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PR1-specific cytotoxic T lymphocytes are relatively frequent in umbilical cord blood and can be effectively expanded to target myeloid leukemia

Lisa S St. John^{1,†}, Liping Wan^{2,†}, Hong He¹, Haven R. Garber¹, Karen Clise-Dwyer¹, Gheath Alatrash¹, Katayoun Rezvani¹, Elizabeth J. Shpall¹, Catherine M. Bollard³, Qing Ma¹, and Jeffrey J. Molldrem¹

¹Section of Transplantation Immunology, Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas M.D. Anderson Cancer Center, Houston, TX. USA

²Department of Hematology, Shanghai Jiao Tong University Affiliated First People's Hospital, Shanghai, China

³Center for Cancer and Immunology Research, Children's Research Institute, Washington DC. USA

Abstract

Background aims—PR1 is an HLA-A2 restricted leukemia-associated antigen derived from neutrophil elastase and proteinase 3, both of which are normally stored in the azurophil granules of myeloid cells but overexpressed in myeloid leukemic cells. PR1-specific cytotoxic lymphocytes (PR1-CTL) have activity against primary myeloid leukemia *in vitro* and *in vivo* and thus could have great potential in the setting of adoptive cellular therapy (ACT). Adult peripheral blood derived PR1-CTL are infrequent but preferentially lyse myeloid leukemia cells. We sought to examine PR1-CTL in umbilical cord blood (UCB) since UCB units provide a rapidly available cell source and a lower risk of graft-versus-host disease (GVHD), even in the setting of mismatched human leukocyte antigen (HLA) loci.

Methods—We first determined the frequency of PR1-CTL in HLA-A2⁺ UCB units and then successfully expanded them *ex vivo* using repeated stimulation with PR1 peptide-pulsed antigen-presenting cells (APCs). After expansion, we assessed the PR1-CTL phenotype (naïve, effector, memory) and function against PR1-expressing target cells.

Results—PR1-CTL are detected at an average frequency of 0.14% within the CD8⁺ population of fresh UCB units, which is 45 times higher than in healthy adult peripheral blood. UCB PR1-CTL are phenotypically naïve, consistent with the UCB CD8⁺ population as a whole. In addition, the cells can be expanded by stimulation with PR1 peptide-pulsed APCs. Expansion

Address correspondence to: Jeffrey J. Molldrem, Section of Transplantation Immunology, Department of Stem Cell Transplantation and Cellular Therapy, University of Texas M.D. Anderson Cancer Center, Unit 900, 1515 Holcombe Boulevard, Houston, TX 77030, Tel. 713-563-3334; Fax. 713-563-3364; jmolldre@mdanderson.org.

[†]These authors contributed equally to this manuscript.

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results in an increased frequency of PR1-CTL, up to 4.56%, with an average 20-fold increase in total number. After expansion, UCB PR1-CTL express markers consistent with effector memory T cells. Expanded UCB PR1-CTL are functional *in vitro* as they are able to produce cytokines and lyse PR1-expressing leukemia cell lines.

Conclusions—This study is the first report showing that T cells specific for a leukemia-associated antigen (LAA) are found at a significantly higher frequency in UCB than adult blood. Our results also demonstrate specific cytotoxicity of expanded UCB-derived PR1-CTL against PR1-expressing targets. Together, our data suggest that UCB PR1-CTL could be useful to prevent or treat leukemia relapse in myeloid leukemia patients.

Keywords

PR1; antigen specific cytotoxic T lymphocyte; umbilical cord blood; leukemia

Introduction

PR1 is an HLA-A*0201 restricted nonameric peptide derived from the serine proteases proteinase 3 and neutrophil elastase, which are both overexpressed in myeloid leukemia cells [1] [2]. PR1 has been effectively targeted in myeloid leukemia using PR1-CTL and an anti-PR1/HLA-A2 antibody [3–7]. PR1-specific memory T cells have been detected at very low frequencies in the peripheral blood (PB) of healthy donors, but are found at significantly higher frequencies in myeloid leukemia patients where their frequency is positively correlated with treatment response [5]. Previous studies have shown that PR1-CTL isolated from patients with myeloid leukemia or elicited from the PB of normal adults demonstrated preferential cytotoxicity against myeloid leukemia cells *in vitro* and *in vivo* [3, 8, 9]. Further, Rezvani et al. [10] showed that PR1-CTL can be transferred from adult donor to recipient, leading to an expansion of the CTL and resulting in a graft-versus-leukemia (GVL) effect in the recipient.

Due to the potential therapeutic value of PR1-CTL in the setting of cellular therapy and the advantages of umbilical cord blood (UCB), including off-the-shelf availability and less stringency in HLA matching, the isolation and/or expansion of UCB-derived PR1-CTL could provide cells capable of targeting, and possibly eliminating, myeloid leukemia. Herein, we investigated the frequency, phenotype, and anti-leukemia function of PR1-CTL derived from UCB. Despite the limited number of mononuclear cells in an average UCB unit ($\sim 5 \times 10^8$ cells) and the low frequency of PR1-CTL in adult PB (1/15,000 to 1/345,000 CD8⁺ T-cells) [5], we discovered that PR1-CTL precursors in UCB are 45-fold more frequent than in adult PB. In addition, we demonstrated the *ex vivo* expansion capacity of UCB-derived PR1-CTL and their specific cytotoxic function *in vitro*. The number of PR1-CTL expanded *ex vivo* is sufficient for adoptive immunotherapy. To our knowledge, this is the first report investigating the phenotype and function of UCB-derived CTLs targeting a myeloid leukemia-associated antigen (LAA).

Materials and Methods

Reagents

HLA-A2 positive fresh UCB units and healthy adult PB were obtained from the Cord Blood Bank of M.D. Anderson Cancer Center and Gulf Coast Regional Blood Center of Houston, respectively. HLA-A2 status was determined serologically by flow cytometry using the monoclonal antibody (mAb), BB7.2 (BioLegend). PR1 peptide (₁₆₉₋₁₇₇VLQELNVTV) was synthesized by Bachem Company. Wild type U937 and K562 cell lines were obtained from American Type Culture Collection. HLA-A*0201 transduced U937 cells (U937-A2) were provided by Dr. Greg Lizee. Specialized K562 antigen-presenting cells (APCs) co-transfected with CD86, CD137L, CD64, CD32, and HLA-A*0201 were kindly provided by Dr. Laurence Cooper of M.D. Anderson Cancer Center. CD4, CD8, CD45RA, and CCR7 mAbs (BD Biosciences) were used for immunophenotyping. Aqua live/dead cell stain (BioLegend) as well as CD14, CD16, and CD19 mAbs were used to exclude monocytes, NK cells, and B cells in the dextramer analyses. PE-conjugated HLA-A*0201 PR1 dextramer as well as WT1, MART1, and Tyrosinase₍₃₆₈₋₃₇₆₎ dextramers were all from Immudex, Denmark.

Ex vivo expansion of PR1-CTL from UCB

Stimulation and expansion of UCB-derived PR1-CTL was performed according to the protocol by Hanley et al. with some modifications [11]. Briefly, UCB mononuclear cells (CBMCs) were isolated and co-cultured with irradiated PR1 peptide-pulsed K562-A2 cells at a ratio of 10:1 in the presence of soluble IL-7 (10 ng/ml), IL-12 (10 ng/ml), and IL-15 (5ng/ml) (R&D Systems, Inc.). CBMCs were stimulated every week for 3 weeks with PR1-pulsed K562-A2 cells in CTL medium (50% Click's (Irvine Scientific) and 50% RPMI 1640 (HyClone) supplemented with 10% human AB serum and 2 mM L-glutamine). IL-15 was again added after the second stimulation. Cells were supplemented with 100 IU/ml recombinant human IL-2 (R&D Systems, Inc.) 3 days after the second and 1 day after the third stimulation. Medium was replenished biweekly. Cells were harvested on day 19 after the first stimulation. CD8⁺ cells were enriched from bulk culture using the MACS CD8⁺ T cell isolation kit (Miltenyi Biotec) for the intracellular cytokine detection and cytotoxicity assays.

Intracellular cytokine and cytotoxicity assays

CD8⁺ T cells from expanded UCB units were incubated with target cells at an E:T ratio of 5:1. Cells were incubated at 37°C, 5% CO₂ for a total of 16 hours (brefeldin A was added at a final concentration of 10 µg/ml after the initial hour). The cells were fixed and permeabilized by FACS Lyse and FACS Perm (BD Biosciences). Cells were stained, washed, and resuspended in 1% PFA. Data were acquired on the LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software. For the cell-mediated cytotoxicity assay, target cells were stained with 5 µg/ml of Calcein AM (Thermo Fisher) for 15 minutes at 37°C, washed 4 times with RPMI 1640 and resuspended at 2×10⁵/ml. Target cells (2,000) in 10 µl were incubated with antigen specific CTL at varying E:T ratios. To quench the reaction, 5 µl of 0.4% Trypan Blue was added after 4 hours [12].

Statistical analysis

GraphPad Prism 6 (GraphPad, San Diego, CA) was used to perform statistical analyses. Student's *t* test was used to test for significance and *P* value < 0.05 was considered significant.

Results

PR1-CTL are found at a relatively high frequency in UCB

As shown in Figure 1A, the frequency of PR1-CTL in UCB was approximately 45 times higher than in healthy adult PB (mean $0.140 \pm 0.104\%$ of CD8⁺ cells in UCB [n=74] vs. 0.0031% of CD8⁺ cells in adult PB [n=15], [p<0.0001]). PR1-CTL with low, intermediate, and high avidities were clearly visible within the UCB CD8⁺ population when stained with PR1-specific multimer followed by flow cytometric analysis (Figure 1B, left). In comparison, PR1-CTL were found at low, sometimes undetectable levels in healthy adult PB (Figure 1B, right) [4]. The relatively high PR1-CTL frequency in UCB was an unexpected finding as T cells specific for self-antigens, like PR1, are subject to central tolerance via deletion in the thymus. Given these data, we sought to examine other tumor antigen specific CTLs in UCB. As shown in Figure 1C, the frequency of other self-antigen specific T cells recognizing WT1- (~0.0796%), MART1- (~0.1316%) and Tyrosinase- (~0.1348%) was comparable to PR1-CTL and was significantly higher in UCB than has been reported in studies of healthy donor adult PB [13–15]. These data complement the results published by Su et al. in which detectable levels of self-antigen specific CD4⁺ T cells were seen in cord blood [16].

UCB PR1-CTL can be successfully expanded *ex vivo*

Although PR1-CTL are detectable in UCB, the absolute number of cells in an unmanipulated UCB unit is too low to be considered a viable option for ACT. We sought to expand PR1-CTL using PR1 peptide-pulsed K562-A2 cells as stimulators, along with a cytokine cocktail of IL-7, IL-12, IL-15, and IL-2 [11]. As shown in Figure 2A, the percentage of PR1-CTL within the CD8⁺ population increased significantly from an average of 0.219% (range: 0.074%–0.456%) prior to expansion to 1.014% (range: 0.155%–4.56%) after three rounds of stimulation (n=17; p=0.001). The bulk CD3⁺CD8⁺ subset also increased from a mean of 21.5% to 56.4% of the lymphocytes during the 19-day protocol (figure 2A, inset). Critically, the absolute number of PR1-CTL also significantly increased during the expansion by an average of 20-fold (95% CI 10.25~38.78) (p<0.001) (Figure 2B). This represents an average of 2×10^5 PR1-CTL per expanded UCB unit. Notably, the total number of CD3⁺CD8⁺ cells increased by only 7.5-fold (95% CI 3.92~14.19) (Figure 2B, inset) (p<0.001), indicating preferential expansion of PR1-CTL. Longitudinal analysis of a representative HLA-A2⁺ UCB unit during the expansion protocol revealed T cells with a range of apparent avidities for PR1/HLA-A2 on Day 19 (Figure 2C).

UCB PR1-CTL transition to an effector memory phenotype during expansion and demonstrate HLA-A2-dependent cytotoxicity

Surface phenotyping of lymphocytes from 8 HLA-A2⁺ UCB units indicated that 95% of PR1-CTL were CD45RA⁺CCR7⁺ prior to expansion (Figure 3A, left). Thus, PR1-CTL directly sorted from UCB were phenotypically naïve, consistent with previous reports of UCB CTL subsets [17–20]. While PR1-CTL directly sorted from UCB were phenotypically naïve, the expanded UCB PR1-CTL lost expression of CD45RA and CCR7, characteristic of effector memory T cells (Figure 3A, right) [21]. We next asked whether the PR1-CTL remained functional after the 19-day *in vitro* expansion protocol. After expansion, UCB PR1-CTL demonstrated a >2-fold increase in secretion of IFN- γ and TNF- α in response to the PR1-expressing target cells U937-A2 when compared to wild type, HLA-A2 negative U937 cells or PR1-CTL alone (Figure 3B). Finally, to measure the specific cytotoxic activity of expanded UCB PR1-CTL, U937 leukemia cells, HLA-A2⁺ and wild type, were used as targets in a CTL assay. As shown in Figure 3C, expanded UCB-derived PR1-CTL cells were capable of killing the U937 leukemia cells in an HLA-A2-dependent manner *in vitro*.

Discussion

In this study, we show that PR1-CTL are present at a significantly higher frequency in UCB than healthy adult PB. While detectable, the frequencies still represent very low overall cell numbers. Thus, in terms of clinical utility, we showed that PR1-CTL from UCB can be effectively expanded *ex vivo*. We were able to achieve, on average, a 20-fold expansion in PR1-CTL after repeated peptide stimulation with specialized APCs. Unlike expansion of PR1-CTL from healthy adult PB, expansion from UCB required the use of robust APCs, additional cytokines, and specific timing to ensure optimal cell proliferation [3]. This is likely due to the naïve nature of the cells, resulting in the need to prime cells before they are capable of extensive proliferation [17, 19]. Once expanded, PR1-CTL lose the phenotypic markers characteristic of naïve cells and take on both phenotypic and functional characteristics indicative of memory CTL. The expanded UCB PR1-CTL produced both IFN- γ and TNF- α in response to PR1-expressing target leukemia cells. In addition, the expanded PR1-CTL were capable of killing PR1-expressing leukemia cells *in vitro* in an HLA-A2 dependent manner.

We presumed that the majority of PR1-CTL undergo deletion in the fetal thymus prior to birth. However, our data suggest that central tolerance is incomplete for PR1-specific T cells, which is in agreement with the recent report by Yu et al. [22] that self-specific T cells can escape complete negative selection in the thymus, resulting in detectable levels of circulating self-specific T cells. We speculate that the presence of PR1-CTL in UCB may, at least in part, be explained by incomplete deletion in the thymus due to the lack of expression of PR1 by medullary thymic epithelial cells (mTECs) [23, 24]. Furthermore, the diminishing number of PR1-CTL in healthy adult PB suggests that these cells are deleted peripherally over time. These results agree with a report by Garderet et al. that the UCB T-cell repertoire is more comprehensively polyclonal than the adult PB repertoire [25].

Our finding of an increased frequency of PR1-CTL in UCB has important clinical implications. Surprisingly, the percentage of PR1-CTL observed in UCB was within one log

of that found in leukemia patients post-immune therapy treatment [5]. Thus, UCB could conceivably represent an ideal source of PR1-CTL, and broadly, a source of CTL that target other tumor antigens in the context of ACT. Overall, our data indicate that PR1-CTL can be expanded from UCB and that the cells are functional against myeloid leukemia *in vitro*. Thus, the adoptive transfer of UCB-derived PR1-CTL could potentially treat myeloid leukemia or prevent leukemia relapse in HLA-A2 positive patients with high-risk myeloid leukemia.

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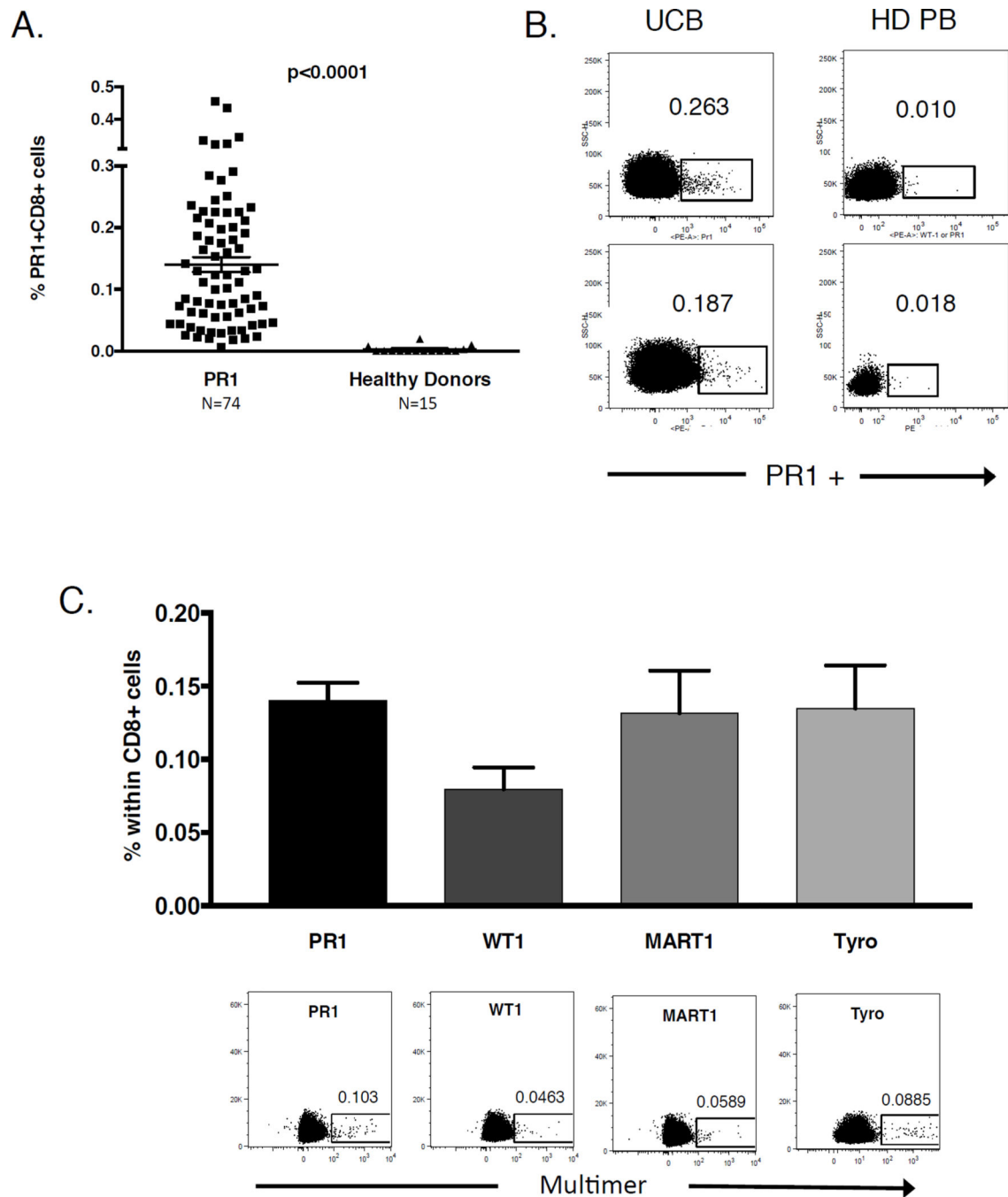


Figure 1. UCB contains a high frequency of PR1-CTL

(A) Summary of the percentage of PR1-CTL in the CD8⁺ population in UCB (0.140%) and healthy adult donor PB (0.0031%). Horizontal bars indicate mean values \pm SD. (B) Representative dextramer staining of PR1-CTL in UCB (left) compared to adult healthy donor PB (right). (C) The average frequency (upper panel) and representative multimer staining (lower panel) of other tumor self-antigen (WT1, MART1, Tyro) specific T cells in UCB (n=5 for non-PR1 antigens).

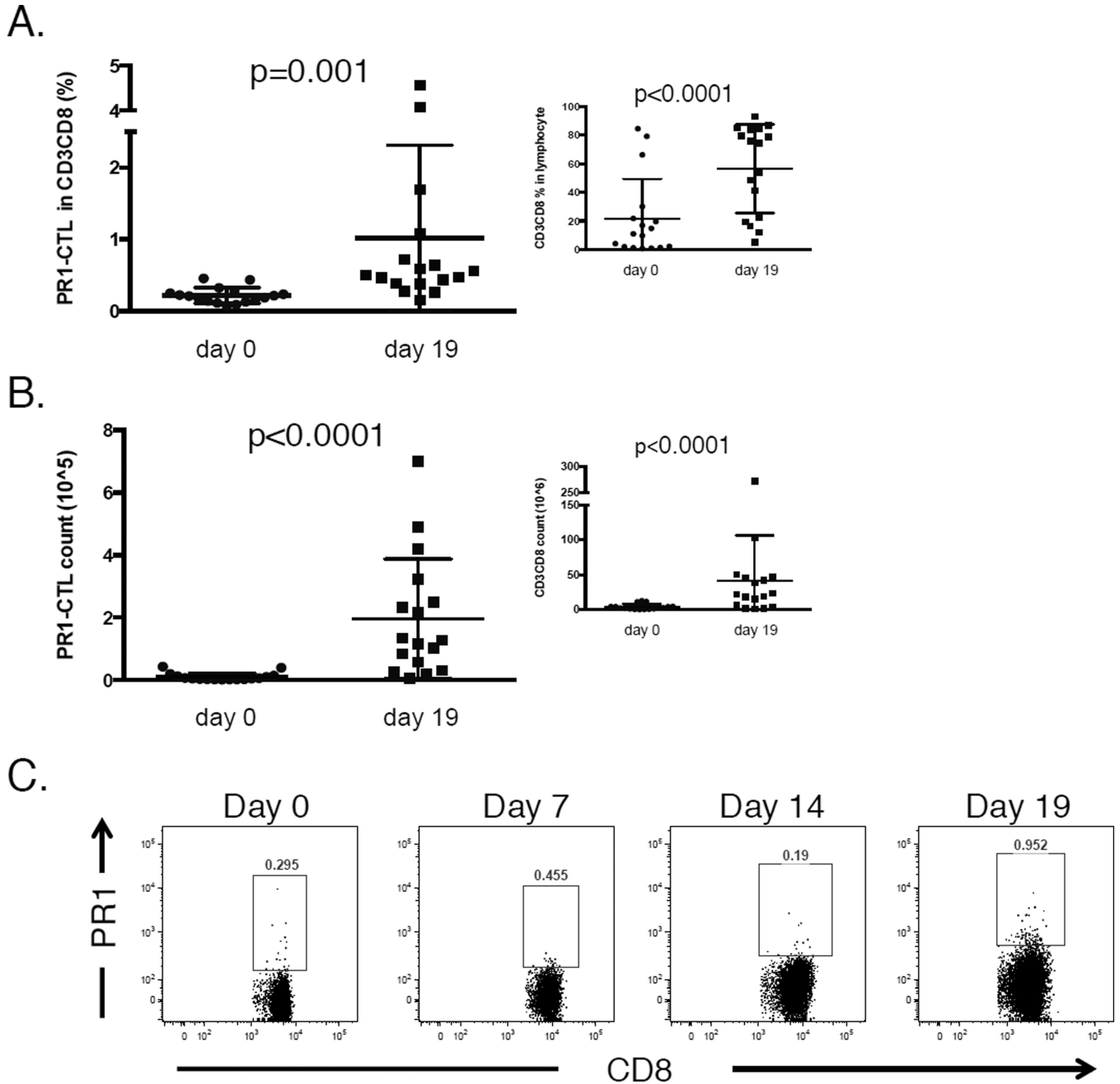


Figure 2. PR1-CTL from UCB units can be expanded 20-fold *ex vivo*
 (A) The mean percentage of PR1-CTL within the CD3⁺CD8⁺ subset increased from 0.219% to 1.014% following stimulation with PR1-peptide pulsed APCs over 19 days (n=17). Inset: The CD3⁺CD8⁺ lymphocyte subset also increased during the expansion (mean 21.5% pre- to 56.4% post-expansion). (B) The absolute number of PR1-CTL increased by an average of 20-fold (95% CI 10.25–38.78) during the expansion while the increase in CD3⁺CD8⁺ T cells was 7.5 fold (inset), indicating preferential expansion of PR1-CTL. (C) Longitudinal PR1 dextramer analyses of a representative HLA-A2⁺ UCB unit undergoing the 19-day *in vitro* PR1-CTL expansion protocol.

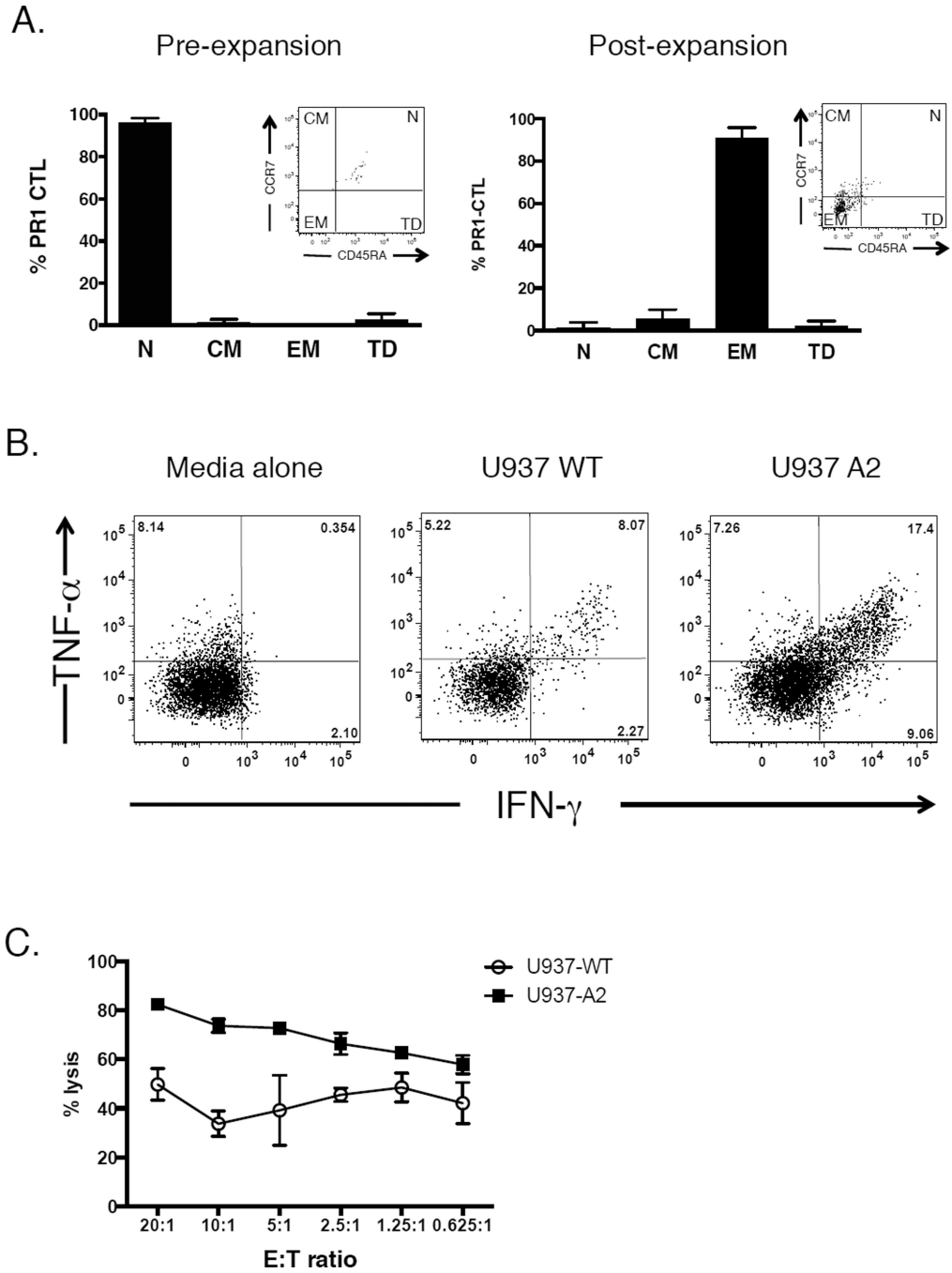


Figure 3. Expanded UCB PR1-CTL are memory CD8⁺ T cells that are functional against an HLA-A2+ leukemia cell line
 (A) Phenotype of UCB PR1-CTL pre- and post-expansion. Phenotype was determined using CCR7 and CD45RA cell surface markers (n=8). N=naïve (CD45RA⁺CCR7⁺), CM=central memory (CD45RA⁻CCR7⁺), EM=effector memory (CD45RA⁻CCR7⁻), TD=terminally differentiated (CD45RA⁺CCR7⁻). Inset: Representative flow cytometry plots of PR1-CTL before and after expansion. (B) Expanded UCB PR1-CTL secrete cytokines in response to a PR1-expressing, A2-transfected leukemia cell line, U937-A2. Expanded UCB cells were incubated either alone or with target cells (U937 WT and U937-A2) and the percentage of T

cells positive for both intracellular IFN- γ ⁺ and TNF- α ⁺ was determined by flow cytometry. (C) Specific cytotoxic activity of expanded UCB PR1-CTL. Target cells U937-A2 and U937 WT were incubated with UCB PR1-CTL in a 4-hr CTL assay at the indicated E:T ratios. Data are presented as mean percent specific killing of target cells \pm SD with experiments done in triplicate.

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