(^{-}\), CXCR3\(^{-}\)), and intermediate T cells 3 (CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{+}\), CD62L\(^{+}\), CXCR3\(^{-}\)) before reverting to stable CD45RA\(^{+}\) central memory T cells (CD45RA\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{-}\), CXCR3\(^{-}\)). If both anti-CD3 and the inflammatory milieu persisted beyond day 10, intermediate T cells 2 gradually developed into effector memory T cells (CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{-}\), CXCR3\(^{-}\)). Furthermore, intermediate T cells 2 or effector memory T cells, when cultured in persistent inflammatory cytokines devoid of anti-CD3, were converted to central memory T cells (CD45RO\(^{+}\), CCR7\(^{-}\), CD62L\(^{-}\)). Overall, these results support ex vivo memory-like T lymphocyte production and favor a developmental pathway including both divergent and linear relationships.

Understanding human T cell development is important for generating different T subsets and designing efficacious therapy in various clinical conditions, including cancer, chronic infections, and autoimmunity. This topic is still being debated after considerable research and has not been entirely resolved. In many previous studies, investigators had taken serial snapshots of T cell development during in vivo infections and constructed various differentiation models, such as (1) the divergent pathway of naïve T cells (T naïve) \(\rightarrow\) effector T cells (T eff) \(\rightarrow\) effector memory T cells (Tem) or central memory T cells (Tcm) separately, as well as (2) the linear pathway of either T naïve \(\rightarrow\) T eff \(\rightarrow\) Tcm, or T naïve \(\rightarrow\) T eff \(\rightarrow\) Tcm \(\rightarrow\) Tem (1, 2). From this earlier work also emerged a common definition of Tcm as CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{+}\), and Tem as CD45RO\(^{+}\), CD127\(^{-}\), CCR7\(^{-}\), CD62L\(^{-}\) (3–5). Whereas most of these studies focused mainly on mapping the intermittent changes in T cells during in vivo viral infections; we hypothesized that if we could replicate conditions similar to these infections and follow the exact development of a single population of naïve CD8 lymphocytes, perhaps we could better delineate the differentiation pathways and produce substantial long-term memory T cells ex vivo.

In our study, the primary aim was to assess the feasibility of generating memory T cells by activating human naïve CD8 through an ex vivo culture program that simulated an acute infection. The choice of an acute infective cycle stemmed from reports demonstrating that memory T cells were produced after acute but not chronic infection, and the two differed primarily in allowing activated cells a complete rest after resolution in acute infection (6, 7). Unfortunately, there are inadequate data in the human infection literature documenting a comprehensive cytokine profile, especially in the ultra early time points of an acute infection. We decided to model after acute murine CMV (mCMV) infection for the following reasons: 1) mCMV infection is similar to human CMV infection; 2) infective agents gain entry into the host cell and are presented as endogenous Ags, which is important for CD8 activation; 3) there is adequate resolution of the acute phase, although latency may develop but is maintained as replicative latency, without productive infection; and 4) a detailed cytokine profile for the entire acute infective period is available (8). On the basis of the published kinetics of cytokines in acute mCMV infection, essential factors, including IL-6, IL-12, IFN-γ, glucocorticoid, TGF-β, IL-15, and IL-7, were identified and their profiles compiled to design our in vitro culture program, designated as simulated infective protocol (SIP). In this SIP program, human naïve CD8 T cells were first enriched from healthy blood donors, cultured in different cytokine conditions with phenotypic and functional features monitored closely. The culture milieu consisted of a common starting condition for the first 10 d, mimicking an early acute infection, but separated into three different arms thereafter. The first central memory CD45RA\(^{+}\) T cell (Tcmra) arm consisted of a culture condition replicating complete resolution, the second Tem arm was maintained with persistent stimulation by anti-CD3 plus inflammatory cytokines, and the third Tcm arm had...
only persistent inflammatory cytokines devoid of anti-CD3 stimulation. For the Tcmra condition, we observed the initial conversion of Tnaive to CD45RO+, which re-expressed as CD45RA later, together with other conventional Tcm phenotypes (Tcmra: CD45RA+", CD127", CCR7", CD62L"). In the second Tem arm, Tnaive developed into conventional Tem (CD45RO+, CD127", CCR7", CD62L") by day 28, whereas in the final Tcm arm, Tcm (CD45RO+, CD127"+, CCR7", CD62L") emerged by day 14. In addition, established Tem at day 28 were convertible to Tcm when recultured in persistent inflammatory cytokines without anti-CD3; and these Tem-derived Tcm would further develop into Tcmra when the culture milieu was finally modified to a complete resting condition. We have also demonstrated better survival potential and proliferative capacity in Tcmra compared with Tem; together, they display features very similar to those of the CD45RA+ stem cell memory T lymphocytes, described recently.

Materials and Methods

Simulated infective protocol

Human PBMCs were collected from healthy donors by layering collected peripheral blood on Ficoll-Paque Plus (GE Healthcare) in 15-ml conical tubes followed by density gradient centrifugation for 30 min at 1350 rpm. The isolated PBMCs were then cryopreserved with 20% DMSO in complete medium consisting of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (HyClone), 1% penicillin/streptomycin (PAA), 1% L-glutamine (PAA), 3% HEPES (Sigma-Aldrich), and 2.4% sodium bicarbonate (B. Braun). For naive CD8 T lymphocyte isolation, cryopreserved cells were thawed in a 37°C water bath and washed using complete medium, and the total number of viable cells was counted using trypan blue solution (Sigma-Aldrich). Cells were then sorted first by untouched CD8+ T cell enrichment (MACS, Miltenyi Biotec), followed by another round of untouched CD45RA+ enrichment (MACS, Miltenyi Biotec). Enriched CD8CD45RA+ cells were activated with 2.5 μl/106 cells anti-CD3 Dynabeads (Invitrogen), 15 μl/106 cells anti-human NKGD2 mAb (clone 149810; R&D Systems), and 15 μl/106 cells goat anti-mouse IgG microbeads (MACS, Miltenyi Biotech) and were cultured in complete medium and a profile of cytokines, described recently.

Flow cytometry analysis

Cultured cells at different time points were harvested and surface stained with mouse anti-human CD8 conjugated to PC5 (clone B9.11; PC5, Beckman Coulter), CD45RA conjugated to FITC (clone HI100; FITC, eBioscience), and various test Abs: CD127 (clone R34.34; PE, Beckman Coulter), CCR7 (clone 150503; PE, R&D Systems), CD62L (clone DREG-56; PE, BD Pharmingen), CD45RO (clone UCHL-1; PE, BD Pharmingen), CD25 (clone M-A251; PE, BD Pharmingen), CD27 (clone M-T271; PE, BD Pharmingen), CD28 (clone CD28.2; PE, BD Pharmingen), CXCR3 (clone 2ST8.5H7; PE, Beckman Coulter), CD27 (clone M-T271; PE, BD Pharmingen), and CD11b (clone 11B8; PE, Beckman Coulter), CD31/4NKGD2 (clone 1D11; PE, BD Pharmingen) and CD279/PD-1 (clone M-H4; PE, BD Pharmingen). Mouse anti-human IgAbs were also included as FITC (clone G18-145; FITC, BD Pharmingen) and PE (clone G18-145; PE, BD Pharmingen) controls. Flow cytometry analysis was performed on a Beckman Coulter XL flow cytometer, and expression of CD45RA against various test Abs was plotted for CD8 gated lymphocytes using WinMDI software.

For intracellular staining, cultured cells were harvested and surface stained first with mouse anti-human CD8-PC5 and CD45RA-FITC (clone HI100; FITC, eBioscience) or PE (clone HI100; PE, eBioscience), fixed and permeabilized with IntraPrep Perm reagent kit (Beckman Coulter), and stained with the following mouse anti-human Abs: BCL2 (clone 124; FITC, DakoCytomation), B27 (clone 2ST8; PE, Beckman Coulter), CD45RA (clone 2ST8.5H7; PE, Beckman Coulter), CD62L (clone DREG-56; PE, BD Pharmingen), granzyme B (clone GB11; FITC, BD Pharmingen), Perforin (clone dG9; FITC, eBioscience), IFN-γ (clone 4S.B3; FITC, BD Pharmingen), IL-2 (clone MQL1-1H1-1FITC, eBioscience), TNF-α (clone MAb111; FITC, eBioscience). For intracellular staining of FOXP3 marker, fixation and permeabilization steps were done using the PE Anti-Human FOXP3 Staining Set (eBioscience) and stained with rat anti-human FOXP3 Ab (clone PCH101; PE, eBioscience). The strength of various test Abs, x, is expressed as δ mean fluorescence intensity (dMFI) = MFIx - MFI of corresponding murine Ig controls.

Morphology by light microscopy

During regular intervals throughout the entire culture period, cultured cells were removed, cytopspun at 1000 rpm for 5 min (Cytospot Cytocentrifuge), and stained with Leishman’s stain for 5–10 min, followed by light microscopic analysis. Photomicrographs were taken at ×100 magnification.

Molecular analysis

According to the manufacturer’s directions, RNeasy spin column (RNeasy Mini Kit, QIAGEN) was used for RNA extraction from cultured lymphocytes. The RNase-free Dnase I (QIAGEN) was used to remove the DNA contamination. At the last isolation step, RNA was eluted with 40 μl RNase-free water from the column. The RNA purity and concentration were detected by a Nanorod 1000 Spectrophotometer at 260 and 280 nm. For each RNA sample, cDNA was amplified from 2 μg total RNA by reverse transcription in 20-μl reactions with the High-Capacity CDNA Reverse Transcription Kit (Applied Biosystems). Measurements of gene expression were performed on a 7500 Fast Real-Time PCR system (Applied Biosystems). The probe and primer set of TaqMan real-time PCR assay includes a pair of unlabeled PCR primers and a TaqMan probe with a FAM dye label on the 5’ end and minor groove binder on the 3’ end. TaqMan Universal PCR Master Mix and TaqMan probe and primers were used to quantify the two gene expressions separately (TBX-21 and EOMES gene). According to the manufacturer’s directions, the TaqMan assay amplification consisted of an activation step (60°C, 2 min and 95°C, 10 min) followed by 40 cycles of denaturation (95°C, 1 s), annealing, and elongation (60°C, 1 min). The amount of mRNA was normalized to an internal control gene (HPRT1 gene). The comparative Cr (2 -ΔΔCt) method was used to calculate the relative quantitation of gene expression for each sample.

Telomere length analysis

Total DNA was extracted from the cultured cells using a DNA extraction kit (QIAGEN). Extracted DNA was digested with Hpll and MnlII, separated by 0.8% agarose gel electrophoresis with 1-DNA ladder (Invitrogen), before being transferred to Hybond-XL membrane (Amersham) for Southern blot analysis. The blot was probed with [32P]-labeled (CCCTAA)6 probe and imaged by Typhoon FLA 9500. Data and measurement of the telomere length were analyzed by the Quantity One program (Bio-Rad). Digitalized gel images were converted to a Spectrum image for accurate determination of the midpoint of each band; the midpoint was back calculated against the DNA ladder to determine the telomere length in bp.

P815 redirected flow-based cytotoxicity assay

Murine P815 cells (American Type Culture Collection), as targets in this assay, were incubated with 50 ng/106 cells OKT3 Ab (Orthoclone; Janssen-Cilag) at 4°C for 1 h and thereafter plated together with cultured human CD8 lymphocytes isolated at diagnosis as effectors in a 96-well plate according to various E:T ratios of 0:1, 0.25:1, 0.5:1, and 1:1 for 4 h in a 5% CO2 incubator at 37°C. After 4 h, cell mixtures were washed first with PBS supplemented with 1% FCS and 1% 5 mM EDTA (Research Instruments) and second with Annexin V binding buffer (BD Pharmingen), then subsequently stained with mouse anti-human CD45-FITC (clone j33; FITC, Beckman Coulter), rat anti-mouse CD45 (clone 804-D3-11-Cy5, BD Pharmingen), Annexin V-PE, BD Pharmingen 7-aminoactinomycin D (BD Pharmingen) for flow cytometric analysis. Flow-Count fluorospheres (Beckman Coulter) were added to the prepared samples for determination of absolute counts. For analysis, all CD8 lymphocytes were selected in region R1 and all Flow-Count fluorospheres were included in R2. Expression of 7-aminoactinomycin D versus annexin V was plotted for gated R1 lymphocytes, with quadrant 3 (Q3) representing all living (l) and absolute (Abs) counts calculated based on the number of recovered fluorospheres. The percent cytotoxicity was determined by (M1-M2)/M1 × 100, where M1 = Abs Q3 (for control with target alone) and M2 = Abs Q3 (for target at different E:T ratio).

CFSE proliferation assay

Tcmra and Tem were stained with 7 μM CFSE dye (CFDA SE Cell Trace kit; Invitrogen) before plating them into a 96-well plate, activated with...
CD3 Dynabeads, anti-human NKG2D, and Goat Anti-Mouse IgG Microbeads and cultured with medium dose concentration of IL-15. They were incubated for 7 d in an incubator at 37°C with 5% CO₂. Flow cytometry was performed on day 7 postculture to determine the proliferation of lymphocytes based on the CFSE peaks distribution. For all analyses, CFSE histograms were plotted for gated CD8 lymphocytes, and the percent proliferation was determined by M2 region, where M2 = all lymphocytes that had undergone at least one round of proliferation.

**CFSE suppression assay**

Postsorted naïve CD8⁺ T cells (EasySep, STEMCELL) were used as responder cells and stained with 7 µm CFSE dye (CFDA SE Cell Tracer kit, Invitrogen), then activated with 50 ng/10⁶ cells OKT3, 15 µL/10⁶ cells monoclonal anti-human NKG2D, and 15 µL/10⁶ cells goat anti-mouse IgG microbeads and cultured with 5 ng/ml IL-15 before plating them in a 96-well plate. Suppressor cells were then added in the suppressor-to-responder ratio of 0.05:1, 0.1:1, 0.2:1, and 1:1. Responder-only 0:1 and suppressory-only 1:1 ratios were included as controls, and the plate was incubated for 7 d in an incubator at 37°C with 5% CO₂. Cell mixtures were harvested on day 7 postculture and surface stained with mouse anti-human CD8 conjugated to PKS5 to determine the proliferation of lymphocytes based on the CFSE peaks distribution on CD8-PC5 gated cells. Flow-Count fluorospheres (Beckman Coulter) were also added to the prepared samples for determination of absolute counts. For analysis, all murine mastocytes were selected in region R1 and all Flow-Count fluorospheres were included in R2. CFSE expression histograms were plotted for gated R1 cells, absolute counts were calculated based on the number of recovered fluorospheres, and percent proliferation was determined by (M2 − M1)/ M2 × 100, where M2 = absolute count of all CFSE⁺ Tnaive cells, M1 = absolute count of first peak of Tnaive cells on the cell gate. Subsequently, the percent suppression = (% proliferation for S:R 1:1) − (% proliferation for S:R 0:1).

**Results**

**Simulated infective protocol**

The key factors identified for SIP were anti-CD3 as first signal, anti-NKG2D as second costimulatory signal, and the third signal from the cumulative culture milieu, including IL-6, IL-12, IFN-γ, dexamethasone, TGF-β, IL-15, and IL-7. The entire cycle lasted ~35 d, with the first 10 d being identical for all three arms (Fig. 1A). During these 10 d, the essential cytokine profile consisted of a single IL12 peak on day 0, double IL6 peaks on day 0 and day 7, double IFN-γ peaks on day 0 and day 8, a single dexamethasone peak on day 1, single TGF-β peak on day 7, and gradual increase in IL-15 from 0 day to 7 day (9–15). Thus, 1) on day 0, high-dose IL-6, IL-12, IFN-γ, but low-dose IL-15, were added; 2) on day 1, high-dose dexamethasone with additional medium and low-dose IL-15 were added; 3) on day 3, all cytokines were washed away and reconstituted with only low-dose IL-15; 4) on day 4, medium-dose IL-6 and TGF-β were added but progressively increased to high-dose IL-6, TGF-β, and IL-15 on day 7; 5) additional anti-CD3 beads were added only on day 7 because it remained attached for ~7 d; 6) on day 8, high-dose IFN-γ and IL15 were added. After day 10, all cytokines were washed off and three different conditions were reconstituted thereafter. In the Tcmra condition, cells were maintained in low-dose IL-6, TGF-β, IL-15, and IL-7, whereas the Tem condition was persistent stimulation by anti-CD3, high-dose IL-6 and medium-dose IL-15, and the Tcm condition was maintenance in high-dose IL-6 and medium-dose IL-15 only (16–21). For clarification of the reagent schedule, please refer to Fig. 1B. Longitudinally, SIP for Tcmra can also be divided into four phases: priming from day 0 to day 3, expansion from day 3 to day 10, contraction from day 10 to day 28, and resolution after day 28.

**Morphology and immunophenotype**

For the morphological changes during priming to activation phase, Tnaive initially divided and developed blastoid features with prominent nucleoli and displayed more vacuolations as well as intracytoplasmic granules (Fig. 2A). By day 10, before conversion to the contraction phase, more lymphocytes began to form cytoplasmic extensions, resulting in an elongated shape. During the contraction and resolution phases of Tcmra development, lymphocytes progressively reverted to round and blastoid morphology. Similarly, Tcm cultured only with inflammatory cytokines also demonstrated blastoid morphology with prominent nucleoli. However, in Tem under the influence of continuous anti-CD3 and inflammatory stimulations, elongated and activated cytromorphology persisted.

After priming, Tnaive (CD45RA⁺, CD127⁻, CCR7⁻, CD62L⁻, CXCR3⁺) developed into Tefg (CD45RA⁻, CD127⁻, CCR7⁻, CD62L⁺, CXCR3⁻) on day 3 (Figs. 2B). By day 7, Tefg expanded into intermediate T cell (Tim) 1 (CD45RO⁺, CD127⁻, CCR7⁺, CD62L⁺, CXCR3⁻) and further converted between day 7 and day 10, into Tim2 (CD45RO⁺, CD127⁻, CCR7⁻, CD62L⁺, CXCR3⁺). For the Tcmra arm between day 10 and day 21, Tim2 gradually changed into Tim3 (CD45RO⁻, CD127⁺, CCR7⁺, CD62L⁻, CXCR3⁻). During the final resolution phase, Tim3 reversed and remained stable Tcmra (CD45RA⁺, CD127⁺, CCR7⁺, CD62L⁺, CXCR3⁺) by day 28 (Fig. 3A). Throughout this period, Tcmra remained CD27⁻ and CD28⁻, but Tem were CD27⁺ and lost CD28 expression progressively. Finally, for the Tcm arm, Tim2 at day 10 will convert to Tcm (CD45RO⁻, CD127⁺, CCR7⁺, CD62L⁻, CXCR3⁻) by day 14.

To assess the development of Tem in the absence of antigenic and/or inflammatory stimulation, the Tem condition was washed off after day 33 and changed to low-dose IL-6, TGF-β, IL-15, and IL-7, similar to the Tcmra resting condition (Fig. 3B). Over the following week, Tem began to upregulate CD45RA, CCR7, and CD62L and demonstrated the ability to revert to Tcmra by day 44. Furthermore, the number of Tem surviving this Tcmra conversion was lower than for those undergoing direct Tcmra generation.

Besides subjecting Tem to the resting milieu, Tem after day 33 were also recultured in the Tcm condition, that is, high-dose IL-6 and medium-dose IL-15 without anti-CD3. Similar to the Tem generated directly after day 10, Tem were converted to the Tcm phenotype by re-expressing CCR7 and CD62L between days 40 and 45. If these Tem-derived Tcm were further cultured in low-dose IL-6, TGF-β, IL-15 and IL-7, they will develop the Tcmra phenotype re-expressing CD45RA.

**Survival potential and secondary activation**

For the comparison of Eomes and Tbet expression, we observed an Eomes/Tbet mRNA ratio of 0.88 ± 0.32, 1.05 ± 0.15, and 0.13 ± 0.03 for day 0 Tnaive, day 33 Tcmra, and day 33 Tem, respectively (Fig. 4A), thus demonstrating comparable Eomes dominance in both Tnaive and Tcmra, but Tbet dominance in Tem. Next, we compared the relative telomere length of day 33 Tcmra and day 33 Tem against Tnaive as baseline and found Tcmra to be 580 ± 118 bp longer than Tem (Fig. 4B). BCL2 expression was also investigated, and the assessment of intracellular BCL2 by flow analysis in day 0 Tnaive, day 33 Tcmra, and day 33 Tem showed a dMFI value of 11.8, 73.8 ± 5.8, and 90.9 ± 6.5, respectively (Fig. 4C). There was a higher BCL2 expression in both memory subtypes of Tcmra and Tem compared with Tnaive lymphocytes.

In determining the proliferative capacity, we first demonstrated the presence of NKG2D on both day 33 populations as a suitable costimulatory signal for secondary activation, then proceeded to compare the proliferative potential between day 33 Tcmra and day 33 Tem by activating both populations with anti-CD3, anti-NKG2D, and...
Each colored curve represents a separate cytokine, as listed in the legend. For the first 10 d, the cytokine profile is identical, but this will be divided into a Tcmra or Tem condition after day 10. During priming, anti-CD3, costimulatory anti-NKG2D, high-dose IL-6, IL-12, IFN-γ, and dexamethasone were the key factors given. In the expansion phase, anti-CD3 and anti-NKG2D were maintained, whereas there was a second peak of high-dose IL-6 and IFN-γ, and a first peak of high-dose TGF-β with a progressive increase to high-dose IL-15. (Ai) For Tcmra development, CD8 T cells were maintained in low-dose IL-6, TGF-β, IL-15, and IL-7 during contraction and resolution. (Aii) For Tem development, CD8 T cells were stimulated continuously with anti-CD3, high-dose IL-6, and medium-dose IL-15. (Aiii) To generate Tcm, CD3 stimulation was removed but continued with high-dose IL-6 and medium-dose IL-15. The x-axis denotes the number of days after the commencement of SIP, and the height of each peak does not correspond to relative strength. (B) This figure translates the cytokine requirements in (A) into a reagent schedule. Cytokines were added in different dose concentrations, as (Figure legend continues)
IL-15 (Fig. 4D). At 7 d post–secondary activation, CD8 gated lymphocytes showed Tcmra to be 81.5 ± 3.5%, whereas Tem was 51.5 ± 7.5% for having undergone at least one cycle of division, thereby supporting a higher proliferative capacity after secondary activation in Tcmra.

**Inflammatory and cytotoxic function**

At 3 days after priming, Tnaive began to increase their expression of IFN-γ (18.4 ± 3.3 dMFI), granzyme B (1.8 ± 0.5 dMFI), and perforin (1.8 ± 1.1 dMFI) (Fig. 5A), which qualified them functionally as Teff (3). These cytolytic granules began to decline over the next few days but demonstrated a second peak on day 14. Comparison of respective dMFI of cytolytic granules among day 0 Tnaive, day 33 Tcmra, and day 33 Tem showed values of granzyme A (0.1 ± 0.1, 0.7 ± 0.1, 1.3 ± 0.1, 1.0 ± 0.1), granzyme B (0.0 ± 0.3, 0.0 ± 0.3, 0.4 ± 0.3, 0.3 ± 0.2), respectively. These results demonstrated a comparable granzyme A and B expression between Tcmra and Tem, as well as comparable perforin expression between Tcmra and Tem. IFN-γ also began to decrease after day 3 but plateaued from day 15 with comparable levels moderate in both Tcmra and Tem. The dMFI of intracellular IFN-γ expression in day 0 Tnaive, day 33 Tcmra, and day 33 Tem was 7.6 ± 0.5, 12.8 ± 1.1, and 14.2 ± 1.4, respectively. Furthermore, both IL-2 and TNF-α (Fig. 5A) were elevated from day 7, which peaked at day 10 and declined to baseline in Tcmra but remained low in Tem up to day 33. Comparative dMFI for day 0 Tnaive, day 33 Tcmra, and day 33 Tem demonstrated values of IL-2 (0.0 ± 0.0, 0.0 ± 0.0, 0.0 ± 0.0, 0.23 ± 0.15) and TNF-α (0.0 ± 0.0, 0.0 ± 0.0, 0.0 ± 0.0, 1.0 ± 0.5), respectively.

For analysis of cytotoxic function (Fig. 5B), both Tcmra and Tem demonstrated in vitro cytotoxic capacity against P815 murine mastocytoma cells. The percent cytotoxicity for Tcmra was in comparison with day 31 Tcmra, the latter remained PD-1+, CD161+, CD8β+, and dim CD25+, whereas day 31 Tem were PD-1−, partial dim CD161, CD8β+, and dim CD25+ (Fig. 6B). The phenotypes of both Tnaive and Tcmra were similar in almost all surface markers except CD25, which was absent in Tnaive but dim positive in Tcmra. Expression of CD161 in Tem on day 31 was interesting because of its association to the Th17 subtype.

We proceeded to examine the suppressive function in both Tcmra and Tem after secondary activation. They were activated with anti-CD3 and anti-NKG2D before coculturing another 7 d with autologous Tnaive, which were similarly activated by both signals (Fig. 6C). At the end of coculture, Tcmra and Tem suppressed 45 ± 28% and 26 ± 13% of Tnaive proliferation at the highest suppressor/responder ratio of 1:1, respectively.

Based on our results, we have proposed a developmental model (Fig. 7), supporting both divergent (Tnaive → Teff → Tcm or Tem) and linear (Tnaive → Teff → Tem → Tcm → Tcmra) relationships.

**Discussion**

CD45RA expression had been regarded traditionally as naive phenotype, but recent evidence suggested that some CD45RA revertants were in fact memory T cells observed to develop after acute infection with activated phenotype (22, 23) and stem cell–like properties (24). Eomes and Tbet mRNA gene products were also found to be determinants for either memory or effector differentiation, and shifting the Eomes/Tbet mRNA ratio toward Eomes dominance will favor memory differentiation (25, 26). Furthermore, true long-term memory T cells were known to 1) re-express telomerase, which extends their telomere length for prolonged survival (27); 2) demonstrate higher proliferative potential compared with chronically stimulated T lymphocytes (24); and 3) express BCL2, which is a key antiapoptotic factor important for sustaining survival of memory T lymphocytes (3) In our experiments, when comparing Tcmra with Tnaive, the former expressed an equivalent Eomes/T-bet mRNA ratio, comparable telomere length, higher BCL2, and similar CD28 expression. In contrast to Tem, Tcmra expressed a higher Eomes/T-bet mRNA ratio, longer telomere length, and comparable BCL2 expression, and remained CD28ab exhibited higher proliferative fraction after secondary activation. Collectively, this evidence strongly supports better survival and proliferative capacity in Tcmra, which corroborates with aforementioned studies reporting CD45RA revertants as memory stem T cells. Besides, Tcmra are also competent cytotoxic T cells, displaying comparable IFN-γ, granzyme A and B, and perforin, and similar ex vivo cytotoxicity at higher E:T ratio as Tem. Furthermore, post–activated Tem were polyfunctional, producing also IL-2 and TNF-α; however, these cytokines were reduced to baseline in Tcm but maintained at a low level in Tem. Superficially, Tcmra are very similar to Tnaive in all tested surface phenotypes, with the exception of dim CD25 being expressed only on Tcmra, which may provide a competitive advantage for IL-2 during secondary activation.

Owing to the use of anti-inflammatory cytokine TGF-β and dexamethasone in SIP, we proceeded to investigate their influence in inducing suppressive characteristics by studying the expression of FOXP3, IL-10, TGF-β, PD-1, CD8αα, CD161, and CD25 and in vitro suppression. On day 3 postactivation, an early peak in FOXP3 expression was observed, which reduced to baseline level subsequently; this is in concordance with other reports demonstrating a transient FOXP3 expression shortly after activation and in itself did not indicate a conversion to regulatory T cells (28, 29). Furthermore, Tcmra remained CD8ααab; did not express significant TGF-β, PD-1, CD161, or high level of CD25; and were all in keeping with a nonregulatory and nonexhausted phenotype (30–34). The initial IL-10 peak at day 3 was similar to the transient FOXP3 expression postactivation; but the gradual rather than abrupt reduction until day 33 in Tcmra was unexpected. However, Cui et al. (35), in their study of giving i.p. injections of anti–IL-10 Ab for 25 d, had shown that IL-10 via the STAT3 pathway was depicted in the lower panel of the table. The top row represents the number of days after commencement of SIP, and the left column represents cytokines with their respective symbols in parentheses. Cytokines were added in an identical manner until day 10, but were washed and reconstituted into three different combinations thereafter: one for Tcmra, another for Tem, and the third for Tcm development. h, High; m, medium; l, low.
FIGURE 2. Morphology and immunophenotype. (A) Microscopic morphology. This figure represents the morphological changes during Tcmra and Tem development throughout SIP by light microscopy at original magnification ×100. The dark, round spheres in the photomicrograph are the anti-CD3 beads used in stimulating CD8 T lymphocytes throughout culture. (B) Immunophenotypic expression. This figure represents the immunophenotypic changes in CD45RA, CD127, CCR7, CD62L, CXCR3, CD27, CD28, and CD45RO by flow cytometry analysis during the entire culture period. The left column displays the number of days after commencement of SIP. From day 0 to day 10, the plots are identical because the culture condition is exactly the same. After day 10, Tcmra development is presented as the subpanel (Bii) up to day 31. These figures are representative of at least three independent experiments.

D, Number of days after commencement of SIP; SS, side scatter.
FIGURE 3. Immunophenotype of other T subtypes. (A) Immunophenotypic expression. This figure represents the immunophenotypic changes in CD45RA, CD127, CCR7, CD62L, CXCR3, CD27, CD28, and CD45RO by flow cytometric analysis during the entire culture period. The left column displays the number of days after commencement of SIP. The initial period of day 0 to day 10 was identical to that in Fig. 2B because the culture condition is exactly the same. After day 10, the subpanel (Ai) refers to Tem, and the subpanel (Aii) to Tcm development, with subsequent conversion to the Tcmra condition from D28 onward. (B) The left panel demonstrates the phenotypic persistence of Tcmra until day 44; the middle panel represents the immunophenotypic features after Tem were converted to Tcmra’s resting condition between day 36 and day 44; and the right panel demonstrates the partial reconversion of Tem to Tcm, and then to Tcmra, when the culture condition was first changed to chronic inflammation, followed by resting milieu after day 43. These figures are representative of at least three independent experiments. D, Number of days after commencement of SIP; SS, side scatter.
important in CD8 maturation and memory formation. Thus, a gradual reduction of IL-10 expression in Tcmra during the immediate postinfection period may have a role in CD8 memory differentiation, which will require further work to elucidate. Functionally, the suppression of naive T proliferation by secondary activated Tcmra was not surprising because this is in keeping with other reports that demonstrated both suppression of bystander Tnaive during secondary activation and suppression of primary allogenic response by CD8+ memory cells. Incidentally, this effect of Tnaive bystander suppression is also IL-10 dependent (36–39). Another noteworthy observation is the CD161 expression in Tem, because more work may be necessary to ascertain the possibility of whether such chronically stimulated Tem are indeed precursors to the Th17 lineage (40).

Many studies have established the differential expression of cell adhesion molecule CD62L, and chemokine receptors CCR7 and CXCR3, as indicators for the migratory tendencies in T lymphocytes. Following this grand schema, T lymphocytes expressing CCR7+/CD62L+ are poised for recirculation between lymphoid tissue and peripheral blood (41). For egress from lymph node, activated T lymphocytes will lose CCR7 to minimize its retention potential yet maintaining CD62L+ in the efferent lymphatics, and when coupled with CXCR3 expression, these exited lymphocytes will preferentially home to inflamed tissue (42–45). In reverse, those
FIGURE 5. Inflammatory and cytotoxic function. (A) Comparison of IFN-γ, cytolytic granules, IL-2, and TNF-α expression. Frozen mononuclear cells were thawed, sorted for Tnaive, and analyzed together with day 33 Tcmra and day 33 Tem. Intracellular expression of IFN-γ, granzyme A, granzyme B, perforin, IL-2, and TNF-α was analyzed by flow cytometry. The figure comprises six corresponding pairs of charts. The graph on the left represents the variation of intracellular cytokine expression versus time over the entire 33 d of culture, whereas the one on the right compares only the expression on day 0 versus day 33. D, number of days after commencement of SIP. dMFI, ΔMFI = MFI(Tcmra or Tem) − MFI of corresponding murine IG control. (B) In vitro cytotoxicity of P815 murine mastocytoma cells. Gate R2 selects the Flow-Count fluorospheres; gate R1 selects all murine plasma-cytoma cells. All data in these charts are presented as mean ± SEM, and these figures are representative of at least three independent experiments. AnnexV, Annexin V; hCD45, anti-human CD45; mCD45, anti-murine CD45.
FIGURE 6. Anti-inflammatory and suppressive function. For both charts (A) and (B), frozen mononuclear cells were thawed, sorted for Tnaive, and analyzed together with day 33 Tcmra and day 33 Tem. (A) Comparison of IL-10, TGF-β, and FOXP3 expression. Intracellular expression of IL-10, TGF-β, and FOXP3 was analyzed by flow cytometry. The figure comprises three corresponding pairs of charts. The graph on the left represents the variation of intracellular cytokine expression versus time over the entire 33 d culture, whereas the one on the right compares only the expression on day 33. D, number of days after commencement of SIP. dMFI denotes \( \frac{\text{MFI}(\text{Tcmra or Tem})}{\text{MFI of corresponding murine IG control}} \). (B) Expression of PD1, CD161, CD8β, and CD25. Expression of PD-1, CD161, CD8β, and CD25 was analyzed by flow cytometry on CD8α+ gated T cells. These dot plots compared the expression of PD-1, CD161, CD8β, and CD25 between day 0 Tnaive, day 31 Tcmra, and day 31 Tem. The presence of CD8α expression is indicated by decreasing CD8β on CD8α+ gated cells. (C) In vitro suppression of activated autologous CD8 Tnaive proliferation. Gate R2 selects the Flow-Count fluorospheres; gate R1 selects all CD8 lymphocytes. All data in these charts are presented as mean ± SEM, and these figures are representative of at least three independent experiments. S.R, Suppressor/responder ratio.
in tissues will upregulate CCR7 to return to lymphoid organs viaafferent lymphatics, and most lymphocytes in afferent lymphatics are, in fact, CCR7+/CD62L− (44, 46). In mirroring these phenotypic variations after T cell activation, Tim1 that expressed CCR7+/CD62L+ would be poised for recirculation during early expansion, Tim2 that downregulated CCR7 but remained CD62L+/CXCR3+ would suggest a tendency to home to inflamed tissue during late expansion, whereas Tim3 that re-expressed CCR7 yet downregulated CD62L during contraction were equipped to return to lymphoid tissues, and finally Tcm that coexpressed CCR7+/CD62L+ would have restored recirculating capability after resolution.

If antigenic and inflammatory stimuli persisted beyond late expansion, activated lymphocytes developed into Tem within downregulation of both CCR7 and CD62L, and they would be trapped in the inflamed tissue. However, when only antigenic signal was removed but the environment remained inflammatory after late expansion, activated lymphocytes were converted to Tcm expressing both CCR7+ and CD62L+, to remain as vigilant CD45RO+ capable of recirculating to hunt for Ags hidden in other inflamed locations.

Interestingly, a similar milieu with inflammation but devoid of antigenic stimulation would also convert Tem to Tcm phenotype. Therefore, observing such matching migratory patterns to their functional requirement during the corresponding developmental phases may indicate a substantial influence of environmental cues in directing lymphocyte homing.

Resident T cells in tumors or chronic infections were reported to harbor a high proportion of Tem, with some specificity to tumor-associated Ags in certain disease models. If these resident Tem equipped with tumor specificity can be converted to long-lasting memory T cells, they may be harnessed for long-term control of these disorders. To assess this potential therapeutic application, we proceeded to investigate the possibility of converting Tem directly to Tcmra, by changing the Tem culture milieu after day 33 to the resting condition of Tcmra. In the absence of both antigenic and inflammatory stimulations, Tem were indeed converted to Tcmra re-expressing CD45RA, CCR7, and CD62L. To extend this observation, we are planning further studies to examine the possibility of converting intratumor Tem to Tcmra, as well as to determine the efficacy of such Tem-derived Tcmra in long-term disease control in appropriate animal models.

Finally, on the basis of our data, we would like to propose the lineage relationships as shown in Fig. 7. This model supports 1) the divergent relationship Tn→Teff→Tem or Tcm separately, when activated T cells are differentially cultured in either persistent stimulation or persistent inflammation, and both subtypes will follow a final common pathway to Tcmra, as well as 2) the linear relationship Tn→Teff→Tem→Tcm→Tcmra, when persistent stimulation is abolished in steps. Overall, our model agrees with two seemingly disparate pathways that culminate in Tcmra as the final long-term memory T cell subtype. It is noteworthy that in both routes, the key to developing Tcmra is to maintain T lymphocytes in the resting condition, the earlier the better. In other words, the transition to memory T cells (the road to memory) does require early relief from both antigenic and inflammatory stimulations (an early rest) to enhance conversion to long-lasting memory T lymphocytes (their long journey).

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Disclosures

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