The receptor for hyaluronic acid-mediated motility induces specific CD8+ T cell response in healthy donors and patients with chronic myeloid leukemia after allogeneic stem cell transplantation

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Received November 8, 2006; Accepted December 27, 2006

Abstract. Recently, we described the receptor for hyaluronic acid-mediated motility (RHAMM) as a leukemia-associated antigen and characterized the RHAMM-derived peptide R3 (pos. 165-173: ILSLELMKL) as a CD8+ T cell epitope. Directing CD8+ T lymphocytes specifically to R3 might help to shape the graft-versus-leukemia effect observed after allogeneic stem cell transplantation (allo-SCT). To detect the potential induction of R3-specific cytotoxic T lymphocytes in chronic myeloid leukemia (CML) patients after allo-SCT and healthy donors, we used mixed lymphocyte peptide culture, enzyme-linked immunospot (ELISPOT) release assays for interferon (IFN)-γ and granzyme B, tetramer staining and ^{51}Cr release cytotoxicity assays. The R3 peptide showed the capacity to elicit specific CD8+ T cell responses characterized by the release of IFN-γ and granzyme B upon stimulation with R3-pulsed T2 cells. Responses to R3 peptide were detected in 67% (6/9) of the CML patients after allo-SCT and 24% (8/34) of healthy donors in ELISPOT assays for IFN-γ and granzyme B. These R3-specific CD8+ T cells comprised predominantly effector cells (CCR7-CD45RA+ or CD27-CD45RA+) in patients with CML after allo-SCT or healthy donors respectively. Cytotoxicity assays demonstrated effective lysis of CML progenitor cells by R3-primed CD8+ T lymphocytes. Imatinib inhibited the functional activation of R3-specific CD8+ T lymphocytes. In summary, we demonstrated R3-specific CD8+ effector T lymphocytes after allo-SCT in CML patients which might have been augumented by R3 peptide vaccination and hampered at least partially by imatinib in this particular patient cohort.

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Key words: chronic myeloid leukemia, stem cell transplantation, cytotoxic T lymphocytes, receptor for hyaluronic acid mediated motility

Introduction

In the past decade great efforts have been devoted to the search of tumor/leukemia-associated antigens (TAAs/LAAs) with the aim to identify molecules selectively expressed on tumor and leukemia cells that might represent optimal targets for cytotoxic immune responses (1,2). The definition of TAAs/LAAs constitutes a corner-stone for the development of vaccines against malignant diseases to be used alone or as a complement to conventional anti-cancer/leukemia therapies. However, this immuno-therapeutic approach has been halted by problems related to the specificity, the distribution among histologically different malignant diseases and the frequency of expression, and especially the immunogenicity of TAAs/LAAs (3,4).

The receptor for hyaluronic acid-mediated motility (RHAMM), also designated CD168, is a receptor for hyaluronan, a glycoaminoglycan that plays a fundamental role in cell growth, differentiation, and motility. RHAMM is a cell-surface receptor (5-7) and is highly expressed in different tumor cell lines (8,9), in multiple myeloma (10), acute myeloid leukemia (AML)/CML (11) and chronic lymphocytic leukemia (CLL) (12), but not at a significant level in adult somatic cells (11,13). For this reason, RHAMM might be considered as a potential 'universal' TAA/LAA (13). The frequency and cytolytic function of circulating anti-RHAMM/CD168 specific CD8+ T lymphocytes constitute a favorable prognostic marker in patients with AML (14,15). R3-specific cytotoxic T lymphocytes (CTLs) were able to kill the leukemia cells and cancer cell lines which express RHAMM (14,15). Earlier, the graft-versus-leukemia (GVL) effect observed after allo-SCT and especially after donor lymphocyte infusion (DLI) in CML patients clearly demonstrated the recognition of CML progenitor cells by T cells in the patient. Proteinase 3 or RHAMM might be potential targets recognized by the donor lymphocytes. However, some CML patients after allo-SCT relapse molecularly and receive imatinib which potentially

decreases the $CD8^+$ T-lymphocyte function (16). These findings have encouraged us to initiate a clinical phase I R3 peptide vaccination trial in patients with hematological malignancies. The existence of CTLs recognizing Bcr-abl and other TAAs/LAAs has been reported within the peripheral T-cell repertoire of patients with CML and healthy individuals (17-19). Given our particular interest in peptide vaccination therapies targeting RHAMM, we performed here a study aimed at analyzing the frequencies and possible induction of R3-specific $CD8^+$ CTLs from patients with CML specifically after allo-SCT and healthy donors. The function of R3-specific $CD8^+$ T lymphocytes was assessed *in vitro* by ELISPOT and ^{51}Cr release assays, and their phenotype was assessed by multi-parameter tetramer-based flow cytometry. This strategy allowed us to obtain data on the expansion and function of R3-specific CTLs both from patients with CML after allo-SCT and healthy donors.

Materials and methods

Cell samples. All samples were taken from HLA-$A2^+$ patients with CML after allo-SCT treated in the framework of clinical studies and healthy blood donors after their informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Biocoll Separation Solution (Biochrom, Berlin, Germany) density gradient centrifugation from EDTA (ethylenediaminetetraacetic acid, Delta-Pharma, Pfullingen, Germany) anti-coagulated blood buffy coat preparations from healthy blood donors or from EDTA anti-coagulated blood from patients with CML after allo-SCT. The viability of obtained PBMCs was always >95% based on Trypan blue dye exclusion (Trypan Blue Solution 0.4%, Sigma-Aldrich, Munich, Germany). The viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany). For cellular assays, Ficoll separated PBMCs were tested freshly or cryopreserved in Roswell Park Memorial Institute medium (RPMI)-1640 (Biochrom AG, Berlin, Germany) containing 20% heat-inactivated AB serum and 10% dimethyl sulfoxide (DMSO), and stored in -192˚C liquid nitrogen. Analysis of HLA-A2 expression was performed by flow cytometry (FACScan, Becton Dickinson) using the mouse anti-human HLA-A2 BB7.2 monoclonal antibody (BD, Heidelberg, Germany). HLA-A2 negative healthy donors were also studied as further controls. The clinical characteristics of the patients with CML after allo-SCT included in this study are displayed in Table I.

Table I. Characteristics of nine patients with CML after SCT.

Patient	Time from SCT to analysis	DLI interval to analysis	Disease status at the time of analysis	T-cell response to R3
1	37 mo	6 mo	Molecular CR	Yes
2	25 mo	8 mo	Molecular CR	No
3	72 mo	5 mo	Molecular CR	Yes
4	53 mo	6 mo	Molecular CR	Yes
5	65 mo	No DLI	Molecular CR	Yes
6	3 mo	No DLI	Hematological CR	No
7	7 mo	No DLI	Molecular CR	No
8	92 mo	No DLI	Molecular CR	Yes
9	39 mo	16 mo	Hematological CR	Yes

SCT, stem-cell transplantation; DLI, donor lymphocyte infusion; mo, month; CR, complete remission.

Selection of $CD8^+$ T cells and $CD34^+$ CML progenitor cells. $CD8^+$ T-lymphocytes and $CD34^+$ CML progenitor cells were selected from PBMCs of HLA-$A2^+$ healthy donors and patients with CML after allo-SCT using anti-CD8 and CD34 MicroBead kits (Miltenyi Biotec, Bergisch Gladbach, Germany) and a MACS device according to the manufacturer's instructions as described previously (16).

Peptides. We used in our experiments the RHAMM-derived peptide R3 (pos. 165-173: ILSLELMKL). The recall peptide derived from influenza matrix protein (IMP) (pos. 58-66: GILGEVFTL) was used as positive control. HER2/neu E75 (pos. 369-377: KIGSFLAFL) or MAGE3 (pos. 271-279: FLWGPRALV) peptides were used as irrelevant peptides. All the peptides were HLA-A*0201-restricted CTL epitopes. The peptides were dissolved in DMSO mixed with PBS (phosphate-buffered saline) at a concentration of 1 μg/μl and stored in aliquots at -20˚C for individual experiments. R3 was chosen because it may target the most immunogenic RHAMM peptide in AML, CML and healthy donors (14). All peptides were synthesized by Thermo Electron Corporation (Ulm, Germany) to a minimum of 95% purity as measured by high performance liquid chromatography (HPLC).

T2 cells. T2 cells (ATCC, the American Type Culture Collection) type positive for HLA-A*0201, but they are unable to present endogenous antigens because they lack the transporter associated protein. These cells were maintained at 37˚C in a humidified 5% CO_2 atmosphere in a standard medium consisting of RPMI-1640 supplemented with 10% (v/v) heat-inactivated AB serum (German Red Cross Blood Center, Ulm, Germany), 2 mM L-glutamine (Biochrom AG), 100 U/ml penicillin (Invitrogen Gibco, Grand Island, USA) and 100 U/ml streptomycin (Invitrogen Gibco). The T2 cell line was used in ELISPOT and ^{51}Cr release assays.

Imatinib. To evaluate the effects on the function of R3-specific $CD8^+$ T lymphocytes, ELISPOT release assays were performed to detect the IFN-γ and granzyme B after MLPCs in the absence or presence of imatinib at different concentrations. Imatinib powder, which was generously provided by Novartis Pharmaceuticals (Basel, Switzerland), was dissolved in DMSO at 10 mM stock. The stock solution was stored at -20˚C and protected from light until use, diluted to the final concentration in X-VIVO 10 medium, and added to MLPCs at a serial concentration (0, 1, 2 and 5 μM) immediately on the first day of eight-day MLPC.

Expansion of IMP and R3-specific CTL by using mixed lymphocyte peptide culture (MLPC). In vitro, expansion of

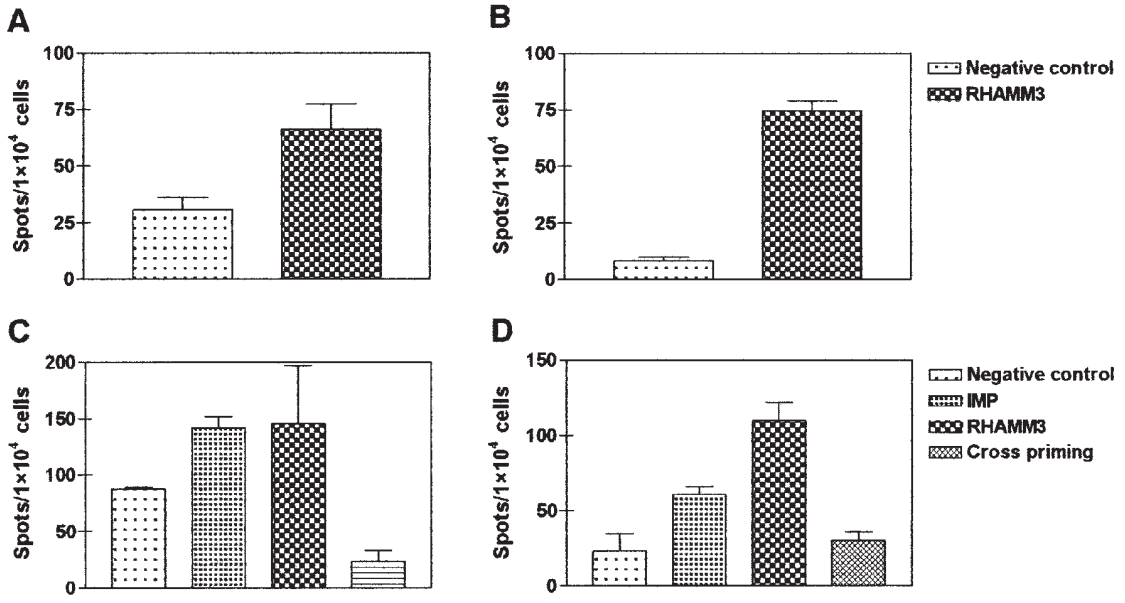

Figure 1. Specific reactivity of cytotoxic CD8+ T-cells (CTLs) from an HLA-A2+ patient with CML after allo-SCT and a healthy donor induced by R3 assessed by ELISPOT assays for IFN-γ and granzyme B. R3-specific CTLs were generated from PBMCs of a patient with CML after allo-SCT (A and B) and a healthy donor (C and D) through one round of stimulation of mixed lymphocyte peptide culture. Resulting CTLs were tested for IFN-γ (A and C) and granzyme B (B and D) using ELISPOT assays. Unpulsed T2 cells (negative control) and T2 cells pulsed with IMP (positive control), R3 or an irrelevant peptide MAGE3 (crossing-priming) were used as stimulators in ELISPOT assays for IFN-γ and granzyme B. Error bars indicate the standard deviation (SD).

IMP and/or R3-specific CD8+ T lymphocytes was performed with PBMCs from patients with CML after allo-SCT and healthy donors by using MLPC which was conducted as described previously (14,15). Briefly, after isolation of CD8+ T lymphocytes, CD8- APCs were irradiated with 30 Gy and pulsed with the IMP or R3 peptides, or the irrelevant MAGE3 or HER2 peptides mentioned above at a concentration of 20 μg/ml for 2 h. After co-incubation with CD8+ T lymphocytes overnight at 37˚C, 5% CO_2 and 99% humidity, the MLPC was supplemented with 10 U/ml IL-2 and 20 ng/ml IL-7 on day +1. Every 3 days, half of the medium was renewed with standard RPMI-1640 medium containing IL-2 and IL-7 at the concentrations indicated above. After a total of 8 days of culture, CTLs were harvested and evaluated for their IFN-γ and granzyme B secreting with ELISPOT assay against T2 cells pulsed with IMP, R3 or cross priming T2 cells pulsed with MAGE3, as described below. The proliferation of IMP or R3-specific CD8+ T lymphocytes was determined after eight days of MLPC by staining with CD8 antibody and HLA-A2/IMP tetramer* phycoerythrin (PE), or HLA-A2/R3 tetramer*PE or HLA-A2/WT-1 tetramer* peridinin chlorophyll protein (PerCP) as described below.

ELISPOT assays for interferon (IFN)-γ and granzyme B. IFN-γ and granzyme B ELISPOT assays were performed according to the manufacturer's instructions and as previously described (15,16). The responses were considered significant if: i) the mean number of cells in triplicate-well experimental peptide was significantly greater ($p<0.05$) than in wells with control, ii) the net spots of triplicate experimental wells (the mean of the triplicate spots/1x10^4 cells in experimental peptide wells - mean of the triplicate spots/1x10^4 cells in control wells) was >10 spots/1x10^4 cells, and iii) the stimulation index (the ratio of mean spots/1x10^4 cells in experimental peptide group to mean spots/1x10^4 cells in control group) was >2.0.

Flow cytometry. IMP tetramer-PE and WT-1 PerCP were used as positive and irrelevant negative controls, respectively. Sample staining was performed using 1x10^6 cells in 50 μl PBS containing 1% FCS. Tetramers (1-2 μg per test with respect to peptide-MHC class I component) were added for 30 min at 37˚C. All tetramers (HLA-A2/IMP tetramer*PE, HLA-A2/R3 tetramer*PE and HLA-A2/WT-1 tetramer*PerCP) were synthesized at the Lausanne Branch of the Ludwig Institute for Cancer Research. Cells were washed once in PBS containing 1% FCS and then stained with titrated panel of directly conjugated antibodies to CD8, CD27, CCR7, CD45RA (BD, Heidelberg, Germany) at 4˚C for 40 min in the dark. Fluorescein isothiocyanate (FITC), allophycocyanin (APC), PE, PerCP, PE-Cy7 and APC-Cy7 were used as fluorophores. This two-step protocol was optimized for these particular tetramers, since it was observed that the simultaneous addition of anti-CD8 antibody and tetramer to the sample reduced the number of tetramer-positive events measured. Cells were stained with appropriate isotope-matched control antibodies and non-specific staining was not observed. After extensive washing twice with PBS, stained cells were fixed with 0.5% paraformaldehyde (Sigma, Germany) for 10 min and then analyzed by flow cytometry. Data acquisition was performed with either a FACSaria or a FACScan (Becton Dickinson) using CellQuest software (BD Biosciences, San Jose, CA) and analyzed using Weasel V. 2.2 software (The Walter and Eliza Hall Institute of Medical Research, Australia). At least 100,000 events were collected. Analysis was performed on tightly gated lymphocytes to exclude dead cells and debris.

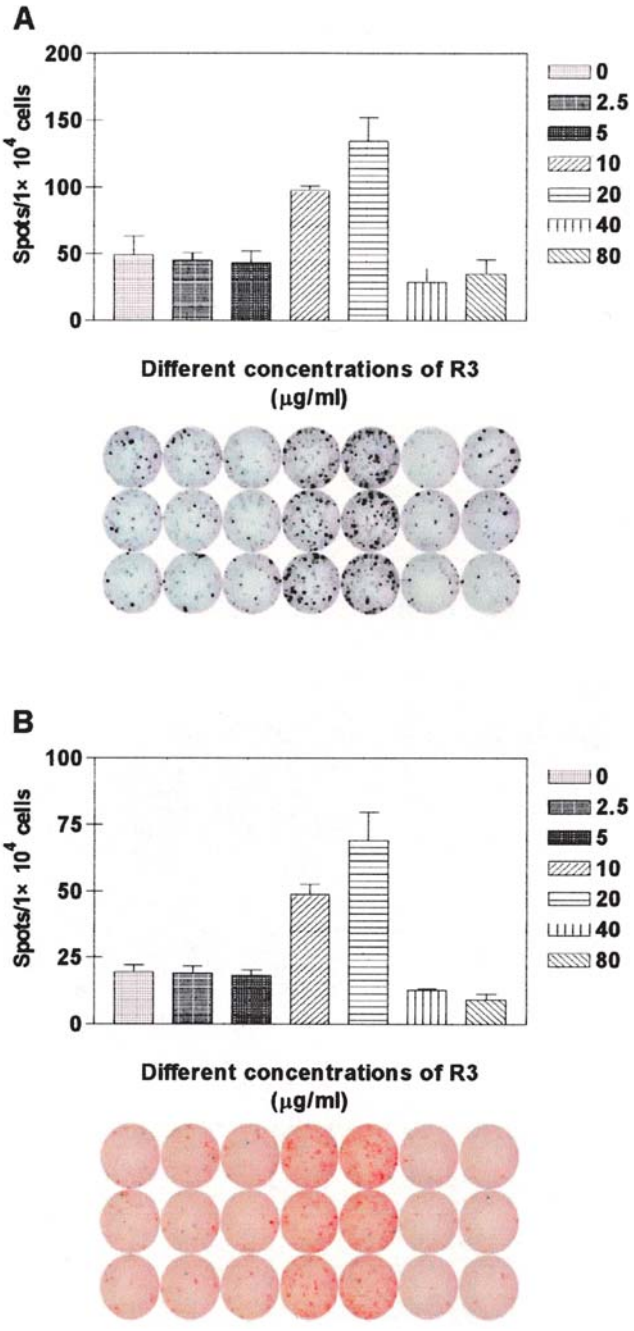

Figure 2. Specific reactivity of CD8+ CTLs to R3 peptide is dose-dependent. R3-specific CD8+ CTLs were generated from the CD8+ T lymphocytes from a healthy donor through one round of stimulation with CD8- APCs pulsed with R3 peptide at different concentrations as indicated. Resulting CTLs were tested for IFN-γ (A) and granzyme B (B) release using ELISPOT assay. T2 cells were pulsed with the indicated concentrations of R3 peptide, and then used as stimulators in IFN-γ and granzyme B assays. The specific CD8+ T lymphocyte activation evaluated by IFN-γ and granzyme B release was dependent on the R3 peptide concentration indicating peptide-specific T lymphocyte answers. The optimal dose of 20 μg peptide per milliliter was used in consecutive assays. The figure shows one representative experiment. Error bars indicate the standard deviation (SD).

^{51}Cr release cytotoxicity assays. R3-specific CTLs from MLPC were tested for cytotoxicity in a standard ^{51}Cr release assay as described earlier (14). Target cells were loaded with 20 μg/ml peptide at 37˚C for 2 h and then labeled with ^{51}Cr for 2 h at 37˚C.

Statistical analysis. All experiments were performed in triplicate. Where applicable, data are reported as the mean ± standard deviation (SD). Statistical significance was determined by two-tailed Student's t-test, analysis of one-way variance (ANOVA). The level of significance was set at p=0.05. All statistical analyses were performed using statistical software package (SPSS Version 11.5 for Windows, SPSS, Chicago, IL).

Results

In vitro generation of R3-specific CD8+ CTLs from patients with CML after allo-SCT and healthy donor PBMCs. To investigate the capacity of R3 to mobilize a specific CTL repertoire, RHAMM-specific CTLs were generated by *in vitro* sensitization of PBMCs from HLA-A2+ patients with CML after allo-SCT and healthy donors with autologous CD8- APCs prepulsed with R3. In nine patients with CML after allo-SCT and 34 HLA-A2+ healthy individuals tested, R3-specific CD8+ T cells were generated. To determine the R3-specificity of *in vitro*-induced CTLs, T2 cells were pulsed with R3 and used as stimulators in ELISPOT IFN-γ and granzyme B assays. CTLs from 6/9 (66.7%) patients with CML after allo-SCT and 8/34 (23.53%) healthy donors produced IFN-γ and granzyme B in response to R3. As expected, very low numbers of spots were detectable for the CD8+ T lymphocyte reaction against T2 cells without peptide (negative control) while strong CD8+ T lymphocyte mediated immune responses (18/34, 52.84%) were detected for IMP peptide used as a positive control. Cross reactivity was excluded by presensitizing CD8+ T cells with autologous CD8- APCs pulsed with the R3 peptide and thereafter evaluating their IFN-γ and granzyme B releases upon presentation of irrelevant MAGE3 derived peptide pulsed T2 cells (cross priming). No spots were detected in freshly isolated CD8+ T lymphocytes. No responses were detected in the HLA-A2 negative subjects (data not shown). Fig. 1 shows representative results of experiments from patients with CML after allo-SCT (Fig. 1A and B) and healthy donors (Fig. 1C and D).

CD8+ T lymphocyte responses to different concentrations of R3 peptide. To determine the specificity of R3 to CD8+ T lymphocytes, the response of CD8+ T lymphocytes to stimulation with different concentrations (0, 2.5, 5, 10, 20, 40 and 80 μg/ml) of R3 was measured by ELISPOT release assays for IFN-γ and granzyme B. A reaction of donor CD8+ T lymphocytes to R3 was demonstrated to be dependent on the peptide concentration as analyzed by ELISPOT release assays for IFN-γ (Fig. 2A) and granzyme B (Fig. 2B). Based on this approach, the optimal dose of 20 μg peptide per milliliter was defined and used in consecutive assays. Similar results were obtained for CD8+ T lymphocytes reactive to the recall peptide IMP (data not showed).

Tetramer staining of CD8+ T cells specifically recognizing the R3 peptide in the context of HLA-A2. R3 tetramer staining was assessed in patients with CML after allo-SCT and healthy subjects following *in vitro* expansion in the presence of the R3 peptide or an irrelevant MAGE derived peptide, or in the absence of any peptide. All subjects were R3 tetramer-negative prior to expansion. Tetramer staining of R3-specific CD8+

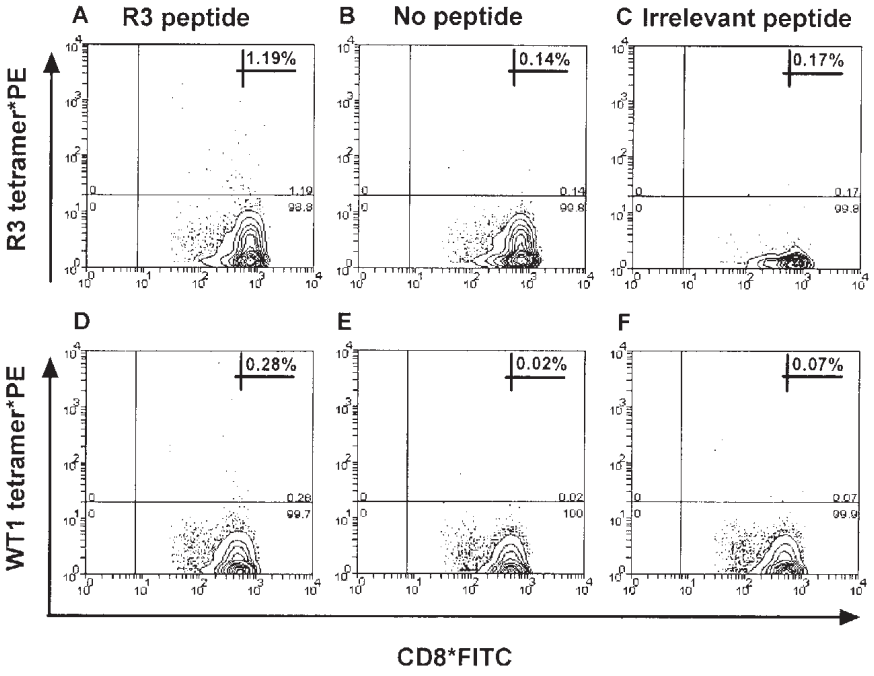

Figure 3. Multicolor staining of R3-specific T lymphocytes from a patient with CML after allo-SCT. $CD8^+$ lymphocytes were subjected to one round of stimulation with autologous $CD8^-$ APCs in the presence (A and D) or absence (B and E) of the R3 peptide or in the presence of an irrelevant MAGE3-derived peptide (C and F) as described in Materials and methods. A difference in the frequency of R3-specific (A-C) and irrelevant WT-1-specific (D-F) $CD8^+$ T lymphocytes was noted. Staining with anti-CD8 antibody is represented on the x-axis, and tetramer staining is shown on the y-axis.

CTLs was observed in 31.58% (6/19) of the healthy donors which is higher than that of ELISPOT assay (23.53%). In those patients/donors whose ELISPOT release assays for IFN-γ and granzyme B were positive in the presence of R3, anti-$CD8^+$ and HLA-A2/R3 tetrameric complexes revealed a frequency of $CD8^+$ T lymphocytes that specifically recognized the R3 peptide ranging from 0.21% to 1.19%, in contrast with up to 0.17% in those whose ELISPOT release assays for IFN-γ and granzyme B were negative in the presence of R3. The $CD8^+$ T lymphocytes recognizing the irrelevant HLA-A2/WT-1 tetramer were <0.28% among those whose HLA-A2/R3 tetramer staining was positive. No tetramer-positive cells were observed in the freshly isolated unstimulated $CD8^+$ T lymphocytes. Fig. 3 shows representative data of a specific HLA-A2/R3 tetramer staining.

Phenotypic analysis of R3-specific $CD8^+$ T lymphocytes compared with IMP-specific $CD8^+$ T lymphocytes. Antigen-specific $CD8^+$ T lymphocytes selected by HLA-A2/R3 tetramer were analyzed for expression of CD45RA, CCR7 and CD27 for characterization of the naïve, memory, and effector phenotype. $CD8^+$ T lymphocytes elicited by stimulation through the R3 peptide were gated for CD8-positive HLA-A2/R3 $tetramer^+$ cells and were further analyzed for expression of CD27, CCR7 and CD45RA for characterization of the naïve ($CD45RA^+CCR7^+$ or $CD45RA^+CD27^+$), memory ($CD45RA^-CCR7^+$ or $CD45RA^-CD27^+$), and effector ($CD45RA^+CCR7^-$ or $CD45RA^+CD27^-$) phenotype. The phenotypic characterization was analyzed in those patients with CML after allo-SCT and healthy donors whose frequencies of R3 tetramer-positive cells were enough. R3 tetramer-specific $CD8^+$ T lymphocytes from the healthy donors showed a predominant phenotype of effector cells and naïve cells (Fig. 4A). In contrast, CML patients after allo-SCT with R3 tetramer-specific $CD8^+$ T lymphocytes displayed a predominant phenotype of effector cells and memory cells (Fig. 4B) comparable to recall peptide IMP tetramer-specific $CD8^+$ T lymphocytes in healthy donors (Fig. 4C).

R3-primed $CD8^+$ T lymphocytes from MLPCs of either patients with CML after allo-SCT or healthy donors lyse CML progenitor cells and T2 cells. ELISPOT release assay detected IFN-γ and granzyme B are not always associated with functional killing. Therefore we tested the CTLs from MLPCs *in vitro* after one round stimulation of R3 peptide in ^{51}Cr release assay using HLA-$A2^+$ CML progenitor cells, and T2 cells pulsed with R3, or irrelevant HLA-A2 matching HER2 peptide or MAGE3 derived peptide and T2 without peptide pulsing as target cells. The HLA-A2 restricted IMP epitope was used as a reference control. ^{51}Cr release assays showed a strong specific lysis of T2 cells pulsed with IMP in contrast to irrelevant HER2 pulsed T2 cells and T2 alone (Fig. 5A). The T2 cells loaded with an irrelevant MAGE3 peptide were not recognized either (data not shown). R3-specific $CD8^+$ T lymphocytes generated from MLPCs of both CML patients (Fig. 5B) and healthy donors (Fig. 5C) were able to recognize and lyse 75% and 50% CML progenitor cells at an E/T ratio of 20:1 respectively. T2 cells pulsed with irrelevant HER2 peptide and T2 cells alone were not able to be lysed by the R3-specific $CD8^+$ CTLs generated from patients with CML after allo-SCT (Fig. 5B).

The functional activation of R3-specific $CD8^+$ T lymphocytes from healthy donors was inhibited by imatinib. To evaluate

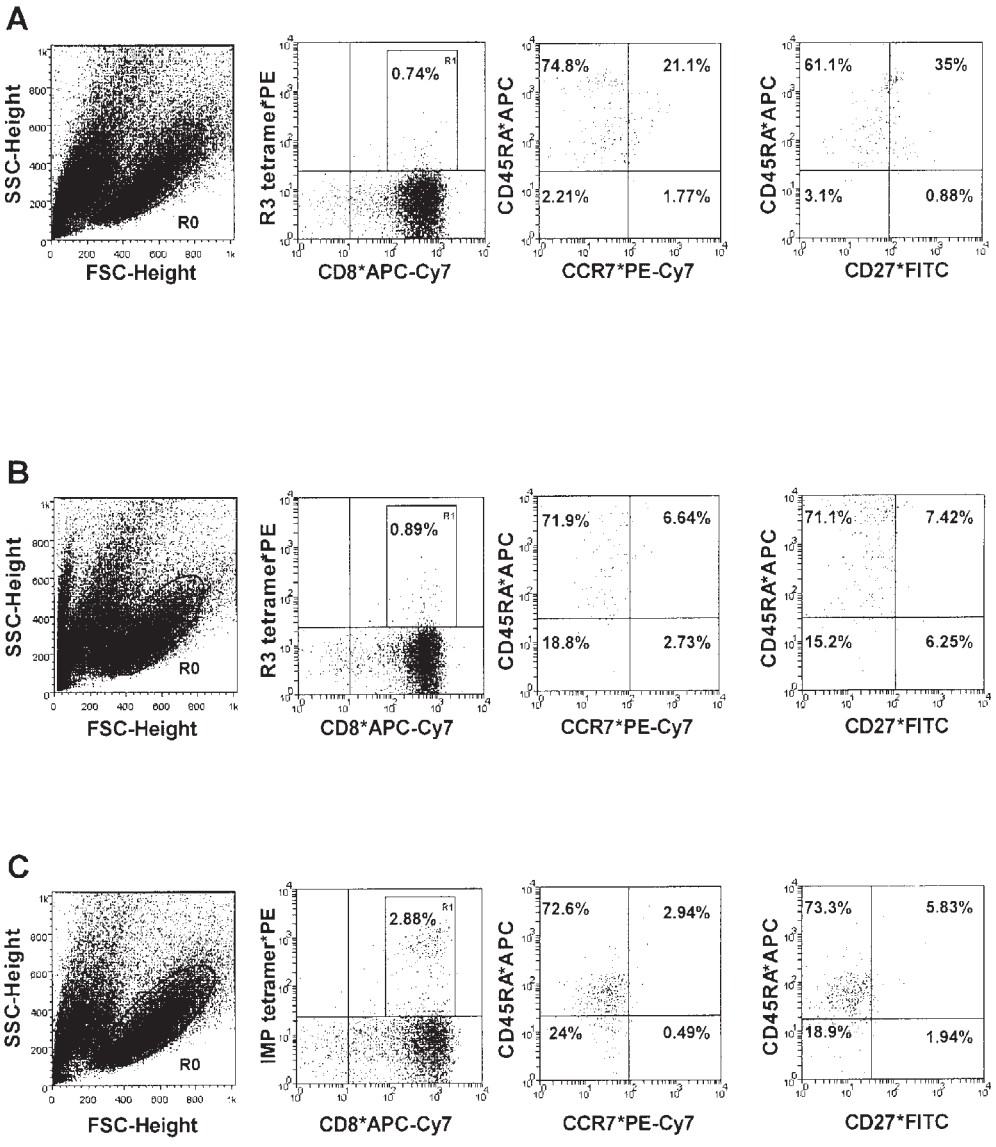

Figure 4. Immunophenotyping of R3 or IMP tetramer-positive CD8+ T lymphocytes. CD8+ lymphocytes from a patient with CML after allo-SCT and a healthy donor were subjected to one round of stimulation with autologous CD8- APCs in the presence of the cognate peptide as described in Materials and methods. Lymphocytes were gated (R0) and stained with HLA-A2/R3 tetramers and anti-CD8 (R1). Lymphocytes from gate R1 were analyzed for their counterstaining of CCR7, CD27 and CD45RA. A, the result for R3 peptide in a representative HLA-A2+ healthy donor of R3 peptide; B, a representative patient with CML after allo-SCT; C, the result for IMP of a representative healthy donor. SSC, side scatter; FSC, forward scatter.

the possible inhibition of functional activation of R3-specific CD8+ T lymphocytes from healthy donors by imatinib, ELISPOT assays were performed to assess the production of IFN-γ and granzyme B secreted by R3-specific CD8+ T lymphocytes upon stimulation with the autologous CD8- APCs pulsed with cognate RHAMM derived R3 peptide. As shown in combined representative results from three independent experiments, increasing concentrations of imatinib gradually reduced the frequency of detectable IFN-γ (Fig. 6A) and granzyme B (Fig. 6B) secretion. The same inhibiting effects were detected in the R3-specific CD8+ T lymphocytes from patients with CML after allo-SCT (data not shown).

Discussion

The present study was based on a combined and simultaneous procedural approach using ELISPOT assays for the release of IFN-γ and granzyme B, tetramer staining, multiparametric flow cytometry, as well as ^{51}Cr release assays to obtain both phenotypic and functional data. CD8+ T lymphocytes able to bind R3 tetramer were found in 31.58% of HLA-A2+ studied healthy subjects and 66.7% of patients with CML after allo-SCT. The present study extends recent studies on TAAs/LAA specific CD8+ memory cells and precursors existing within patients with CML and healthy individuals (17-19). We did not observe significant HLA-A2/R3 tetramer binding to cells derived from any of the control HLA-A2- subjects; thus indicating that the positivity we observed in HLA-A2+ subjects was related to the expansion of the reactive R3-specific T lymphocyte subjects.

Tetramer staining is an appropriate technology to assess CD8+ T lymphocytes able to interact with the specific peptide-HLA complex. However, it does not provide information on the functional status of the cells (20). To verify the presence

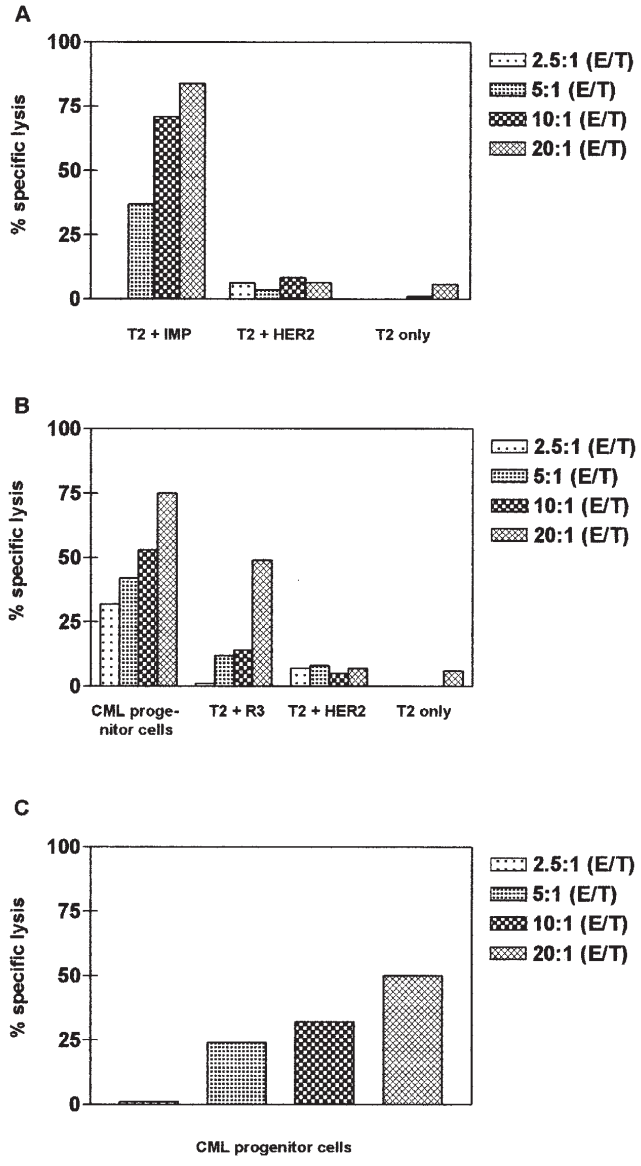

Figure 5. Specific lysis of R3-pulsed T2 cells and CML progenitor cells as evaluated by ^{51}Cr release assay. (A) Stimulated IMP-primed CD8$^+$ T cells from a representative HLA-A2$^+$ healthy donor used as a reference control lysed T2 cells pulsed with IMP, but could not lyse the T2 cells pulsed with irrelevant HER2 peptide and T2 cells only. (B) Stimulated R3-primed CD8$^+$ T lymphocytes from a representative patient with CML after allo-SCT were able to recognize CML progenitor cells and T2 cells pulsed with R3 at a certain level, while these CD8$^+$ T cells could not lyse T2 cells pulsed with an irrelevant HER2 peptide and T2 cells only. (C) Stimulated R3-primed CD8$^+$ T lymphocytes from a representative HLA-A2$^+$ healthy donor were able to recognize and to kill the CML progenitor cells to a percentage as high as 50% at an E/T ratio of 20:1.

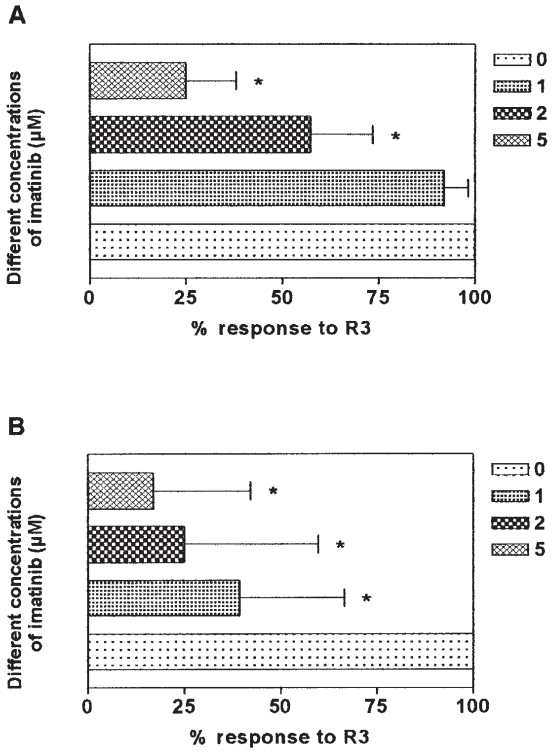

Figure 6. Addition of imatinib in different concentrations to the MLPCs from healthy donors resulted in the inhibition of R3-specific CD8$^+$ T lymphocytes by imatinib in a dose-dependent manner. To evaluate the functional inhibition of imatinib on R3-specific CD8$^+$ T lymphocytes from healthy donors, CD8$^+$ T lymphocytes were subjected to one round of stimulation with irradiated autologous CD8$^-$ APCs pulsed with R3 peptide in the presence of escalating doses of imatinib (0-5 μM) as described in Materials and methods. ELISPOT assays for release of IFN-γ (A) and granzyme B (B) were performed to explore the functional inhibition of R3-specific CD8$^+$ T lymphocytes by imatinib. All assays were performed in triplicate. The figures show the combined results of three independent experiments with similar results which were expressed as mean values of the % maximal response ± SD at each imatinib concentration. Error bars indicate standard deviation (SD). Significant differences from the group not treated with imatinib were observed, *P<0.01.

of R3 specific cytotoxic cells in patients with CML after allo-SCT and healthy donors, we used MLPC, ELISPOT release assays for IFN-γ and granzyme B, multiparametric flow cytometry, as well as ^{51}Cr release assays, for the analysis of the functional status of these cells and the generation of R3-specific CTLs. ELISPOT release assays for IFN-γ and granzyme B showed 66.7% (6/9) of HLA-A2$^+$ patients with CML after allo-SCT and 23.8% (8/34) of HLA-A2$^+$ healthy donors to be reactive to R3, which is a lower frequency than measured by tetramer staining. This suggests that tetramer analysis may be a more sensitive assay and likely detects lower numbers of antigen-specific CD8$^+$ T lymphocytes. Since tetramer analysis does not measure T-cell function, it is plausible that a CTL population carrying a low-affinity T-cell receptor was expanded *in vitro* but failed to be detected by tetramer staining. Alternatively, a potent cytotoxic population may have been expanded, but due to cell activation (and therefore T cell receptor down-regulation) tetramer constructs may have failed to bind to the T cells at a detectable level. Overlapping markers such as CD45RA, CD45RO, CCR7, CD27, CD28 and CD57 have been used to identify the differentiation state of antigen-specific CD8$^+$ T lymphocytes (21-24). On the basis of CD45RA, CCR7, and CD27 expression and analysis of replicative history and clonality of the T cell population, phenotypically distinct and sequential stages of CD8$^+$ T lymphocyte differentiation have been proposed. We found that, both in patients with CML patients after allo-SCT and healthy donors, RHAMM reactive CD8$^+$ T lymphocytes displayed a predominantly mixed CCR7$^-$CD45RA$^+$ or CD27$^-$CD45RA$^+$ phenotype, similar to the IMP reactive CD8$^+$ T lymphocytes, corresponding to the terminally differentiated effector phenotypes (25,26). The percentage of CCR7$^-$CD45RA$^+$CD8$^+$R3 tetramer$^+$ cells was higher in the CML

patients after allo-SCT than in the healthy subjects. It is indicated that, following allo-SCT, there was a shift toward an effector phenotype, implying an ongoing GVL effect. These R3-specific $CD8^+$ T cells generated from either patients with CML after allo-SCT or healthy donors were able to kill HLA-$A2^+$ CML progenitor cells, as assessed by the ^{51}Cr release assay, suggesting that RHAMM derived R3 peptide could induce significant CTL responses *in vitro* from patients with CML after allo-SCT and healthy donors and the CTL memory cells and precursor population against RHAMM exist within the $CD8^+$ T lymphocyte repertoire of patients with CML and healthy individuals.

The ABL tyrosine kinase inhibitor imatinib mesylate is highly effective in the treatment of CML and is increasingly used in the SCT setting (17-19,21). Since ABL-dependent intracellular signaling molecules are involved in T-cell activation, imatinib may affect T-cell response *in vivo*, thus affecting T-cell function, disrupting immune reconstitution after SCT and/or impeding the GVL effect. Here we demonstrate that imatinib inhibits the functional activation of R3-specific $CD8^+$ T lymphocytes generated from healthy donors *in vitro* at concentrations (1-5 μM) representative of the pharmacological doses used therapeutically *in vivo* (27) assessed by ELISPOT release assays for IFN-γ and granzyme B. Because of the imatinib-induced suppression of R3-specific $CD8^+$ T cells observed in this study, and other results (16,28-30), imatinib-free therapeutic windows during $CD8^+$ T lymphocyte-based immunotherapies may be required to enable expansion of leukemia-specific T lymphocyte responses.

In conclusion, we showed that: i) HLA-$A2^+$ patients with CML after allo-SCT and healthy donors indeed have circulating $CD8^+$ effector T cells specific for the RHAMM derived R3 peptide; ii) induced CTLs specific for R3 peptide from both patients with CML after allo-SCT and healthy donors were expanded *in vitro* and efficiently killed HLA-$A2^+$ CML progenitor cells; iii) functional activation of R3-specific $CD8^+$ CTLs was inhibited by the Bcr-Abl tyrosine kinase inhibitor imatinib.

These results encourage peptide vaccination trials for CML patients after allo-SCT as a means to augment the R3/leukemia specific GVL effect.

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