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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

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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-CTLs) 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-CTLs are infrequent but preferentially lyse myeloid leukemia cells. We sought to examine PR1-CTLs in umbilical cord blood (UCB) because UCB units provide a rapidly available cell source and a lower risk of graft-versus-host disease, even in the setting of mismatched human leukocyte antigen (HLA) loci. *Methods.* We first determined the frequency of PR1-CTLs 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 (naive, effector, memory) and function against PR1-expressing target cells. *Results.* PR1-CTLs 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-CTLs are phenotypically naive, consistent with the UCB CD8$^+$ population as a whole. In addition, the cells can be expanded by stimulation with PR1 peptide-pulsed APCs. Expansion results in an increased frequency of PR1-CTLs, up to 4.56%, with an average 20-fold increase in total number. After expansion, UCB PR1-CTLs express markers consistent with effector memory T cells. Expanded UCB PR1-CTLs 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 to show that T cells specific for a leukemia-associated antigen are found at a significantly higher frequency in UCB than adult blood. Our results also demonstrate specific cytotoxicity of expanded UCB-derived PR1-CTLs against PR1-expressing targets. Together, our data suggest that UCB PR1-CTLs could be useful to prevent or treat leukemia relapse in myeloid leukemia patients.

Key Words: *antigen specific cytotoxic T lymphocyte, leukemia, PR1, umbilical cord blood*

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-specific cytotoxic lymphocytes (PR1-CTLs) and an anti-PR1/HLA-A2 antibody [3–7]. PR1-specific memory T cells have been detected at 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-CTLs 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-CTLs can be transferred from adult donor to recipient,

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leading to an expansion of the CTLs and resulting in a graft-versus-leukemia effect in the recipient.

Because of the potential therapeutic value of PR1-CTLs 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-CTLs could provide cells capable of targeting, and possibly eliminating, myeloid leukemia. Herein, we investigated the frequency, phenotype and anti-leukemia function of PR1-CTLs derived from UCB. Despite the limited number of mononuclear cells in an average UCB unit (~5×10^8 cells) and the low frequency of PR1-CTLs 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.

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 ($_{169\text{-}177}$VLQELNVTV) 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$_{(368\text{–}376)}$ dextramers were all from Immudex, Denmark.

Ex vivo expansion of PR1-CTL from UCB

Stimulation and expansion of UCB-derived PR1-CTLs 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 interleukin (IL)-7 (10 ng/mL), IL-12 (10 ng/mL) and IL-15 (5 ng/mL) (R&D Systems). 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 mmol/L 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) 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% CO_2 for a total of 16 h (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 min at 37°C, washed four times with RPMI 1640 and resuspended at 2×10^5/mL. Target cells (2000) 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 h [12].

Statistical analysis

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

Results

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

As shown in Figure 1A, the frequency of PR1-CTLs in UCB was approximately 45 times higher than in healthy adult PB (mean 0.140 ± 0.104% of $CD8^+$ cells in UCB ($n = 74$) versus 0.0031% of $CD8^+$ cells in adult PB ($n = 15$, $P < .0001$)). PR1-CTL with low, intermediate and high avidities were clearly visible within the UCB $CD8^+$ population when stained with