Priming of PRAME- and WT1-specific CD8\(^+\) T cells in healthy donors but not in AML patients in complete remission: Implications for immunotherapy

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**Abbreviations:** CR, complete remission; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FCS, fetal calf serum; HD, healthy donor; LAA, leukemia-associated antigen; LF, leukapheresis; MRD, minimal residual disease; MUTZ3-DC, DCs derived from the MUTZ-3 cell line; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PRAME, preferentially expressed antigen in melanoma; RA, retinoic acid; Tm, tetramer; WT1, Wilms’ tumor 1

Active immunotherapy may prevent the relapse of acute myeloid leukemia (AML) by inducing leukemia-specific T cells. Here, we investigated whether Wilms’ tumor 1 (WT1) and preferentially expressed antigen in melanoma (PRAME)-specific T cells could be induced upon the priming of healthy donor- and AML patient-derived T cells with HLA-A2-matched, peptide-loaded allogeneic dendritic cells. AML-reactive, tetramer (Tm)-binding and interferon-producing, cytotoxic T lymphocytes specific for PRAME could readily be isolated from healthy individuals and maintained in culture. In this setting, priming efficacy was significantly higher for PRAME than for WT1. The priming of T cells from patient-derived material proved to be near-to-impossible: No leukemia-associated antigen (LAA)-specific T cell could be primed in 4 patients that had recently achieved a complete response (CR), and in only 1 out of 3 patients exhibiting a sustained CR we did observe WT1-specific T cells, though with a low frequency. These findings suggest that the functionality and/or repertoire of T cells differ in healthy subjects and AML patients in CR, and may have repercussions for the implementation of active vaccination approaches against AML.

Introduction

Therapeutic vaccination with dendritic cells (DCs) is regarded as a viable option for the treatment of minimal residual disease (MRD) in acute myeloid leukemia (AML) patients. DC-based vaccination aims at eliciting a leukemia-associated antigen (LAA)-specific immune response against residual leukemic cells, thus preventing relapse.\(^1\) In this respect, peptides carrying defined LAA-epitopes are an attractive source of antigens. Among various LAAs, Wilms’ tumor 1 (WT1) and preferentially expressed antigen of melanoma (PRAME) appear to be overexpressed in various hematological malignancies and share the potential for the induction of specific T-cell responses.\(^2,3\) These LAAs are often expressed by AML cells, with expression rates ranging from 60–90%.\(^4\) Of note, both PRAME and WT1 are involved in leukemogenesis. PRAME influences disease progression by interfering with retinoic acid (RA) receptor signaling,\(^5\) while WT1 inhibits cell differentiation through hitherto unknown mechanisms.\(^6,7\) The involvement of PRAME and WT1 in leukemogenesis and their expression by malignant stem cells make them attractive candidates for the detection of MRD and valuable targets for immunotherapy.\(^8-12\) Cytotoxic T-lymphocyte (CTL) epitopes of WT1 and PRAME have been characterized for both HLA-A2 and HLA-A24 molecules.\(^13-16\)
Moreover, T cells recognizing endogenous PRAME- and WT1-derived peptides that are presented by HLA-A2 molecules on the surface of tumor cells have been documented in patients affected by various malignancies, including AML.2,17,18

Here, we investigated the relative priming efficiencies of CTLs obtained from the peripheral blood (PB) of either healthy donors (HD), as leukapheresis (LF) material, or AML patients in first complete remission (CR).

**Results**

**WT1 and PRAME Tm-specific T cells can be primed in HDs but not in AML patients.** Primed Tm+ T cells were detectable in samples from HDs for both LAA tested, i.e., PRAME and WT1 (Fig. 1). PRAME and WT1 Tm+ T cells were induced in 3 out of 4 and in 2 out of 5 HDs, respectively (Table 1). Multiple parallel priming cultures of CD8+ T cells were set up in separate wells with a fixed number of CD8+ T cells per well (1 x 10^6 cells). WT1 Tm+ CD8+ T cells were detected in 4 out of a total of 104 cultures (Table 1; 3/28 for donor 1; 1/20 for donor 2). The priming efficiency of PRAME CD8+ T cells in HDs was significantly higher, with 20 Tm+ cultures out of 51 in total (Table 1; 8/12 for donor 1; 6/15 for donor 2; 6/9 for donor 3). Of note, the same HD-derived T cells were used for PRAME and WT1 priming experiments, and WT1- and PRAME-specific T cells were both detected in donor 1 and 2. The highest frequency of PRAME or WT1 Tm+ CD8+ T cells was detected after 1 to 5 rounds of (re-)stimulation (Table 1). Tm+ CD8+ T-cell frequencies ranged from 0.02 to 1.07% for the PRAME epitope and from 0.01 to 0.09% for the WT1 epitope.

Next, we analyzed the priming efficiencies of CD8+ T cells obtained from AML patients shortly after the achievement of a CR or patients in sustained CR (Table 2). No PRAME and WT1 Tm+ T cells could be expanded from CD8+ T cells isolated from AML patients that had recently achieved a CR (1–6 mo duration, Fisher’s exact test for PRAME in HD vs. AML patients in CR; p < 0.001) (Table 1) and only one patient undergoing a sustained CR exhibited WT1-specific T cells, though with low frequencies in 2 out of 6 wells (UPN 1) (Table 1).

**PRAME-specific T cells kill AML cell lines and patient-derived leukemic cells in a dose-dependent manner.** Tm+ T cells recognizing the PRAME epitope were sorted from donor 1 and then cultured in limiting dilution conditions for the generation of PRAME-specific CTL clones. One of such T-cell clones was immortalized by retroviral hTERT transduction,19 followed by the characterization of its function. To confirm the ability of the PRAME-specific CTL clone to produce IFNγ upon peptide recognition. Tm+ CD8+ T cells obtained from the PBMCs of HDs produced IFNγ upon recognition of PRAME or WT1 peptides (Fig. 1). PRAME Tm+ T cells were tested for intracellular IFNγ expression upon stimulation with PRAME100–108 and Tm+ populations were indeed able to produce IFNγ (range = 24–71% of Tm+ T cells) (Fig. 1C). CD8+ T cells from two HDs were found to stained positively for the WT1 Tm after priming (Table 1). IFNγ production upon re-stimulation with WT1126–134 was tested in one HD only, exhibiting 27.7% IFNγ cells within the Tm+ cell fraction (data not shown).

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To confirm the ability of the PRAME-specific CTL clone to eliminate AML blasts, PRAME-specific T cells were co-cultured with titrated amounts of HLA-A2+ AML target cells, resulting in the killing of both MUTZ-3 cells (PRAME: AML precursors) (Fig. 2C) and HLA-A2-matched allogeneic primary AML blasts (Fig. 2D, one representative example out of two patient samples tested) in a dose-dependent manner.
T cells recognizing WT1 and proteinase 3 have previously been detected in AML patients at diagnosis based on their reactivity in a very sensitive ELispot assay and IFNγ production upon antigen-specific stimulation.18 However, Tm staining-based sorting offers a means of isolation and more extensive functional characterization of specific CTL clones. Since T cells from HDs have not encountered leukemic cells, the outgrowth of PRAME- and WT1-specific CD8+ T cells should be the result of naive T-cell priming. PRAME-specific Tm+ T cells could be readily detected after priming in HDs, whereas the frequency WT1-specific cells was low. Others have studied WT1126–134-loaded DCs for priming, finding that 10 out of 10 low-frequency Tm+ T cells from HDs could be primed, reaching up to 10% Tm+ T cells upon extensive expansion in vitro.21 Such a difference in efficiency between these results and our data may stem from variations in experimental protocols,22 most likely the use of 2-d “fast” DCs and PBMCs in expansion rounds. However, employing the same protocol that we detail here, we have previously observed higher priming efficiencies for other tumor-associated antigens such as hTERT, ERBB3-binding protein 1 (EBP1) and carcinoembryonic antigen (CEA).23 Moreover, Quintarelli and coworkers observed priming efficacy.28 Similar observations have been made in CML patients.24 Conversely, we were able to induce the outgrowth of WT1-specific, but not PRAME-specific, CD8+ T cells upon the stimulation (with WT1126–134-loaded MUTZ-DCs) of material from one (out of 3) AML patient undergoing sustained CR (Table 1). Remarkably, no PRAME-specific T cells could be primed in any of the patients, suggesting that either no PRAME-specific naive T cells were present or PRAME-specific CTLs were anergic and hence unable to expand. Although not conclusive, since we could not expand T cells to numbers compatible with functional tests, these findings strongly suggest that even in the setting of prolonged CR AML-reactive T cells remain relatively dysfunctional (or their repertoire exhausted) and may never fully recover sufficiently to respond to WT1 or PRAME. It remains to be investigated whether this phenomenon is specific for WT1 and PRAME or whether it applies to a wide range of AML-derived antigens and epitopes. Of note, in the case of allogeneic stem cell transplantation (SCT), the immunosuppressive effects of chemotherapy and AML do not affect donor-derived hematopoietic cells, implying that—under

**Discussion**

The success of immunotherapeutic strategies relies on an adequately responding immune system. It is not yet clear at what time point the immune system of AML patients has sufficiently recovered from disease and chemotherapy to respond to immunotherapy. Therefore, we analyzed the priming efficiencies in the PB of AML patients that had recently achieved a CR and patients undergoing a sustained CR. Remarkably, no PRAME- and WT1-specific Tm+ T cells could be expanded starting from the CD8+ T cells of patients recently achieving a CR. Such a priming failure can be explained either by a general immunosuppressed state of the patient or by a profound disturbance of the T-cell repertoire. It can take indeed up to six months before normal numbers of T cells are restored once the depleting effects of abundant AML blasts and chemotherapy are relieved.26,27 Furthermore, granulocyte colony-stimulating factor (G-CSF), which is normally given before leukapheresis, can induce T-cell tolerance and hence might have hampered priming efficacy.28 Similar observations have been made in CML patients.24 Conversely, we were able to induce the outgrowth of WT1-specific, but not PRAME-specific, CD8+ T cells upon the stimulation (with WT1126–134-loaded MUTZ-DCs) of material from one (out of 3) AML patient undergoing sustained CR (Table 1). Remarkably, no PRAME-specific T cells could be primed in any of the patients, suggesting that either no PRAME-specific naive T cells were present or PRAME-specific CTLs were anergic and hence unable to expand. Although not conclusive, since we could not expand T cells to numbers compatible with functional tests, these findings strongly suggest that even in the setting of prolonged CR AML-reactive T cells remain relatively dysfunctional (or their repertoire exhausted) and may never fully recover sufficiently to respond to WT1 or PRAME. It remains to be investigated whether this phenomenon is specific for WT1 and PRAME or whether it applies to a wide range of AML-derived antigens and epitopes. Of note, in the case of allogeneic stem cell transplantation (SCT), the immunosuppressive effects of chemotherapy and AML do not affect donor-derived hematopoietic cells, implying that—under

| **Table 1. Priming efficiencies of CD8+ T cells obtained from healthy donors or acute myeloid leukemia patients** |
|---|
| **PrAME(100–108)** | **WT1(126–134)** | **WT1(126–134)** | **WT1(126–134)** |
| HD | AML-Cr1 | AML-Cr1 | AML-Lt-Cr1 |
| Tm+ rates after co-culture (per no. of tested HD/patients) | 3/4 | 0/4 | 0/3 |
| Total no. of Tm+ co-cultures | 21/50 | 4/104 | 0/14 |
| Median % Tm+ cells per positive culture (range) | 0.10% (0.02–1.07) | 0.07% (0.03–0.09) | 0.00% |
| Number of restimulations at maximum of Tm+ | 1–5 | 1–4 | - |

*Patient material was obtained from individuals undergoing short-term (1–6 mo, AML-Cr) or long-term (> 1.5 y, AML-Lt-Cr) complete remission. Priming efficiency was determined upon stimulation with leukemia-associated antigen (LLA)-derived peptide-loaded, HLA-A2-matched, MUTZ-3 cell-derived dendritic cells.

| **Table 2. Characteristics of acute myeloid leukemia patients in long-term (Lt) or short-term (St) complete remission (CR)** |
|---|
| **AML subtype** | **Duration CR at start priming** |
| AML-LT-CR1 UPN 1 | MDS-RAEB | 1.5 y |
| AML-LT-CR1 UPN 2 | M1 | 12 y |
| AML-LT-CR1 UPN 3 | M4 | 14 y |
| AML-CR1 UPN 1 | AML, not further classified | 6 mo |
| AML-CR1 UPN 2 | AML-M2 | 1 mo |
| AML-CR1 UPN 3 | Therapy related AML | 1 mo |
| AML-CR1 UPN 4 | AML-M5B | 2 mo |
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Patient and donor materials. Peripheral blood cells were drawn from 9 AML patients after obtaining the patients’ informed consent. Peripheral blood mononuclear cells (PBMCs) from 3 patients in first CR for 1.5 y or longer (long-term CR1, LT-CR1) were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech). PBMCs (containing > 80% leukemic cells) from 2 patients at diagnosis were isolated using Ficoll-Paque (Amersham Pharmacia Biotech) and stored for later use. Cells were cryopreserved at a controlled rate in liquid nitrogen using RPMI 1640 medium (Gibco) supplemented with 20% heat-inactivated fetal calf serum (FCS) (Greiner) and 10% dimethylsulphoxide (Merck). Autologous leukapheresis products from 4 patients (AML-CR1) were cryopreserved at a controlled rate according to institutional guidelines. Before use, cryopreserved material was rapidly thawed and washed twice in RPMI-1640 supplemented with 40% FCS. Cells were then resuspended in culture medium as described below.

Materials and Methods

In conclusion, we show here that expanding LAA-specific T cells remains a challenge in AML patients recovering from disease and treatment, suggesting that active immunotherapy may be more effective in early-stage AML, including myelodysplastic syndrome (MDS) patients. In this setting, active immunotherapy may be combined with demethylating agents such as 5-azacytidine, as these might augment PRAME expression by leukemic cells and hence PRAME-specific recognition and lysis by CTL clones. In addition, the ability of PRAME Tm+ T cells derived from HDs to recognize and kill target cells indicates that PRAME is a promising candidate for adoptive T-cell transfer approaches, for instance strategies based on the transduction of TCR-coding genes, and deserves further investigation for the development of induction and consolidation therapeutic regimens against AML.

Figure 2. Cytotoxic activity of a healthy donor-derived PRAME100–108-specific cytotoxic T lymphocyte clone toward leukemic cell lines and patient samples. (A and B) Cytotoxic activity of a healthy donor-derived PRAME100–108-specific cytotoxic T lymphocyte (CTL) clone against chronic myeloid leukemia (CML) PRAME+ K562 cells (either HLA-A2– or upon transduction with HLA-A2), acute myeloid leukemia (AML) PRAME HLA-A2+ ME1 cells, unloaded JY cells and PRAME100–108-loaded JY cells, as monitored in a flow cytometry-based assay with 10:1 effector-to-target (E:T) cell ratios. (B) Cytotoxic activity of PRAME100–108-specific CTLs against HLA-A2-expressing K562 (K562-A2+) cells, in the presence of an MHC class I-blocking antibody (MHC) or appropriate isotype control antibodies (iso). (A) and (B) depict the percentage of viable cells upon incubation with PRAME100–108-specific CTLs. (C and D) Percentage of viable MUTZ-3 cells (C) and patient-derived AML cells (one representative example out of two HLA-A2 matched patients tested) (D) upon incubation with PRAME100–108-specific CTLs at the indicated E:T cell ratios.
Cell lines. The CD34+ human AML cell line MUTZ-3 [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)] was cultured in MEMα medium containing ribonucleosides and deoxyribo nucleosides (Life Technologies) supplemented with 20% FCS (Perbio), 100 IU/mL penicillin sodium (Yamanouchi Pharma), 100 μg/mL streptomycin sulfate (Radiumfarma- Fisipharma), 2.0 mM l-glutamine (Invitrogen), 0.01 mM 2-mercaptoethanol (Merck) and 10% β2-microglobulin (Sigma-Aldrich) in the presence of 3 μg/mL β2-microglobulin (Sigma-Aldrich) for 2–4 h at room temperature and irradiated (50 Gy). PRAME100–108 was synthesized according to the FMOCL technology (HB-LUMC) while WT1126–134 was obtained from Sanquin.

Isolation of CD8+ T cells from buffy coats and generation of MUTZ-3-derived DCs. Buffy coats were obtained from healthy volunteers according to institutional guidelines in accordance with the Helsinki Declaration of 1975. PBMCs were isolated by density centrifugation using Ficoll-Paque. Subsequently, CD8+ T cells were obtained by negative depletion using an untouched magnetic Microbead-based kit (Miltenyi Biotec GmbH). MUTZ-3-derived DCs (MUTZ3-DCs) were generated as previously described.23 Briefly, MUTZ-3 progenitors were cultured in 12-well tissue culture plates at a concentration of 1 × 10^7 cell/mL in MEM-α medium in the presence of 100 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF, from Strathmann Biotec), 1,000 U/ml interleukin-4 (IL-4, from Strathmann Biotec) and 1,000 U/ml interleukin-12 in a 24 well tissue-culture plate. At day 1, 10 ng/mL IL-10 (Sigma-Aldrich) was added. From day 10 on, CTL cultures were stimulated every week for 5 weeks with 1 × 10^5 fresh peptide-loaded antigen-presenting cells (10 ng/mL) in the presence of 5 ng/mL IL-7 (Strathmann Biotec). For the first re-stimulation MUTZ3-DCs were used as antigen-presenting cells, while for the subsequent and re-stimulations re-stimulations HLA-A2+ JY cells were employed. One day prior to each re-stimulation, a sample was taken and analyzed by flow cytometry using both PE- and APC labeled Tms presenting the relevant epitope. Two days after each re-stimulation, 10 U/mL IL-2 (Strathmann Biotec) was added. PRAME Tm+ CTLs were then isolated by Tm+ flow sorting and subsequently cloned by limiting dilution. For this purpose, CTLs were weekly stimulated with irradiated feeder-mixes consisting of allogeneic PBMCs (1:10 ratio with CTLs) and JY cells (1:100 ratio with CTLs) in Yssel’s medium supplemented with 100 ng/mL phytohemagglutinin (PHA, from Murex Biotech) and 20 U/mL IL-2. Human telomerase reverse transcriptase (hTERT) was introduced in a PRAME-specific CTL clone as described previously. Briefly PRAME-specific CTLs, stimulated for 48 h with the feeder-mix as described above, were transduced with a retrovirus encoding LZRS-hTERT-IRE5-DNGFR, in fibronectin-coated cell culture plates (Retronectin) in the presence of 100 U/ml IL-2. During transduction, plates were centrifuged at 2,000 g for 90 min at 37°C and subsequently incubated at 37°C for 4.5 h. Then, cells were washed and cultured overnight in Yssel’s medium containing 20 U/mL IL-2. Retroviral transduction was repeated one day later, and after 48 h transduction efficiency was determined by the nerve growth factor receptor (NGFR) expression on the cell surface (monitored by flow cytometry).

Intracellular interferon γ detection. To determine the ability of CTL clones to produce interferon γ (IFNγ) upon recognition of a specific target, intracellular IFNγ staining was performed. HLA-A2/PRAME+, HLA-A2/PRAME+ cancer cell lines and JY cells or T2 cells pulsed with either relevant or irrelevant peptides were used as target cells. CTLs were cultured with target cells at an effector:target (E:T) cell ratio of 2:1 in 96-well round-bottom plates, and 0.5 μL of GolgiPlug (BD Biosciences) was added to each well. After 6 h, cells were harvested, washed and stained.
with PE-labeled tetramers and APC-labeled anti-CD8 monoclonal antibodies. After fixation with 4% paraformaldehyde (Merck) and permeabilization with the BD Perm/wash solution (BD Biosciences), cells were labeled with FITC-conjugated anti-IFN-γ antibodies (BD Biosciences) and analyzed by flow cytometry.

**FACS-based cytotoxicity assays.** The cytotoxic activity of PRAME-specific CTLs was assessed by a flow cytometry-based cytotoxicity assay, as described previously. In brief, effector T cells were labeled with 1 μM CFSE (Molecular Probes) for 10 min and subsequently co-cultured with 1 × 10^5 target cells (cell lines or patient samples) in E:T ratios of 20:1, 10:1 and 5:1, in sterile round-bottom polystyrene tubes (BD Biosciences). All E:T ratios were tested in duplicate. Cell suspension was adjusted to 100 μl with complete IMDM. Separate control cultures of effector and target cells alone were performed to check spontaneous apoptosis and secondary necrosis. Co-cultures were kept for 6 h in a 37°C humidified incubator.

At the end of cytotoxic contact, target cells were stained at 37°C with PE-conjugated anti-CD34 antibodies plus SYTO62 and 7-AAD. MHC-restriction was tested by the addition of 2.5 μg/mL MHC class I-blocking antibodies (W6/32, a kind gift of Dr. S.M. van Ham, Department of Immunopathology, Sanquin Research) or the same amount of appropriate isotype controls (mouse IgG2a, from Sanquin).

**Statistical analysis.** Statistical significance was determined by two-tailed, paired Student’s t-tests for the results of cytotoxicity assays and by the Fisher’s exact test for priming efficiencies. p values < 0.05 were regarded as statistically significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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