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A PR1-Human Leukocyte Antigen-A2 Tetramer Can Be Used to Isolate Low-Frequency Cytotoxic T Lymphocytes from Healthy Donors That Selectively Lyse Chronic Myelogenous Leukemia[1]

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ABSTRACT

We previously showed (E. Clave *et al.*, J. Immunother., *22:* 1–6, 1999; J. Molldrem *et al.*, Blood, *88:* 2450–2457, 1996) that PR1, a human-lymphocyte-antigen (HLA)-A2.1-restricted peptide from proteinase 3, could be used to elicit CTLs from normal individuals. These CTLs showed HLA-restricted cytotoxicity and colony inhibition of myeloid leukemia cells that overexpress proteinase 3. In this study, we constructed a phycoerythrin-labeled PR1-HLA-A2 tetramer to identify PR1-specific CTLs by flow cytometry. No peripheral blood lymphocytes from three HLA-2.1^+ donors stained with the tetramer, but, after 20 days in culture with weekly PR1 stimulation, 2–8% became tetramer$^+$. Tetramer staining identified up to 40-fold more PR1-specific CTLs than were identified by limiting dilution analysis and correlated better with lysis of PR1-coated T2 cells ($R^2 = 0.95$ *versus* $R^2 = 0.76$). Tetramer$^+$ CTLs were memory phenotype (91% CD45RO$^+$), and most (58% CD95$^+$) were activated. Tetramer-sorted allogeneic CTLs produced 83% lysis of HLA-$A2.1^+$ chronic myelogenous leukemia (CML) blasts at an E:T ratio of 2.5:1, compared with 23% lysis by nonsorted CTLs, with no background lysis of HLA-$A2.1^+$ normal cells. Cytoplasmic proteinase-3 expression was one log greater in CML blasts than in normal granulocytes. These results show that a PR1-HLA-A2 tetramer can be used to identify and select CTLs from normal donors that preferentially lyse CML cells, which could be used for leukemia-specific adoptive immunotherapy.

INTRODUCTION

We have previously shown that the nonpolymorphic (1) HLA-A2.1-restricted[3] self-peptide PR1 (aa 169–177) derived from proteinase 3 could be used to elicit CTL lines from healthy donors (2). These PR1-specific CTLs lysed myeloid leukemia cells (2) and inhibited CFU-GM from HLA-$A2^+$ patients with CML (3). The amount of leukemia lysis and colony inhibition was proportional to proteinase-3 overexpression in leukemia cells compared with HLA-matched normal marrow cells. In support of our findings, Papadopoulos *et al.* recently found that peptides derived from proteinase 3 were naturally processed and presented on MHC I molecules from CD34$^+$ CML blasts (4). On the basis of these results and because proteinase 3 is aberrantly expressed in many cases of myeloid leukemia (5), proteinase 3 may serve as a leukemia-associated antigen. Lymphocytes with PR1-specificity may, therefore, be useful in adoptive immunotherapy strategies to treat patients with myeloid leukemia if they (the lymphocytes) could be easily isolated and expanded.

A recently developed technique to identify low-frequency peptide antigen-specific CTLs by flow cytometry uses fluorescence-labeled HLA-peptide tetramers as a staining reagent (6). These tetramers are constructed by folding the HLA heavy chain, β_2-microglobulin, and peptide, followed by biotinylation at the COOH terminus of the HLA heavy chain and conjugation with fluorochrome-labeled avidin at a 4:1 molar ratio (6). Using viral peptide tetramers, CTLs with LCMV-, EBV-, and HIV-reactive specificities have been identified in patients infected with these viruses (7–9). Tetramer labeling has allowed sensitivity down to 0.02% CD8$^+$ cells to identify antigen-specific CTLs, and correlative studies with conventional LDA show that LDA may underestimate the true number of peptide-specific CTLs (10). This may occur because LDA relies on CTLs that can survive in culture for 2 weeks, whereas tetramer staining labels all peptide-specific CTLs regardless of their proliferative potential. This also calls into question which assay may best correlate with lytic function and possible clinical relevance.

In this study, we set out to determine the relationship between tetramer labeling of PR1-specific CTLs and the CTLp frequency determined by LDA and to adapt a cell-sorting technique using the PR1-tetramer to select CTLs that could be used in adoptive immunotherapy of myeloid leukemias. We first established short-term cultures of PR1-reactive CTLs from PBMCs of HLA-$A2.1^+$ healthy donors. The number of PR1-reactive CTLp in the short-term cultures as measured by LDA underestimated by as much as 40-fold the number of PR1-specific CTLs measured using the PR1-HLA-A2.1 tetramer. Specific lysis of PR1-coated target cells by bulk culture CTL correlated best with the number of PR1-HLA-A2.1 tetramer positive cells contained in the culture. Moreover, the PR1-specific CTLs separated from a short-term culture by FAC sorting with the PR1-HLA-A2.1 tetramer showed 56% greater lysis of CML blasts compared to the nonsorted bulk culture CTLs, with no lysis of HLA-matched normal marrow progenitors at an effector to target (E:T) ratio of 10:1.

MATERIALS AND METHODS

Patients and Donors. All donors and patients were treated at the University of Texas M. D. Anderson Cancer Center on protocols approved by the Institutional Review Board. After informed consent, cells from a patient with blast crisis CML (P1, Table 1) as well as the paired HLA-identical healthy PBPCs from the donor for that patient (D1), were obtained before bone marrow transplant. Cells from three other unrelated HLA-$A2.1^+$ healthy donors were also obtained from peripheral blood for the generation of the PR1-specific cell lines CTL1–3. The cells were separated using Ficoll-Hypaque gradient-density (Organon Teknika Co., Durham, NC) and subsequently frozen in RPMI 1640 complete (CM, Life Technologies, Inc., Gaithersburg, MD) supplemented with 20% heat-inactivated pooled human AB serum (Sigma Chemical, St. Louis, MO) and 10% DMSO according to standard protocols. Before use, cells were thawed, washed, and suspended in CM + 10% AB. HLA-A2 phenotype was confirmed using the BB7.2 mouse monoclonal antibody (ATCC, Rockville, MD).

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[3] The abbreviations used are: HLA, human leukocyte antigen; aa, amino acid; CFU-GM, granulocyte-macrophage colony-forming unit; CML, chronic myelogenous leukemia; CTLp, precursor CTL(s); CTLe, effector CTL(s); LDA, limiting dilution analysis/analyses; PBMC, peripheral blood mononuclear cell; PBPC, peripheral blood progenitor cell; CM, 25 mM HEPES buffer, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin; CMV, cytomegalovirus; ATCC, American Type Culture Collection; FBS, fetal bovine serum; IL, interleukin; rhIL-2, recombinant human IL-2; BSP, substrate peptide; PHA, phytohemagglutinin; DLI, donor lymphocyte infusion.

Table 1 *Patient and donor HLA types*[a]

Patient/Donor	Cell description	*HLA-A*[b]	*HLA-B*[b]	*HLA-DR*[b]
ND1	CTL1	2,2	44,60	4,4
ND2	CTL2	2,3	35,44	1,4
ND3	CTL3	2,24	35,39	4,4
D1	Normal marrow	2,26	44,45	4,7
P1	CML-BC	2,26	44,45	4,7

[a] CTL1–3, CTLs derived from three separate healthy donors (ND1–3); CML-BC, CML blast crisis.
[b] Alleles of the gene loci.

Peptide Synthesis. PR1 (aa 169–177) peptide ^{169}VLQELNVTV^{177}, derived from the azurophilic granule protein proteinase 3, and Influenza B nuclear protein (Flu, aa 85–94) peptide ^{85}KLGEFYNQMM^{94} were synthesized by Biosynthesis (Lewisville, TX) to a minimum of 95% purity as measured by high-performance liquid chromatography. CTLs specific for PR1, a peptide with high binding affinity to HLA-A2.1, have previously been shown to lyse leukemia cells from patients with CML and acute myelogenous leukemia (2) and to inhibit CFU-GM from patients with CML (3). The CMV peptide NLVPMVATV, derived from the pp65 protein, was synthesized by the Stanford Protein and Nucleic Acid Facility to a minimum of 90% purity. The pp65 protein is the immunodominant antigen from CMV (11, 12).

Cell Lines. T2 cells (ATCC) are a hybrid human cell line that lacks most of the MHC class II region including the known transporter proteins for antigenic peptide and proteasome genes. They contain the gene *HLA-A*0201* but express very low levels of cell surface HLA-A2.1 and are unable to present endogenous antigens (13, 14). These cells were maintained in culture in CM + 10% FBS (Atlanta Biologicals, Norcross, GA).

Generation of Peptide-specific T Cells from Healthy Human PBMCs. PBMCs from three healthy HLA-A2.1$^+$ donors (ND1–3, Table 1) were stimulated *in vitro* with PR1 or pp65 using a protocol adapted from previous studies (2). Briefly, T2 cells were washed three times in serum-free CM and by incubating with peptide at 10 μg/ml for 2 h in CM. The peptide-loaded T2 cells were then irradiated with 7500 cGy, washed once, and suspended with freshly isolated PBMCs at a 1:1 ratio in CM supplemented with 10% AB. After 7 days in culture, a second stimulation was performed; and the following day, 10 IU/ml rhIL-2 (Biosource International, Camarillo, CA) were added. After 14 days in culture, a third stimulation was performed, followed on day 15 by the addition of rhIL-2. After a total of 20 days in culture, the peptide-stimulated T cells were harvested and tested for peptide-specific lysis of human bone marrow cells derived from a patient with CML (P1) or from PBPCs from an HLA-matched normal donor (D1). Peptide-specific CTLs were also tested for lysis of peptide-coated T2 cells.

Production of MHC/Peptide Tetramers. The production of MHC/peptide tetramers was described in detail previously (6). Briefly, a 15-amino acid BSP for BirA-dependent biotinylation has been engineered onto the COOH terminus of HLA-A2. The A2-BSP fusion protein and human β2-microglobulin were expressed in *Escherichia coli* and were folded *in vitro* with the specific peptide ligand. The properly folded MHC-peptide complexes were extensively purified using FPLC and anion exchange and were biotinylated on a single lysine within the BSP using the BirA enzyme (Avidity, Denver, CO). Tetramers were produced by mixing the biotinylated MHC-peptide complexes with PE-conjugated Neutravidin (Molecular Probes) at a molar ratio of 4:1. PR1 tetramers were validated by staining against a CTL line specific for PR1. CMV tetramers were validated by staining with PBMCs from a CMV-immune individual. Specificity was demonstrated by the lack of staining of irrelevant CTLs. By titrating positive CTLs into PBMCs from normal controls, we established our limit of detection to be as low as 0.01% of CD8$^+$ cells. Each tetramer reagent was titered individually and used at the optimum concentration, generally 20–50 μg/ml.

Antibodies and Flow Cytometry. Cytoplasmic proteinase-3 staining was determined by the following procedure: (*a*) cells were permeabilized and fixed with Ortho PermeaFix (Ortho Diagnostics, Raritan, NJ) according to the

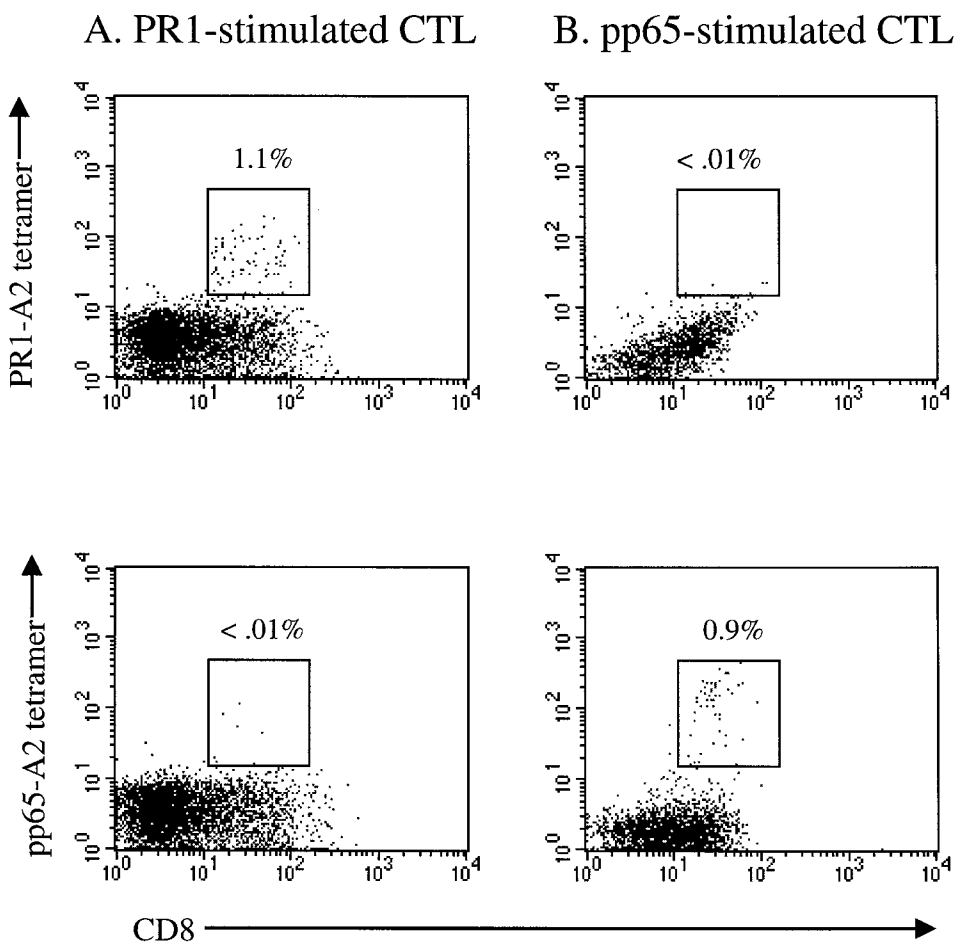

Fig. 1. PR1 tetramer validated. PBMCs from ND2 were stimulated weekly with 10 μg/ml PR1 or pp65 peptides. On day 20, lymphocytes were washed twice and incubated with FITC-labeled anti-CD8 for 30 min. Cells were washed and incubated with PE-labeled peptide-tetramers for 40 min and analyzed by flow cytometry. Plots display CD8 and tetramer staining with the tetramer$^+$ cells boxed. Analysis of PR1-stimulated CTLs shows staining with the PR1 tetramer but not with the pp65 tetramer (*A*). Lymphocytes stimulated weekly with pp65 did not stain with the PR1 tetramer but did stain with the pp65 tetramer (*B*).

Table 2 *Comparison of tetramer staining, CTLp frequency, and specific lysis*

Healthy donor line	% tetramer$^+$ (day 21)	CTLp frequency[a]		% specific lysis[b] (day 21)
		Day 0	Day 21	
CTL1	2	345,000	1,446	63
CTL2	4	115,098	905	79
CTL3	8	15,781	106	94

[a] CTLp frequency $= \frac{1}{n}$.

[b] Healthy donor CTLe at 20:1 ratio with PR1-coated T2 cells as target cells.

manufacturer's directions; (*b*) the cells were stained with 5 μl of mouse antibody to human proteinase 3 (Accurate Chemical and Scientific Corporation, Westbury, NY) and incubated for 40 min at room temperature; (*c*) the cells were washed once with PBA (PBS with 0.1% NaN_3 and 0.1% BSA); (*d*) the cells then were labeled with FITC-labeled goat antimouse IgG (PharMingen, San Diego, CA) for 30 min at room temperature and washed with PBA; and (*e*) the cells were then fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Mouse monoclonal anti-HLA-A2.1 antibody BB7.2 was derived from culture supernatant of a hybridoma cell line (ATCC) and was not labeled.

For surface antigen staining, roughly 100,000 CTLs were incubated at 4°C for 1 h with the antibody. PR1-HLA-A2.1 tetramer was incubated at 50 μg/ml final concentration in PBS plus 2% FCS. After washing, cells were incubated with FITC-labeled CD8 (Caltag, Burlingame, CA) or with purified unlabeled CD8 (Caltag) plus APC-labeled goat antimouse (Caltag) before adding either FITC-labeled CD95 (Becton Dickinson, San Jose, CA) or FITC-labeled CD45RO (Becton Dickinson) for 30 min on ice. Cells were washed and fixed in 2% paraformaldehyde and analyzed on a FACScan (Becton Dickinson), and data were analyzed using CELLQuest (Becton Dickinson) software. Cell sorting was performed on the FACS Vantage (Becton Dickinson), and cells were selected for both PE-labeled PR1-HLA-A2.1 tetramer and FITC-labeled CD8 (Caltag) after gating on live cells.

CTL Precursor Frequency by Microtiter-LDA The previously frozen PBMC samples were thawed and then washed three times in serum-free CM and counted. Nineteen and one-half million cells (7.5 million stimulator cells and 12 million responder cells) were prepared for the assay. One million additional PBMCs were used to generate PHA (Sigma Chemical)-stimulated blasts that were used as target cells on day 10 of the assay. One million unrelated HLA-disparate PBMCs (third party) were used as a positive control, with one-half of the total number used as stimulator cells and the other half as responder cells.

PHA blasts were generated by placing 10^6 cells in 1.0 ml of CM supplemented with 10% FBS (Sigma Chemical) in a 25-cm^2 flask. Five μl/ml PHA were added, and the cells were cultured in 5% $C0_2$ at 37°C. On days 4, 6, and 8 of the culture, additional CM + 10% FBS with 5 μl/ml PHA (Sigma Chemical) and 500 IU/ml rhIL-2 (Biosource International) were added. Cells were counted, and cultures were maintained at 10^6 cells/ml. These cells were used as target cells on day 10 of the CTLp assay.

PBMC responder cells (from ND1–3) were plated into high-profile Terasaki Trays (Robbins Scientific, Sunnyvale, CA) at 6 dilutions (from 5×10^4 to 5×10^5 PBMCs/well) using 24 replicates at each dilution in CM + 10% human AB serum (Sigma Chemical). Third party responders were plated at a single dilution (5×10^4 PBMCs/well). Patient PBMC stimulator cells were either pulsed with peptide (PR1 or Flu as a positive control peptide) at 20 μg/ml or with no peptide for 90 min at 37°C, washed once with serum-free CM, and then irradiated with 2500 cGy. They were then plated into the wells containing the responder cells as well as 24 additional wells of stimulators alone. On days 3 and 7, 60 IU/ml IL-2 were added to each well. On day 10, the previously prepared PHA blasts from the same patient (as target cells) were either pulsed with PR1 or Flu at 20 mg/ml or no peptide for 90 min at 37°C, washed once with serum-free CM, and then labeled with Calcein AM (Molecular Probes, Eugene, OR) as described in the cytotoxicity experiments below. One thousand target cells per well were plated, lightly centrifuged at 800 rpm for 1 min, and then cultured in 5% $C0_2$ at 37°C for 4 h. Five μl of FluoroQuench EB Stain-Quench Reagent (One Lambda Inc., Canoga Park, CA) were added to each well, and the plates were analyzed for fluorescence emission using an automated CytoFluor II plate reader (PerSeptive Biosystems, Framingham, MA).

The mean plus three SDs of the 24 wells containing the stimulators alone was determined as the cutoff value for background fluorescence. Any experimental well less than the cutoff value was considered positive for lysis; and from the fraction of negative wells at the various responder cell dilutions, the frequency of CTLp was calculated using the maximum likelihood method based on Poisson probabilities.

CTL Cytotoxicity Assay. A semi-automated mini-cytotoxicity assay was used to determine specific lysis as described previously (2). Effector cells (CTL1–3 cells) were prepared in doubling dilutions from 6×10^3 to 25×10^3 cells/well and were plated in 40-μl, 60-well Terasaki trays (Robbins Scientific) with six replicates per dilution. Target cells (T2 cells ± peptides, marrow-derived leukemic cells from P1 or PBPCs from D1) at a concentration of 2×10^6 cells/ml were stained with 10 μg/ml Calcein-AM (Molecular Probes Inc.) for 60 min at 37°C. After washing three times in CM + 10% AB, target cells were resuspended at 10^5 cells/ml (*i.e.*, 10^3 target cells in 10 μl of medium were added to each well containing effector cells). Wells with target cells alone and medium alone were used for maximum (max) and minimum (min) fluorescence emission, respectively. After 4-h incubation at 37°C in 5% CO_2, 5 μl of FluoroQuench (One Lambda, Inc.) were added to each well, and the trays were centrifuged for 1 min at $60 \times g$ before the measurement of fluorescence (excitation at 485 nm, emission measured at 530 nm) using an automated CytoFluor II plate reader (PerSeptive Biosystems). A decrease in the fluorescence emission is proportional to the degree of lysis of target cells, once the released dye is quenched by the hemoglobin contained in the FluoroQuench reagent. The percentage of lysis was calculated as follows:

$$\%\text{lysis} = \left\{1 - \left(\frac{\text{Mean experimental emission} - \text{mean min}}{\text{Mean max} - \text{mean min}}\right)\right\} \times 100\%$$

RESULTS

PR1-HLA-A2 Tetramer Validation. PBMCs from a normal healthy donor homozygous for HLA-A2 (ND1, Table 1) were stimulated with either PR1- or pp65-pulsed T2 cells as positive controls. The donor was seropositive for CMV by latex agglutination (data not shown). After 20 days in culture with weekly peptide restimulation, CTLs were tested for staining with the PR1-HLA-A2 tetramer and a pp65-HLA-A2 tetramer.

The specificity of the PR1-HLA-A2 tetramer is demonstrated by its ability to stain T lymphocytes from ND1 elicited with weekly PR1 stimulation but not lymphocytes from ND1 elicited with pp65. As

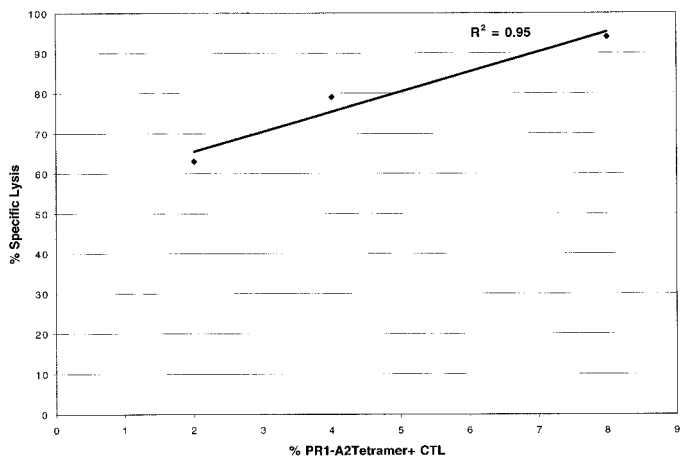

Fig. 2. The number of lymphocytes stained with the PR1 tetramer correlates with the amount of specific lysis of PR1-coated T2 cells. PBMCs from donors ND1–3 were stimulated weekly with 10 μg/ml PR1; the corresponding day-20 CTL1–3 cell lines were then stained with FITC-labeled anti-CD8 and PE-labeled PR1 tetramer; and the percentage of dual-positive cells was analyzed by flow cytometry. The CTL1–3 cell lines were also incubated at 37°C with PR1-coated T2 cells at an E:T ratio of 20:1 in a 4-h microtiter cytotoxicity assay, and specific lysis was determined. Specific lysis correlated with the percentage of CTLs that stained with the PR1 tetramer by linear regression analysis ($R^2 = 0.95$).

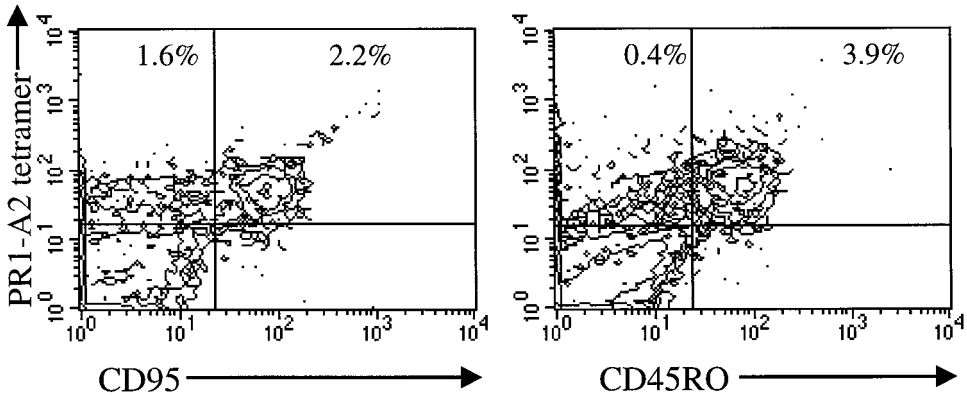

Fig. 3. Phenotype of PR1-stimulated CTLs. PBMCs from ND2 were stimulated weekly with 10 μg/ml PR1, and on day 20, cells were washed and incubated with unlabeled anti-CD8 for 30 min. Cells were then incubated for 30 min with APC-labeled secondary antibody. Cells were then washed and incubated with PR1 tetramer for 40 min, washed again, and incubated with either FITC-labeled anti-CD95 or FITC-labeled anti-CD45RO for another 30 min. Only $CD8^+$ cells were gated as CTLs for analysis. Of the PR1 tetramer-staining CTLs, 58% displayed an activated phenotype ($CD95^+$), and 95% were of memory phenotype ($CD45RO^+$). Seventy-five percent log density contour plots were generated to demonstrate the small populations that stain positive for the PR1 tetramer. The percentage of cells in the lymphocyte gate is shown for the quadrants containing cells that stain positively with the PR1 tetramer.

shown in Fig. 1, the PR1-HLA-A2 tetramer stained approximately 2% of PR1-stimulated lymphocytes and < 0.1% of pp65-stimulated lymphocytes, whereas the pp65-HLA-A2 tetramer stained 3% of pp65-stimulated lymphocytes and < 0.1% of the PR1-stimulated lymphocytes.

Comparison of Lymphocyte Staining with the PR1-HLA-A2 Tetramer and PR1-specific CTLp Frequency by LDA. PBMCs from $HLA\text{-}A2^+$ healthy donors (ND1–3, Table 1) were stimulated with PR1-pulsed T2 cells. The T2 cells were incubated with 10 mg/ml PR1 at 37°C for 2–4 h, irradiated with 7500 cGy, and combined with PBMCs to induce PR1-specific CTLs as described previously (2). On the day the cultures were set up (day 0) and again after 20 days in culture with weekly restimulation, lymphocytes were tested for CTLp frequency against the PR1 peptide by LDA using a microtiter method. Lymphocytes on days 0 and 20 were also tested for the percentage of CTLs that stained with the PR1-HLA-A2 tetramer. Lymphocytes from day 20 were tested for specific lysis of Calcein AM-labeled T2 cells loaded or not loaded with 10 μg/ml PR1.

As shown in Table 2, the CTLp frequency against PR1 on day 0 from each of the three donors ND1–3 ranged from as low as 1/345,000 to 1/15,781. At the end of 20 days of PR1 restimulation, the precursor frequency of PR1-specific CTLs had increased in each of the three cultures by as much as 2.5 logs, with the greatest increase in the culture established from ND1 (CTL 1). Although the percentage of PR1-HLA-A2 tetramer-staining CTLs was undetectable on day 0, by day 20, cultures from all of the three donors contained 2–8% PR1-specific CTLs.

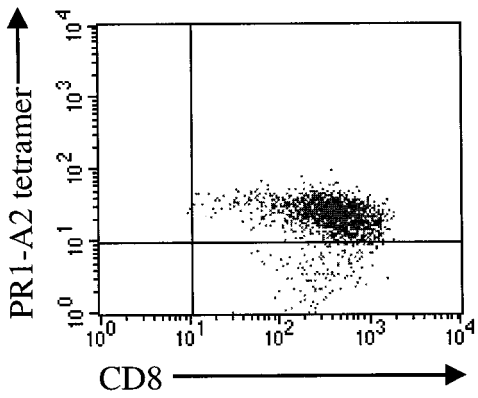

Fig. 4. Phenotype of CTL2 sorted with the PR1 tetramer. PBMCs from donor ND2 were stimulated weekly with 10 μg/ml, and on day 20, the cells were washed and incubated with FITC-labeled anti-CD8 for 30 min. Cells were washed and incubated with PE-labeled PR1 tetramer for 40 min. Cells were washed and analyzed by flow cytometry. The cells were then selected using a FACS Vantage cell sorter for dual-positive cells. After collection, cells were reanalyzed by flow cytometry. CTL sorting resulted in a 78% yield of dual-positive staining cells, with 95% purity.

Table 3 *Yield of CD8 and PR1-tetramer double positive after FACS sorting*

Presort		Postsort		
Total cells	No. of $CD8^+$/PR1-$tetramer^+$ cells (%)	Total cells	No. of $CD8^+$/PR1-$tetramer^+$ cells (%)	Yield[a]
8×10^6	1.6×10^5 (2)	1.23×10^5	1.17×10^5 (95)	78%

[a] Actual yield of CD8 and PR1-tetramer double positive cells.

Lysis of T2 cells coated with 10 μg/ml PR1 by all of the three donor CTLs was less than 20% at an E:T ratio of 20:1 on day 0. By day 20, however, each of the three resulting cell lines showed 63–94% specific lysis of PR1-coated T2 cells. The lysis of T2 cells coated with 10 μg/ml Flu (control peptide) was less than 20% at E:T ratio of 20:1 for all three of the CTL lines (data not shown).

The number of PR1-specific CTLp on day 20 measured by LDA ranged from 9-fold (CTL 3) to 40-fold (CTL 2) less than the percentage of PR1-specific CTLs measured with the PR1-HLA-A2.1 tetramer. In Fig. 2, the amount of specific lysis of PR1-coated T2 cells by all three of the CTL lines is plotted against the percentage of lymphocytes that stained with the PR1-HLA-A2 tetramer. Specific lysis correlated better with the percentage of PR1-specific CTLs contained in the lymphocyte bulk culture ($R^2 = 0.95$) than with CTLp frequency determined by LDA ($R^2 = 0.76$).

Phenotype of PR1-specific CTLs. Fig. 3 shows the phenotype of the CTL2 cell line on day 20. All of the cells were labeled with unconjugated anti-CD8 plus the PE-labeled PR1-HLA-A2 tetramer and FITC-labeled anti-CD95 or CD45RO. A secondary antibody conjugated to APC and directed against the anti-CD8 antibody was used to gate only $CD8^+$ cells. Of the total $CD8^+$ lymphocyte population, 2.2% stained with antibody to CD95 (58% of all of the PR1-specific CTLs), which indicated an activated cell phenotype. Nearly all (91%) of the PR1-specific CTLs stained with antibody to CD45RO, which is consistent with a memory T-cell phenotype.

PR1-HLA-A2 Tetramer-sorted CTLs Efficiently Lyse CML. We next sought to determine whether PR1-HLA-A2 $tetramer^+$ lymphocytes separated from bulk culture could lyse allogeneic CML cells from the bone marrow of an $HLA\text{-}A2.1^+$ individual (P1, Table 1). CTL2 cells were stained with the PR1-HLA-A2 tetramer and FITC-labeled antibody to CD8, and the dual-labeled lymphocytes were selected using a FACS Vantage cell sorter. Fig. 4 shows the phenotype of the sorted population. The yield of PR1-HLA-A2 $tetramer^+$ cells was 78%, with 95% purity of PR1-specific lymphocytes as shown in Table 3.

The day-20 bulk culture CTL2 were selected using the FACS Vantage flow sorter for $CD8^+$ PR1 $tetramer^+$ cells and placed back into culture with 20 IU/ml IL-2. After 48 h, the CTLs were washed twice and compared with nonsorted CTLs in a 4-h cytotoxicity ex-

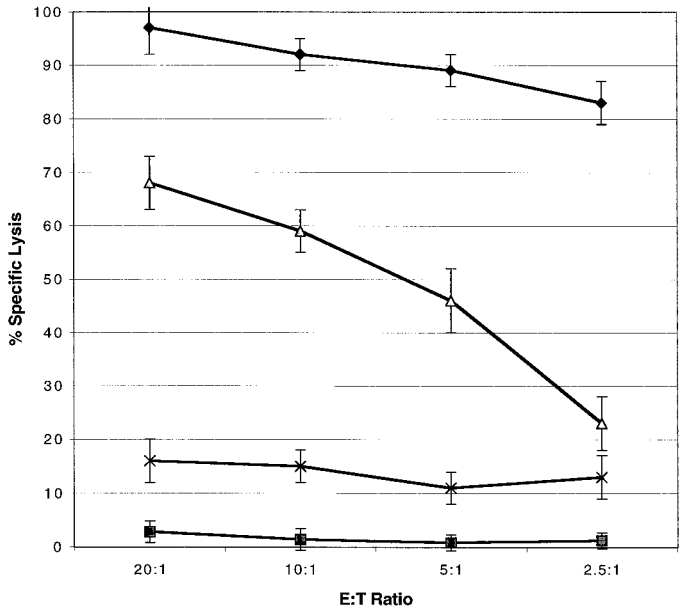

Fig. 5. Specific lysis of CML cells by PR1 tetramer-sorted CTLs is greater than lysis by the nonsorted bulk culture CTLs. PBMCs from donor ND2 were stimulated weekly with 10 μg/ml PR1, and on day 20, the resulting CTL2 were stained with FITC-labeled anti-CD8 and PE-labeled PR1 tetramer. An aliquot of dual-staining CTLs was selected using a FACS Vantage cell sorter, and a separate aliquot of day-20 CTL2 was not stained and did not undergo cell sorting (nonsorted cells). PR1 tetramer-sorted and nonsorted CTL effector cells were then plated with bone marrow cells from a patient with blast crisis phase CML (P1, Table 1) or PBMCs from a HLA-matched donor (D1) in a 4-h cytotoxicity assay at E:T ratios from 20:1 to 2.5:1. Data were pooled from two separate experiments, and six replicate wells were used for each dilution of effector cells. Data are displayed as mean specific lysis ± SD. —◆—, tetramer-sorted CTL2 *versus* P1; —△—, nonsorted CTL2 *versus* P1; —□—, tetramer-sorted CTL2 *versus* D1; —×—, nonsorted CTL2 *versus* D1.

periment. Bone marrow cells from a patient with blast crisis CML (P1, Table 1) were readily lysed by bulk culture CTL2 cells (Fig. 5), whereas granulocyte-colony-stimulating-factor-mobilized PBPCs from an HLA-identical healthy donor (D1) showed only background lysis of less than 20%. By comparison, the PR1-HLA-A2 tetramer-sorted CTLs showed nearly 50% greater lysis of CML cells than the nonsorted CTLs at every E:T ratio tested, with no background lysis of normal PBPC, even at an E:T ratio of 20:1. The addition of antibody to HLA-A2.1 (BB7.2) during the 4-h cytotoxicity assay partially blocked lysis at every E:T ratio (data not shown), as demonstrated previously (2). The PR1-HLA-A2 tetramer-sorted cells were cultured in the presence of 20 IU/ml IL-2 for 48 h before testing for cytotoxicity.

In a second experiment, 1×10^5 PR1-HLA-A2 tetramer-sorted CTL2 cells of 95% purity were cultured in CM supplemented with 100 IU/ml of IL-2 for 72 h. The cells were then stained with the PR1-HLA-A2 tetramer and FITC-labeled antibody to CD8, and the dual-labeled lymphocytes were analyzed. Of 1.4×10^6 total lymphocytes in culture at the end of 72 h, 81% or 1.13×10^6 cells stained dual positive, yielding a 10-fold expansion of dual-positive CTLs (data not shown).

Proteinase 3 Is Overexpressed in CML Blasts. We have previously shown (2, 3) that both the amount of target cell lysis and the amount of CFU-GM inhibition correlate with the intracellular level of proteinase 3 in the leukemia cells. We, therefore, tested cells from P1 and D1 for the amount of cytoplasmic proteinase 3. Cells were first stained with PE-labeled anti-CD34 antibodies. Then, after permeabilizing the cell membrane, indirect staining was performed using a mouse antibody to proteinase 3 and a second FITC-labeled goat antimouse antibody, followed by flow cytometry. Small mononuclear PBPCs from the normal donor D1 were gated to analyze the $CD34^+$ progenitors, and a separate gate for granulocytes was also examined. As shown in Fig. 6*A*, proteinase 3 was expressed only in the granulocytes and not in the $CD34^+$ progenitors of normal PBMCs from D1. However, bone marrow blasts from P1 expressed one log higher cytoplasmic proteinase 3 than normal granulocytes from D1, which normally express a maximal amount of proteinase 3.

DISCUSSION

We have previously shown (2) that allogeneic CTL lines from healthy donors that are specific for the HLA-A2-restricted peptide PR1 preferentially lyse myeloid leukemia targets and suppress CFU-GM from CML patients compared with HLA-matched normal marrow cells (3). We showed this was not due to polymorphic differences in the PR1-coding region of the proteinase 3 gene between the donor CTL lines and the target cells (3). Instead, the amount of target cell lysis and CFU-GM inhibition correlated with the amount of cytoplasmic overexpression of proteinase 3 in the leukemia cells. We chose to examine proteinase 3 as a potential T-cell target because of its overexpression in leukemia cells (5) and because T cells from patients with the autoimmune disease Wegener's granulomatosis proliferated in response to purified proteinase 3, which suggested that it might be easier to elicit T-cell responses to peptides derived from the protein (15).

These studies suggested that PR1 might be a powerful therapeutic target for adoptive allogeneic cellular strategies to treat CML or other myeloid leukemias. We speculated that large numbers of such leukemia-selective CTLe might not be necessary because as few as 1×10^7 T cells/kg unselected allogeneic peripheral blood T cells are sufficient to induce remissions in patients with CML (with estimated leukemic burdens of 10^{10} to 10^{12} cells; Ref. 16). Leukemia-reactive CTLp within the unselected DLI could expand *in vivo* to sufficient numbers of CTLe to eliminate the leukemia. The observation that chronic phase CML responds more frequently to DLI than accelerated phase or blast crisis CML is consistent with the idea that leukemia growth outpaces the expansion of donor antileukemia CTLp (17, 18). Rapidly growing CML and acute myelogenous leukemia might be eliminated with lymphocyte infusions if a sufficient number of preselected leukemia-specific CTLe could be infused to overwhelm the kinetic growth of the leukemia while avoiding any graft-*versus*-host disease.

To better select only the leukemia-reactive CTLs, we studied whether a PR1-HLA-A2 tetramer could be used to identify PR1-specific CTLs in the PBMCs of healthy adult donors. Because we expected the number of CTLs with self-peptide specificity in healthy donors to be low and perhaps below the limit of detection by tetramer staining, we first examined the PR1-specific CTLp frequency in the PBMCs of healthy $HLA\text{-}A2.1^+$ donors using a limiting dilution assay. Although the CTLp frequency was low in two donors, one donor had a surprisingly high frequency of 1 in 15,781. Still, the small number of PR-specific CTLs was below the detection threshold in peripheral blood when up to 100,000 cells were analyzed using the PR1-HLA-A2 tetramer. After only 20 days of *in vitro* expansion with weekly PR1 restimulation, however, the percentage of CTLs that stained with the PR1-HLA-A2 tetramer increased to between 2 and 8%.

The number of PR1-specific CTLp determined by LDA underestimated by as much as 40-fold the number of PR1-specific CTLs when examined by PR1-HLA-A2 tetramer staining in each of the three donors, although the two methods showed a strong correlation ($R^2 = 0.91$). Although the amount of PR1-coated target-cell lysis by day-20 CTL lines did not correlate well with the CTLp frequency as determined by LDA ($R^2 = 0.76$), it did show a strong correlation with

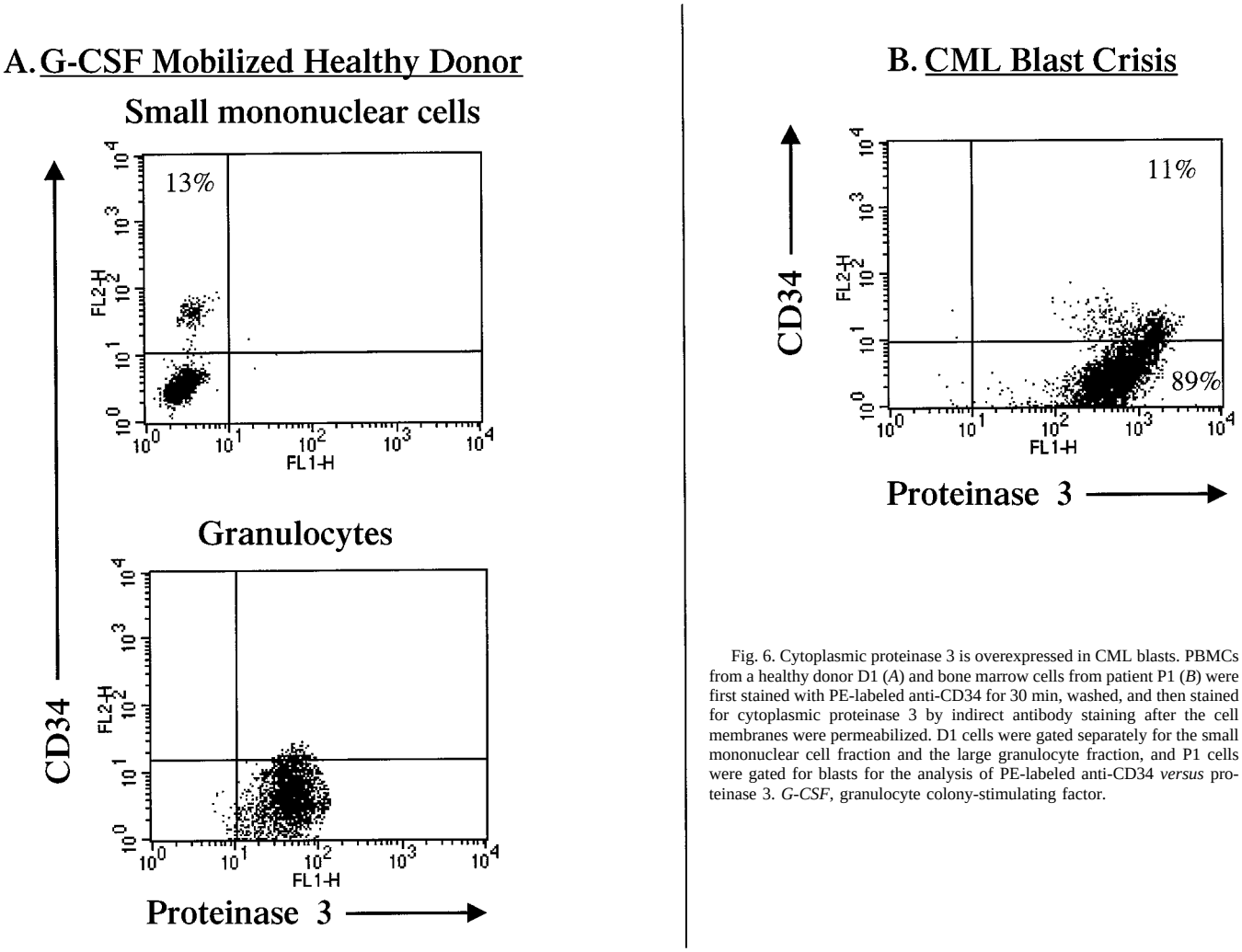

Fig. 6. Cytoplasmic proteinase 3 is overexpressed in CML blasts. PBMCs from a healthy donor D1 (*A*) and bone marrow cells from patient P1 (*B*) were first stained with PE-labeled anti-CD34 for 30 min, washed, and then stained for cytoplasmic proteinase 3 by indirect antibody staining after the cell membranes were permeabilized. D1 cells were gated separately for the small mononuclear cell fraction and the large granulocyte fraction, and P1 cells were gated for blasts for the analysis of PE-labeled anti-CD34 *versus* proteinase 3. *G-CSF*, granulocyte colony-stimulating factor.

the number of PR1-HLA-A2 tetramer-staining CTLs ($R^2 = 0.95$). This suggests that tetramer staining identifies a higher percentage of CTLe than does LDA, perhaps because of the requirement of an adequate number of CTLp that must first survive and proliferate during the 10-day LDA assay.

When PR1-specific CTLs were selected by flow sorting using the PR1-HLA-A2 tetramer, a very homogeneous CTL population was collected. The tetramer-sorted CTLs showed significantly higher lysis of HLA-A2.1$^+$ leukemia cells than nonsorted CTLs, with none of the background lysis of HLA-matched normal marrow cells observed using the nonsorted CTLs. Importantly, the tetramer-sorted CTLs could be easily expanded *in vitro* by another 10-fold over 3 days in the presence of 100 IU/ml of IL-2 while retaining peptide-specificity. Adding to our previous studies, proteinase 3 was overexpressed in both CD34$^+$ and CD34$^-$ leukemia cell targets compared with both normal granulocytes and CD34$^+$ cells.

It remains unclear why the PR1 self-peptide is retained within the T-cell receptor repertoire of healthy individuals who show no evidence of Wegener's granulomatosis or other autoimmune vasculitis. This phenomenon has been observed by investigators studying other self-peptides, which strongly suggests that peripheral tolerance mechanisms must prevail to keep potentially self-reactive CTLs in check (19, 20). One postulated mechanism of peripheral T cell tolerance to self-antigens depends on the antigen activation threshold (21). Therefore, the level of antigen expression by normal myeloid cells may be below the threshold necessary to trigger T-cell recognition and effector function. In the case that leukemia cells overexpress proteinase 3, the threshold of antigen expression may be exceeded resulting in T-cell activation. However, clonal deletion may result if there is a high avidity interaction between the T-cell receptor and the peptide-MHC combination, or anergy may result if the leukemia cells lack costimulatory molecules (21–24). Anergy may not be relevant when designing adoptive immunotherapy strategies using allogeneic CTLs because *ex vivo* T-cell activation would have already occurred during *in vitro* expansion, and a second stimulus from the leukemia cell would no longer be necessary. T-cell expansion strategies may also be designed to expand low avidity CTLe, thereby avoiding clonal deletion of reactive CTLs (21).

A potential pitfall concerning adoptive immunotherapy with only the single peptide PR1-specific CTLs is that this may allow development of resistance by leukemia cells that mutate or down-regulate expression of proteinase 3. However, tetramers used to study immune reactions against EBV have demonstrated that CTLs with single-epitope reactivity may comprise 44% of the entire CD8$^+$ T-cell population in peripheral blood at the height of an effective immune response to the virus (8). If similarly high numbers of PR1-specific T cells could be infused into patients with leukemia, this might have the effect of overwhelming the leukemia before selection pressure could result in resistance.

This study highlights the utility of PR1-HLA-A2 tetramers to identify leukemia-reactive CTLs. Because LDA assays are time-consuming and difficult to perform, this technique offers the advan-

tages of more rapid identification of CTL and better correlation with effector function against leukemia. Tetramers also allow for the selection of a homogeneous population of CTLs that may be useful as adoptive immunotherapy in the relapse setting after BMT or as prophylaxis in high-risk patients for early relapse. We are currently using PR1-HLA-A2 tetramers to investigate the clinical relevance of PR1-specific T-cell expansions in patients after allografting and after DLI. In the future, tetramers could also be used to add back or deplete selective T-cell immunity to many antigens after marrow allografting or as a tool to tolerize T cells to minor antigens that contribute to graft-*versus*-host disease.

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A PR1-Human Leukocyte Antigen-A2 Tetramer Can Be Used to Isolate Low-Frequency Cytotoxic T Lymphocytes from Healthy Donors That Selectively Lyse Chronic Myelogenous Leukemia

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