The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy

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CHAPTER 3

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Detection and Functional Analysis of CD8$^+$ T Cells Specific for PRAME: a Target for T-Cell Therapy

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Abstract

Purpose: Preferentially expressed antigen on melanomas (PRAME) is an interesting antigen for T-cell therapy because it is frequently expressed in melanomas (95%) and other tumor types. Moreover, due to its role in oncogenic transformation, PRAME-negative tumor cells are not expected to easily arise and escape from T-cell immunity. The purpose of this study is to investigate the usefulness of PRAME as target for anticancer T-cell therapies.

Experimental Design: HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for CD8$^+$ T cells directed against previously identified HLA-A*0201-binding PRAME peptides by IFN-γ enzyme-linked immunosorbent spot assays and tetramer staining. PRAME-specific T-cell clones were isolated and tested for recognition of melanoma and acute lymphoid leukemia (ALL) cell lines. PRAME mRNA expression was determined by quantitative real-time reverse transcription-PCR.

Results: In 30% to 40% of healthy individuals and patients, PRA$^{100-108}$-specific CD8$^+$ T cells were detected both after *in vitro* stimulation and directly *ex vivo* after isolation by magnetic microbeads. Although CD45RA$^-$ memory PRA$^{100-108}$-specific T cells were found in some individuals, the majority of PRA$^{100-108}$-tetramer$^+$ T cells expressed CD45RA, suggesting a naive phenotype. PRA$^{100-108}$-tetramer$^+$ T-cell clones were shown to recognize and lyse HLA-A*0201$^+$ and PRAME$^+$ melanoma but not ALL cell lines. Quantitative real-time reverse transcription-PCR showed significantly lower PRAME mRNA levels in ALL than in melanoma cell lines, suggesting that PRAME expression in ALL is below the recognition threshold of our PRA$^{100-108}$-tetramer$^+$ T cells.

Conclusion: These data support the usefulness of PRAME and in particular the PRA$^{100-108}$ epitope as target for T-cell therapy of PRAME-overexpressing cancers.

Tumor antigens that are used as targets in clinical studies belong to the melanocyte differentiation antigens, cancer-testis antigens, or antigens overexpressed in tumors (1–5). Cancer-testis antigens are expressed in different tumors but not in normal tissues, except for testis, and are therefore useful targets for T-cell therapy. Most cancer-testis antigens, however, are expressed at low frequencies (30-50%).

Preferentially expressed antigen on melanomas (PRAME) has been identified as an antigen recognized by an HLA-A24-restricted CTL isolated from a melanoma patient (6). Semi-quantitative reverse transcription-PCR (RT-PCR) analysis showed frequent PRAME expression in melanomas (95%) and other tumor types, including lung and breast tumors and leukemias but not in healthy tissues, except for testis and low expression in endometrium, ovaries, and adrenals. DNA microarray analysis revealed the gene encoding PRAME as one of the genes of an expression profile for poor prognosis in breast carcinoma (7). Recently, the function of PRAME has been elucidated by Epping et al. (8). PRAME binds to retinoic acid receptor α, thereby inhibiting retinoic acid–induced differentiation, growth arrest, and apoptosis. Suppression of high levels of endogenous PRAME in retinoic acid–resistant melanoma cells by RNA interference restores sensitivity to the antiproliferative effects of retinoic acid, suggesting that PRAME overexpression contributes to oncogenesis by inhibiting retinoic acid signaling.

In this study, HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for T cells directed against four previously identified HLA-A*0201-binding PRAME epitopes (9) by IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays and tetramer staining. T cells specific for PRA$^{100-108}$ were most frequently found both after *in vitro* stimulation and directly *ex vivo* after isolation by magnetic microbeads. Furthermore, PRA$^{100-108}$-tetramer$^+$ T-cell clones

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Note: S. Osanto and C. Melief contributed equally to the work.

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were isolated and shown to recognize and lyse tumor cells expressing high levels of PRAME, supporting the usefulness of PRAME as target for immunotherapy.

Materials and Methods

Cell lines and culture conditions. Melanoma cell lines 453A0, 513D, 518A2, and IGR39D were established in our laboratory. Cell lines FM6 and FM3 were kindly provided by J. Zeuthen (Copenhagen, Denmark). Breast carcinoma cell line MDA-231 was obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured in DMEM (Invitrogen, Breda, the Netherlands) supplemented with 8% FCS, 4 mmol/L L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin. The Leiden acute lymphoid leukemia (L-ALL) cell lines L-ALL-BV, L-ALL-RL, L-ALL-VG, L-ALL-CR, L-ALL-HP, L-ALL-KW, L-ALL-PH, L-ALL-SK, L-ALL-VB, and L-CML-B were cultured as described (10). L-CML-B is derived from a patient with lymphoblastic crisis of chronic myeloid leukemia (CML) but has phenotypic characteristics of ALL. K562-A2 and K562-A3 are generated by transduction of cell line K562 with retroviral vectors encoding HLA-A*0201 and A*0301. K562-A2, K562-A3 and EBV-B cell lines were cultured in RPMI with penicillin/streptomycin and 8% FCS. The T2 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) with penicillin/streptomycin and 5% FCS.

In vitro *stimulation of peripheral blood mononuclear cells.* Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood from healthy individuals and melanoma patients (American Joint Cancer Committee stages III and IV) by Ficoll gradient centrifugation. The study was approved by the local Medical Ethical Committee, and informed written consent was given by all individuals. Individuals were subtyped as HLA-A*0201 by PCR. PBMCs were seeded at 3×10^6 per well in 24-well plates in T-cell medium [IMDM with penicillin/streptomycin; 116 μg/mL L-arginine, 36 μg/mL L-asparagine, and 215 μg/mL L-glutamine; 10% human serum; and 150 IU/mL human recombinant interleukin-2 (IL-2)], containing 2 μg/mL of PRAME ($PRA^{100-108}$, $PRA^{142-151}$, $PRA^{300-309}$, and $PRA^{425-433}$) or influenza (FLU^{58-66}) peptides (9, 11). At day 11, PBMCs were collected, washed, and seeded at 3×10^6 per well in 24-well plates in T-cell medium without peptides. At day 13, PBMCs were tested for specific $CD8^+$ T cells by IFN-γ ELISPOT assays and tetramer staining.

IFN-γ ELISPOT assay. The IFN-γ ELISPOT assay was done as described (12) using 96-well nylon Silent Screen plates (Nunc Gmbh and Co. KG, Weisbaden, Germany) and AP-conjugate substrate kit (Bio-Rad, Hercules, CA). Peptide-stimulated PBMCs were seeded at 5×10^4, 2.5×10^4, and 1×10^4 per well together with 5×10^4 (peptide pulsed) T2 cells in triplicate in IMDM with 5% human serum overnight at 37°C. T2 cells were pulsed with peptides (5 μg/mL) in IMDM for 2 hours at 37°C.

Tetramer staining. Peptide-stimulated PBMCs ($1\text{-}5 \times 10^5$) and $CD8^+$ T-cell clones ($1\text{-}5 \times 10^4$) were washed with PBS supplemented with 0.5% bovine serum albumin (PBA) and incubated with phycoerythrin-labeled $PRA^{100-108}$, $PRA^{142-151}$, $PRA^{300-309}$, and FLU^{58-66} or APC-labeled $PRA^{425-433}$ tetrameric complexes (10 pmol/mL) in PBA for 1 hour at room temperature. After subsequent incubation with anti-CD8-FITC for 20 minutes at 4°C, cells were washed and resuspended in PBA for fluorescence-activated cell sorting analysis. Propidium iodide (PI) was added (5 μg/mL) to exclude dead cells.

Isolation of tetramer$^+$ T cells by magnetic microbeads. Total PBMCs (30×10^6) were stained with phycoerythrin-labeled $PRA^{100-108}$-tetramer (10 pmol/mL) in PBA for 1 hour at room temperature. After washing, cells were incubated with magnetic anti-phycoerythrin microbeads and applied on MS^+ columns, according to manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total PBMCs before isolation (1×10^6) and all PBMCs after isolation were stained with anti-CD8-FITC and anti-CD45RA-APC in PBA for 30 minutes at 4°C. After washing, cells were analyzed by fluorescence-activated cell sorting. PI was added (5 μg/mL) to exclude dead cells.

Generation and isolation of $PRA^{100-108}$-specific $CD8^+$ T cells. Adherent monocytes from HD13 were cultured in AIM-V (Invitrogen, Breda, the Netherlands) with L-arginine/L-asparagine/L-glutamine and penicillin/streptomycin supplemented with 500 units/mL IL-4 (Peprotech, Inc., Rocky Hill, NJ) and 800 units/mL granulocyte macrophage colony-stimulating factor (Behringwerke, Marburg, Germany) for 8 days. Dendritic cells were pulsed with 10 μg/mL $PRA^{100-108}$ peptide in IMDM with penicillin/streptomycin for 4 hours, washed, and subsequently incubated with autologous nonadherent PBMCs in IMDM with penicillin/streptomycin, L-arginine/L-asparagine/L-glutamine, 10% human serum, 10 ng/mL IL-7 (Peprotech), and 100 pg/mL IL-12 (Sigma-Aldrich, Zwijndrecht, the Netherlands). T-cell cultures were weekly restimulated with autologous dendritic cells pulsed with $PRA^{100-108}$ as described above in T-cell medium. At day 21, $PRA^{100-108}$-tetramer$^+$ $CD8^+$ T cells were sorted by fluorescence-activated cell sorting and seeded at 1 per well in 96-well plates, each well containing 1×10^5 irradiated (50 Gy) allogeneic PBMCs, 5×10^3 irradiated (100 Gy) EBV-B cells, and 5×10^3 irradiated (100 Gy) $PRA^{100-108}$-pulsed EBV-B cells. Growing T-cell clones were isolated and weekly restimulated as described above.

Cocultures of tumor cells and $PRA^{100-108}$-specific $CD8^+$ T cells. Tumor cells (5×10^4) were seeded together with $PRA^{100-108}$-specific T cells (5×10^4) in triplicate in 96-well flat-bottomed plates in T-cell medium. As controls, triplicate wells were incubated with tumor cells (5×10^4) in the absence of T cells. After overnight incubation at 37°C, pools of supernatants of triplicate wells were analyzed for release of cytokines by human Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences, Franklin Lakes, NJ). After 48 hours of incubation, all individual cocultures were collected, washed with PBA, and stained with anti-CD3-FITC. Total numbers of viable tumor cells were analyzed by fluorescence-activated cell sorting after addition of PI (5 μg/mL). The percentage of viable tumor cells (% cell viability) was calculated as follows: [mean number of viable tumor cells (PI^- and $CD3\text{-}FITC^-$) after coincubation with T cells / mean number of viable tumor cells in the absence of T cells] × 100.

Quantitative real-time RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Random-primed cDNA was synthesized according to manufacturer's instructions (Roche, Indianapolis, IN). The PRAME 5′-TCACTTGTTGCCACGCACGTCTGA-3′ and housekeeping porphobilogen deaminase (PBGD) 5′-CTCATCTTTGGGCTGTT-TTCTTCCGCC-3′ probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and labeled with reporter dye TET at the 5′ end and quencher dye TAMRA at 3′ end (Eurogentec, Seraing, Belgium). The forward PRAME primer 5′-GTCAACAGCAACTTCGCGGT-3′ was selected on the junction of exons 1 and 2 at position 94 bp (GeneID 23532), and the reverse PRAME primer 5′-AATGGAACCCCA-CAAACGC-3′ was selected on the junction of exons 3 and 4 at position 265 bp. The forward and reverse primers for PBGD (GeneID 3145) are 5′-GGCAATGCGGCTGCAA-3′ and 5′-GGGTACCCACGCGAATCAC-3′, respectively. PCR was done using the qPCR core kit (Eurogentec). Amplification was started with 10 minutes at 95°C followed by 55 cycles of 15 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 65°C. The 171-bp PRAME product was confirmed by DNA sequencing. PRAME expression was normalized to expression of the *PBGD* gene.

Results

In vitro *expansion of PRAME-specific $CD8^+$ T cells.* To investigate whether human individuals have circulating T cells specific for PRAME, total PBMCs from HLA-A*0201-subtyped healthy individuals and melanoma patients were stimulated *in vitro* with four previously identified HLA-A*0201-binding PRAME peptides (9) and tested for PRAME-specific T cells by IFN-γ ELISPOT assays and tetramer staining.

Of the four PRAME peptides, T cells specific for PRA$^{100\text{-}108}$ were most frequently found by ELISPOT and tetramer staining (Fig. 1; i.e., in 4 of 14 healthy individuals and 4 of 11 patients). None of the donors and patients had measurable T cells specific for PRA$^{142\text{-}151}$ (data not shown). T cells specific for PRA$^{300\text{-}309}$ were detected in one healthy individual and one patient by ELISPOT but not by tetramer staining (data not shown). T cells specific for PRA$^{425\text{-}433}$ were detected in two healthy individuals by tetramer staining but not in ELISPOT assays (data not shown). FLU$^{58\text{-}66}$-specific T cells were detected in 13 of 14 healthy individuals and 8 of 11 patients by ELISPOT and in two additional patients by tetramer staining (data not shown).

Ex vivo ***detection of PRA$^{100\text{-}108}$-specific CD8$^+$ T cells.*** To investigate the frequency and phenotype of PRA$^{100\text{-}108}$-specific T cells, total PBMCs (30×10^6) from the same healthy individuals and melanoma patients were stained with phycoerythrin-labeled PRA$^{100\text{-}108}$-tetramer and subsequently isolated by magnetic anti-phycoerythrin microbeads. By this method, FLU$^{58\text{-}66}$-tetramer$^+$ CD8$^+$ T cells were enriched ~200-fold (Fig. 2A). Of the FLU$^{58\text{-}66}$-specific T cells, 70% to 80% were CD45RA$^-$, indicating a memory phenotype. Figure 2B shows the results of all individuals with percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than the mean percentage + 3× SD as obtained in eight HLA-A*0201$^-$ individuals. PRA$^{100\text{-}108}$-specific CD8$^+$ T cells were detected directly *ex vivo* after isolation by magnetic microbeads in 4 of 14 healthy individuals and 4 of 11 patients. The percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$ after isolation are 0.0 (HD1, 2, 4-6, 8-10 and P2, 8, 10), 0.1 (HD3, 12 and P4-6, 9) 0.5 (P7), 0.9 (HD14), 1.2 (P3), 1.3 (HD11), 1.5 (HD7), 1.8 (HD13), 4.3 (P11), and 6.5 (P1). The individuals with detectable PRA$^{100\text{-}108}$-tetramer$^+$ T cells *ex vivo* (Fig. 2B) were the same as those showing expansion of PRA$^{100\text{-}108}$-specific T cells after *in vitro* stimulation (Fig. 1). Variable numbers of memory (CD45RA$^-$) PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in two healthy individuals (37% in HD7 and 89% in HD14) and one patient (57% in P1). The majority of PRA$^{100\text{-}108}$-tetramer$^+$ T cells, however, expressed CD45RA, suggesting a naive phenotype. Frequencies ranged between 13 and 111 PRA$^{100\text{-}108}$-specific T cells isolated from 30×10^6 total PBMCs, indicating a low frequency of ~4 to 40 antigen-specific T cells in 1×10^6 CD8$^+$ cells.

Generation and isolation of PRA$^{100\text{-}108}$-specific CD8$^+$ T cell clones. Because PRAME and in particular the PRA$^{100\text{-}108}$ epitope are interesting targets for T-cell therapy, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were further investigated for their ability to recognize and lyse tumor cells. PRA$^{100\text{-}108}$-tetramer$^+$ T cells were induced *in vitro* by stimulating monocyte-depleted PBMCs from HD13 with autologous dendritic cells pulsed with PRA$^{100\text{-}108}$ peptide (Fig. 3). After two *in vitro* stimulations, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were sorted at 1 per well in the presence of irradiated feeder cells. Fourteen growing PRA$^{100\text{-}108}$-specific T-cell clones were isolated, and four clones (clones 3, 7, 33, and 34) were tested for specific cytokine release by Cytometric Bead Array. All clones expressed CD8, CD45RO, and TCRVβ7.1 but not CD27, CD28, and CCR7 (data not shown).

Figure 4A shows that PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clones released significant levels of IL-4, IL-5, and IFN-γ and low

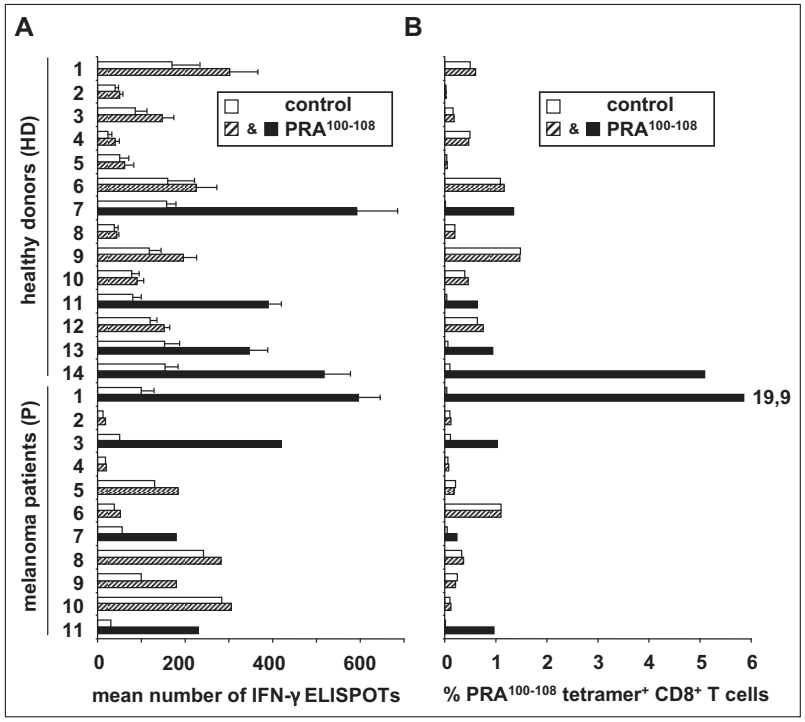

Fig. 1. Detection of PRA$^{100\text{-}108}$-specific CD8$^+$ T cells after *in vitro* stimulation. Total PBMCs from HLA-A*0201-subtyped healthy individuals and melanoma patients were stimulated *in vitro* with PRA$^{100\text{-}108}$ peptide. At day 13, PBMCs were tested for specific T cells by IFN-γ ELISPOT assays and tetramer staining. *A,* PRA$^{100\text{-}108}$-stimulated PBMCs were seeded at 5, 2.5, and 1×10^4 per well together with 5×10^4 nonpulsed (control) or PRA$^{100\text{-}108}$-pulsed T2 cells in triplicate wells in IFN-γ ELISPOT assays. Columns, mean numbers of spots produced by 10^5 peptide-stimulated PBMCs of triplicate wells; bars, SD. Solid columns are shown for mean numbers of specific spots higher than the mean number of background spots + 3× SD and $P < 0.05$ using the Student's *t* test for unpaired samples. *B,* PRA$^{100\text{-}108}$-stimulated PBMCs and PBMCs stimulated with other peptides (control = PRA$^{142\text{-}151}$, PRA$^{300\text{-}309}$, or PRA$^{425\text{-}433}$) were stained with anti-CD8 and PRA$^{100\text{-}108}$-tetramer. Percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$. Solid columns are shown for PBMC cultures containing percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than 4× the percentages in PBMC cultures stimulated with control peptides.

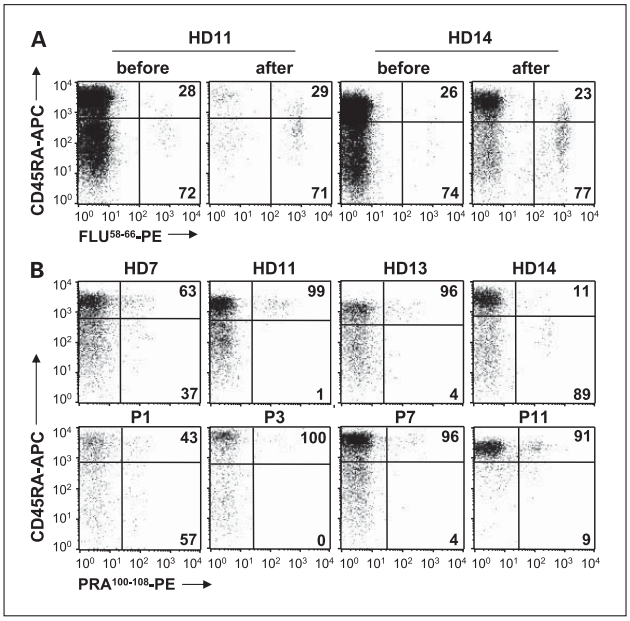

Fig. 2. *Ex vivo* detection of tetramer$^+$ CD8$^+$ T cells. *A,* total PBMCs (30×10^6) from two healthy individuals were stained with phycoerythrin-labeled FLU$^{58\text{-}66}$-tetramer and subsequently isolated by magnetic anti-phycoerythrin microbeads. Total PBMCs (2.5×10^5) before isolation and all PBMCs after isolation were stained with anti-CD8-FITC and anti-CD45RA-APC and analyzed by fluorescence-activated cell sorting. FLU$^{58\text{-}66}$-PE tetramer and anti-CD45RA-APC staining for gated viable (PI$^-$) CD8$^+$ cells. Percentages of FLU$^{58\text{-}66}$-tetramer$^+$ T cells that are CD45RA$^+$ and CD45RA$^-$. *B,* total PBMCs (30×10^6) from HLA-A*0201-subtyped healthy individuals and melanoma patients were stained with phycoerythrin-labeled PRA$^{100\text{-}108}$ tetramer and isolated by magnetic anti-phycoerythrin microbeads. All isolated cells were stained with anti-CD8-FITC and anti-CD45RA-APC and analyzed by fluorescence-activated cell sorting. PRA$^{100\text{-}108}$-PE tetramer and anti-CD45RA-APC staining for gated viable (PI$^-$) CD8$^+$ cells. Percentages of PRA$^{100\text{-}108}$-tetramer$^+$ T cells that are CD45RA$^+$ and CD45RA$^-$. For HD7, 11, 13, 14 and P1, 3, 7, 11 with percentages of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells higher than the mean percentage + 3× SD as obtained in eight HLA-A*0201$^-$ healthy donors ($0.06 + 3 \times 0.04 = 0.18$).

levels of IL-2, IL-10, and tumor necrosis factor-α upon incubation with breast carcinoma cell line MDA231 (HLA-A*0201$^+$, PRAME$^-$) pulsed with PRA$^{100\text{-}108}$ peptide or infected with an adenoviral vector encoding PRAME (data not shown). Nonpulsed MDA231 cells and MDA231 cells pulsed with control peptide (PRA$^{300\text{-}309}$) or empty adenoviral vector were not recognized. PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clones also released cytokines upon incubation with K562 (MHC class I$^-$, PRAME$^+$) transduced with HLA-A*0201 (K562-A2), whereas K562 transduced with HLA-A*0301 (K562-A3) was not recognized. These results show that PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells recognize endogenously processed PRA$^{100\text{-}108}$ in the context of HLA-A*0201.

Specific cytokine release upon stimulation with PRA$^{100\text{-}108}$ peptide and K562-A2 cells was also shown for PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cell clones isolated from peptide-stimulated PBMCs from HD7 (five clones) and P3 (two clones) as well as PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells from HD14 tested immediately after isolation from peptide-stimulated PBMCs (data not shown). However, all T cells isolated from peptide-stimulated PBMCs, as shown in Fig. 1, failed to expand to sufficient numbers for detailed analysis.

The avidities of the PRA$^{100\text{-}108}$-tetramer$^+$ T cell clones were determined by titrating the PRA$^{100\text{-}108}$ peptide on HLA-A*0201$^+$ T2 cells (Fig. 4B). Specific cytokine release could be measured at 1 ng/mL of PRA$^{100\text{-}108}$ peptide. Moreover, at suboptimal peptide concentrations, PRA$^{100\text{-}108}$-specific T cell clones released more IL-4 and IL-5 than IFN-γ.

Recognition of melanoma and ALL cell lines by PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T-cell clones. Next, the PRA$^{100\text{-}108}$-tetramer$^+$ T cell clones were tested for recognition of a panel of melanoma and ALL cell lines. The T-cell clones released IL-4, IL-5, IFN-γ (Fig. 4C), and granzyme B (data not shown) upon incubation with HLA-A*0201$^+$ and PRAME$^+$ melanoma (453A0, FM6, 513D, 518A2, and FM3), but not ALL (L-ALL-BV and L-CML-B) cell lines. Negative control cell lines lacking expression of HLA-A*0201 (IGR39D and L-ALL-VG) or PRAME (MDA231 and L-ALL-RL) were not recognized. Finally, PRA$^{100\text{-}108}$-specific T cells not only recognized but also lysed HLA-A*0201$^+$ and PRAME$^+$ melanoma cell lines, as shown by reduced numbers of viable tumor cells after 48 hours of coincubation

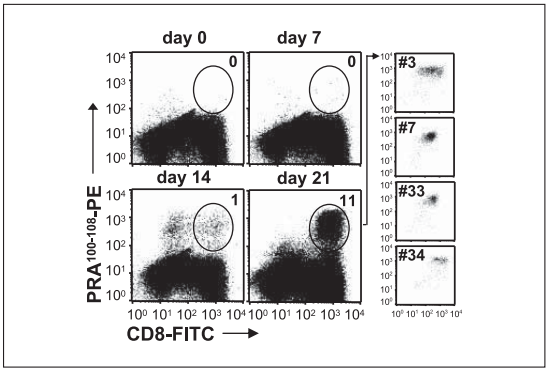

Fig. 3. Generation and isolation of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells. Dendritic cells from HD13 were pulsed with PRA$^{100\text{-}108}$ peptide and incubated with autologous nonadherent PBMCs at a ratio of 1:10. At days 7 and 14, T-cell cultures were restimulated with PRA$^{100\text{-}108}$-pulsed dendritic cells and analyzed for numbers of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells by fluorescence-activated cell sorting. Numbers indicate percentages of CD8$^+$ cells that are PRA$^{100\text{-}108}$-tetramer$^+$. At day 21, PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T cells were sorted at 1 per well. Fourteen growing T-cell clones, among which clones 3, 7, 33, and 34, were isolated.

with PRA$^{100\text{-}108}$-tetramer$^+$ T cells (Fig. 4D) and increased uptake of PI (data not shown).

PRAME mRNA expression by quantitative real-time RT-PCR. Total mRNA was isolated from melanoma and ALL cell lines to investigate PRAME expression levels by quantitative real-time RT-PCR (data not shown). All melanoma cell lines expressed high levels of PRAME mRNA (~10-fold lower than in K562). PRAME mRNA levels in ALL cell lines, however, were significantly lower (~100- to 1,000-fold) than in melanoma cell lines.

Discussion

The human PRAME protein is frequently expressed in tumors of various histologic origins. In this report, we investigated the usefulness of PRAME as target for anticancer T-cell therapy. HLA-A*0201-subtyped healthy individuals and advanced melanoma patients were screened for CD8$^+$ T cells directed against four previously identified PRAME epitopes (9). The results show that T cells specific for PRA$^{100\text{-}108}$ are most frequently found in 30% to 40% of healthy individuals and melanoma patients after *in vitro* stimulation. PRA$^{100\text{-}108}$-tetramer$^+$ T cells could not be detected directly *ex vivo* before isolation by magnetic microbeads due to the low frequency of these T cells in peripheral blood (~4 to 40 antigen-specific T cells in 1×10^6 CD8$^+$ cells). After isolation by magnetic microbeads, however, PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in the same individuals as after *in vitro* stimulation, showing that magnetic microbeads allow rapid *ex vivo* isolation and phenotyping of low-frequency tetramer$^+$ T cells.

Variable numbers of memory (CD45RA$^-$) PRA$^{100\text{-}108}$-tetramer$^+$ T cells were found in two healthy individuals and one melanoma patient. The majority of PRA$^{100\text{-}108}$-tetramer$^+$ T cells, however, expressed CD45RA, suggesting a naive phenotype. The presence of naive PRA$^{100\text{-}108}$-tetramer$^+$ T cells in melanoma patients indicates that these T cells have not been activated *in vivo* by PRAME-expressing tumor cells possibly due to lack of danger signals, defective dendritic cell function, or production of immunosuppressive factors (13–15). The presence of memory PRA$^{100\text{-}108}$-tetramer$^+$ T cells in a minority of individuals remains unclear. These T cells might have been activated *in vivo* by PRAME-expressing benign or malignant cells or by foreign antigen mimicry, as shown for the MART-1/Melan-A$^{27\text{-}35}$ peptide (16).

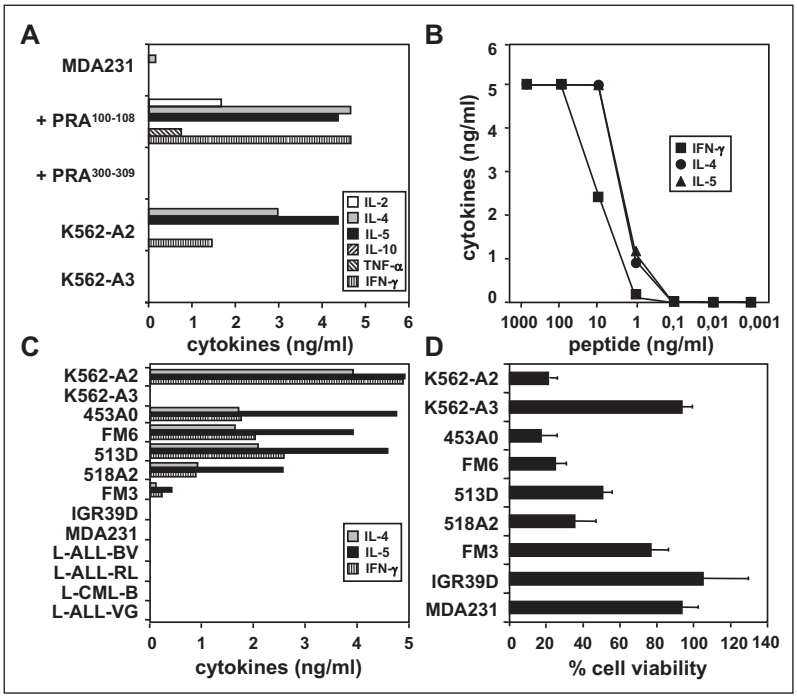

Fig. 4. Functional analysis of PRA$^{100\text{-}108}$-tetramer$^+$ CD8$^+$ T-cell clones. *A,* PRA$^{100\text{-}108}$-tetramer$^+$ T-cell clone 34 was incubated with breast carcinoma cell line MDA231 (PRAME$^-$, HLA-A*0201$^+$) pulsed with 10 μg/mL PRA$^{100\text{-}108}$ or control (PRA$^{300\text{-}309}$) peptide and with K562 (PRAME$^+$) cell lines expressing HLA-A*0201 (K562-A2) and HLA-A*0301 (K562-A3). After overnight incubation, cytokine release in supernatants was measured by human Th1/Th2 cytokine CBA. Similar results have been obtained with T-cell clones 3, 7, and 33 (data not shown). *B,* peptide sensitivity of T-cell clone 34 was tested by titrating PRA$^{100\text{-}108}$ peptide on T2 cells. After overnight incubation, cytokine release in supernatants was measured by Cytometric Bead Array. Similar results have been obtained for T-cell clone 3 (data not shown). *C,* T-cell clone 7 was incubated together with human tumor cell lines in triplicate wells. K562-A2; melanoma cell lines 453A0, FM6, 513D, 518A2, and FM3; and ALL cell lines L-ALL-BV and L-CML-B are PRAME$^+$ and HLA-A*0201$^+$. K562-A3, melanoma cell line IGR39D, and cell line L-ALL-VG are PRAME$^+$ and HLA-A*0201$^-$. Cell lines MDA231 and L-ALL-RL are PRAME$^-$ and HLA-A*0201$^+$. After overnight incubation, supernatants were analyzed for release of cytokines by Cytometric Bead Array. Similar results have been obtained for T-cell clone 3 (data not shown). *D,* after 48 hours of incubation, all cocultures, as described in (*C*), were collected, stained with anti-CD3-FITC, and analyzed for numbers of viable (PI$^-$) tumor cells by fluorescence-activated cell sorting. Columns, mean % cell viability = [number of viable tumor cells (PI$^-$ and CD3-FITC$^-$) after coincubation with T cells / mean number of viable tumor cells in the absence of T cells] × 100 (triplicate wells); bars, SD. Similar results have been obtained for T-cell clone 3 (data not shown).

Natural T-cell immunity against tumor cells seems to occur frequently (17), but the tumor antigens recognized are often unknown. MART-1/Melan-A-tetramer$^+$ T cells are readily detectable directly *ex vivo* in melanoma patients and healthy individuals due to the high frequency of these T cells in peripheral blood ($\sim$400 to 4,000 antigen-specific T cells in 1×10^6 CD8$^+$ cells). These MART-1/Melan-A-specific T cells have been shown to display a memory or naive phenotype (18, 19). It has been suggested that MART-1/Melan-A-specific T cells with a memory phenotype are induced *in vivo* upon tumor progression due to the presence of high antigen load (20). At that stage, the antigen-specific T cells apparently fail to counter tumor progression; therefore, therapies boosting naturally acquired antitumor responses are not expected to be very effective. Because circulating PRA$^{100\text{-}108}$-specific T cells display a naive phenotype in most patients, *in vivo* immunization with peptides covering the PRA$^{100\text{-}108}$ epitope may be an effective strategy to stimulate antitumor T-cell reactivity.

To investigate whether PRA$^{100\text{-}108}$-tetramer$^+$ T cells are able to recognize and lyse tumor cells, PRA$^{100\text{-}108}$-specific T cells were induced *in vitro* by stimulating PBMCs from HD13 with peptide-pulsed dendritic cells. All T-cell clones released type 1 (IFN-γ) and type 2 (IL-4 and IL-5) cytokines. This mixed Tc1/Tc2 cytokine pattern may be associated with the naive phenotype of these T cells in nonstimulated PBMCs from HD13, because PRA$^{100\text{-}108}$-specific T cells from HD14, displaying a memory phenotype in nonstimulated PBMCs, were shown to have a more Tc1 cytokine profile, predominantly releasing IFN-γ and tumor necrosis factor-α (data not shown).

All PRA$^{100\text{-}108}$-specific T-cell clones recognized HLA-A*0201$^+$ and PRAME$^+$ melanoma but not ALL cell lines. Quantitative real-time RT-PCR showed significantly lower ($\sim$100- to 1,000-fold) PRAME mRNA levels in ALL than in melanoma cell lines. These results do not support previous studies showing high levels of PRAME expression in ALL (21, 22). A possible explanation for this discrepancy may be the use of different primer pairs in PCR reactions. A 171-bp PRAME fragment from positions 94 to 265 bp was amplified by our real-time RT-PCR, whereas others amplified a 560-bp fragment from positions 662 to 1,222 bp (21, 22). Five PRAME mRNA variants have been described, all encoding the same PRAME protein. Two of these variants have an alternative exon 1 and may have been missed in our RT-PCR reactions due to partial homology to the forward primer. It can be speculated that high PRAME mRNA expression in ALL is caused by selective overexpression of these two PRAME variants. The differential recognition of melanoma and ALL cell lines by our PRA$^{100\text{-}108}$-specific CD8$^+$ T cell clones, however, makes this possibility unlikely and supports the finding that PRAME mRNA expression is significantly lower in ALL than in melanoma cell lines.

In standard 4-hour ^{51}Cr-release assays, we consistently observed low specific lysis of K562-A2 (10-20%) versus K562-A3 (0%) by PRA$^{100\text{-}108}$-tetramer$^+$ T cells (data not shown), whereas these cells were lysed up to 80% after 2 days of coincubation. Similarly, with the exception of cell line 453A0, no specific lysis of melanoma cell lines could be measured in ^{51}Cr-release assays, whereas all HLA-A*0201$^+$ and PRAME$^+$ melanoma cell lines were recognized in Cytometric Bead Array assays and killed after 2 days of coincubation with PRA$^{100\text{-}108}$-tetramer$^+$ T cells. The slow killing mechanism might be attributed to release of low levels of granzyme B and perforin. Because the T-cell clones clearly recognise HLA-A*0201$^+$ and PRAME$^+$ tumor cell lines in cytokine release assays, indicating that the affinity of the TCR is sufficiently high to recognize endogenously processed PRAME, transfer of the PRA$^{100\text{-}108}$-specific TCR to CD8$^+$ T cells with more powerful cytolytic machineries might be an effective strategy to generate potent tumor-specific T cells.

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