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PRAME-SPECIFIC ALLO-HLA-RESTRICTED T-CELLS WITH POTENT ANTITUMOR REACTIVITY USEFUL FOR THERAPEUTIC T-CELL RECEPTOR GENE TRANSFER

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Clin Cancer Res. 2011 Sep 1;17(17):5615-25

ABSTRACT

Purpose: In HLA matched stem cell transplantation (SCT) it has been demonstrated that beneficial immune response mediating graft versus tumor (GVT) responses can be separated from graft versus host disease (GVHD) mediating immune responses. In this study we investigated whether it would be possible to dissect the beneficial immune response of allo-HLA reactive T-cells with potent anti-tumor reactivity from GVHD inducing T-cells present in the detrimental immune response after HLA mismatched SCT.
Experimental Design: The presence of specific tumor reactive T-cells in the allo-HLA repertoire was analyzed at the time of severe GVHD after HLA-mismatched SCT using tetramers composed of different tumor associated antigens (TAA).
Results: High avidity allo-HLA restricted T-cells specific for the TAA preferentially expressed antigen on melanomas (PRAME) were identified that exerted highly single peptide specific reactivity. The T-cells recognized multiple different tumor cell-lines and leukemic cells, whereas no reactivity against a large panel of non-malignant cells was observed. These T-cells however also exerted low reactivity against mature dendritic cells (DCs) and kidney epithelial cells, which was demonstrated to be due to low PRAME expression.
Conclusions: Based on potentially beneficial specificity and high reactivity, the T-cell receptors of these PRAME specific T-cells may be effective tools for adoptive T-cell therapy. Clinical studies have to determine the significance of the reactivity observed against mature DCs and kidney epithelial cells.

TRANSLATIONAL RELEVANCE

Adoptive cell therapy using T-cells expressing transgenic T-cell receptors (TCRs) with antitumor reactivity is a promising therapy for cancer patients. However, identification of high affinity TCRs specific for tumor associated antigens (TAAs) is a critical bottleneck in this strategy since high avidity TAA specific T-cells are deleted by negative selection in the thymus. Since allo-HLA reactivity of T-cells is not subjected to negative selection we investigated whether beneficial high avidity TAA specific T-cells could be identified within an allo-HLA directed immune response. Our search for TAA specific T-cells within a patient experiencing GVHD led to the discovery of two preferentially expressed antigen on melanomas (PRAME) specific T-cell clones. Based on their single peptide specificity and high antitumor reactivity, the TCRs of these PRAME specific T-cells may be effective tools for adoptive T-cell therapy.

INTRODUCTION

Alloreactive T-cells can mediate detrimental graft versus host disease (GVHD) as well as beneficial graft versus tumor (GVT) response after allogeneic stem cell transplantation (allo-SCT). The alloreactivity of T-cells after HLA matched allo-SCT is mainly directed against peptides derived from polymorphic proteins that differ between patient and donor, and are processed and presented in the context of HLA class I and II molecules. Previously, we and others have demonstrated that GVT reactivity can be separated from the GVH reactivity within polyclonal alloreactive immune responses mediating GVHD[1]. T-cells that are reactive against polymorphic peptides derived from proteins expressed in multiple tissues induce GVHD, whereas T-cells directed against polymorphic peptides derived from proteins exclusively expressed in hematopoietic cells steer the allo-immune response towards GVT[2] response. Analysis of the clinical responses in patients after allo-SCT and of the T-cell receptor (TCR) repertoires within these alloreactive immune responses demonstrated that beneficial GVT response occurs more frequently when the severity of the GVHD response increases, due to the activation of polyclonal responses[3]. This phenomenon was illustrated by the work of Goulmy *et al.* in which was demonstrated that a mismatch for the polymorphic peptide HA-1 was associated with a high risk of severe GVHD, initially suggesting that HA-1 specific T-cells may induce GVHD responses[4]. However, in later studies it was demonstrated that HA-1 specific immune responses also dominated in patients that experienced beneficial GVT responses[5], and that the expression profile of HA-1 was in accordance with a relative specific anti-leukemia reactivity of the T-cells, since HA-1 is exclusively expressed in cells of the hematopoietic lineage[6]. These results imply that despite an overall detrimental clinical phenotype, beneficial GVT reactivies can appear in GVHD, and that these beneficial immune reactivities can be isolated from GVHD responses.

Treatment of patients with HLA-mismatched allo-SCT is associated with GVHD. Also in this transplantation setting beneficial GVT alloreactivity is associated with GVHD, and, depending on the HLA mismatch, GVT is clinically observed[7]. The alloimmune responses after HLA-mismatched allo-SCT will mainly be directed against peptides derived from monomorphic proteins presented in the context of allo-HLA molecules. T-cells present in the polyclonal alloreactive immune response and directed against allo-HLA molecules presenting peptides derived from proteins that are ubiquitously expressed may induce GVHD. In contrast, T-cells directed against allo-HLA molecules presenting peptides derived from monomorphic proteins exclusively expressed in tumor cells, and therefore capable of inducing selective GVT reactivity may also be part of this immune response. This may imply that despite occurrence of a clinical detrimental immunological war after HLA mismatched SCT, characterization of the fine specificity of allo-reactive T-cells may result in the isolation of allo-HLA reactive T-cells that mediate beneficial GVT responses.

As allo-HLA molecules are not expressed within the thymic environment during lymphopoiesis, T-cells are able to recognize peptides with high affinity in the context of the allo-HLA, since allo-HLA reactivity of T-cells is not subjected to negative selection. Therefore, in contrast to self-restricted T-cells, allo-HLA reactive T-cells with anti-tumor reactivity may exert high avidity for the allo-HLA presenting tumor-associated antigens (TAA) derived from over-expressed or tissue specific self-proteins. Many research groups have tried to isolate or generate high affinity TAA specific TCRs. Stauss and colleagues[8], as well as other research groups have confirmed that high affinity T-cells specific for TAA can be derived from the allo-HLA repertoire[8-10]. However, these studies demonstrated that stringent selections have to be performed to identify high affinity single peptide specific T-cells from these *in-vitro* stimulations. In addition, selection from the murine repertoire can be alternative options to isolate high affinity TCRs[11;12]. Examples of over-expressed tumor antigens that may be of potential interest in human leukemia are Wilms' tumor 1 (WT-1), and proteinase 3 (Pr3). In addition, the preferentially expressed antigen on melanomas (PRAME) is of potential interest since it is highly expressed in many different cancers, including acute and chronic myeloid and lymphoid leukemia's[13], whereas normal tissues have low PRAME expression[14]. High affinity TCRs specific for TAA that are shared between various tumors would be attractive tools for TCR gene therapeutic strategies[15;16].

In this study we hypothesized that within the detrimental immunological war that is induced after HLA-mismatched SCT by donor T-cells reacting against patient allo-HLA, donor T-cells that mediate beneficial GVT responses may also selectively be activated and expanded. For this purpose we investigated whether high avidity T-cells directed against allo-HLA presenting TAAs could be observed in a patient experiencing severe GVHD after HLA-A2 mismatched SCT, using HLA-A2 tetramers composed of different TAA peptides. We observed polyclonal allo-HLA restricted T-cells specific for PRAME. These T-cells were demonstrated to exert single peptide specificity and recognition was strictly correlated with PRAME expression. The isolated allo-HLA restricted T-cell clones were in contrast to self-restricted T-cell clones highly reactive against multiple different PRAME positive tumor cell-lines as well as freshly isolated metastatic melanoma and primary leukemic cells, whereas no reactivity against a large panel of non-malignant cells was observed. However, the clones exerted limited on-target reactivity against mature dendritic cells (mDCs) and kidney epithelial cells. Based on their potential beneficial specificity and high reactivity against numerous different tumors, the high affinity TCRs from the allo-restricted PRAME specific T-cells may be effective tools for broad application of TCR gene therapy.

MATERIALS AND METHODS

Cell collection and preparation

Stable Epstein–Barr virus (EBV)-transformed B cell-lines (EBV-LCLs) were generated using standard procedures, and cultured in IMDM and 10% FBS. K562, COS, T2, renal cell carcinoma cell-lines (RCC 1257, RCC 1774, RCC 1851), lung carcinoma cell-lines (A549, NCI-H292), melanoma cell-lines (518A2, FM3, FM6, SK2.3, MI-3046, BML, 1.14), cervix carcinoma cell-lines (SIHA, HELA, CASKI), breast carcinoma cell-lines (MCF7, BT549, MDA231) and colon carcinoma cell-lines (SW480, HCT116, LS411, LS180) were cultured in IMDM and 10% FBS. K562, COS, H292, A549, SIHA and HELA not expressing HLA-A2 were transduced with a retroviral vector encoding for HLA-A2 as previously described[17]. In addition, melanoma cells were freshly isolated from an HLA-A2 positive patient with lymph node metastatic melanoma by ficol isolation of minced tumor cells and subsequent FACS sort of the CD45, CD3, CD19, CD14, CD56 negative cells. For selected experiments COS-A2 cells were transfected with pcDNA3.1 expression vector encoding for wild-type human PRAME. Peripheral blood of HLA-A2 positive patients with primary AML cells (>80% blasts) were cultured for 1 day in IMDM and 10% FCS and used as stimulator cells. Primary AML cells were activated for 1 day with GM-CSF (100 ng /ml; Novartis), TNFα (10 ng/ml; R&D Systems), IL-1b (10 ng/ml; Immunex), IL-6 (10 ng/ml; Cellgenix), PGE-2 (1 µg/ml; Sigma-Aldrich), and IFNγ (500 IU/ml; Immukine, Boehringer Ingelheim). HLA-A2 positive ALL cell-lines were generated as previously described[18]. B cells were isolated from PBMCs by MACS using anti-CD19 coated magnetic beads (Miltenyi Biotec). ALL cell-lines and freshly isolated B cells were activated by culturing the cells for 48 h at a concentration of 10^6 cells/ml in 24-well plates in the presence of IL-4 (500 U/ml; Schering-Plough), CpG oligodeoxynucleotide (10 µg/ml; Eurogentec) and $1x10^5$/ml murine fibroblasts transfected with the human CD40 ligand[19]. In-vivo activated B cells were derived from inflamed tonsils. T-cell blasts were generated by stimulation of PBMCs with PHA and IL-2 (120 IU/ml) for 7 days. Monocytes were isolated from PBMCs by MACS using anti-CD14 coated magnetic beads (Miltenyi Biotec). Macrophages (MØ) were generated by culturing CD14+ cells for 6 days in IMDM and 10% HS at a concentration of $0.5x10^6$cells/ml in 24-well plates. Pro-inflammatory macrophages (MØ1) were obtained by culture in the presence of GM-CSF (5 ng/ml) and anti-inflammatory macrophages (MØ2) cells were cultured with M-CSF (5 ng/ml, Cetus Corporation). Monocyte derived DCs were generated by culturing CD14+ cells for 48 h in IMDM and 10% HS at a concentration of $0.5x10^6$ cells/ml in 24-well plates in the presence of IL-4 (500 U/ml) and GM-CSF(100 ng/ml). For maturation of the CD14 DCs, cells were cultured for another 48 h in IMDM and 10% HS supplemented with GM-CSF (100 ng / ml), TNFα (10 ng/ml), IL-1b (10 ng/ml), IL-6 (10 ng/ml), PGE-2 (1 µg/ml), and IFNγ (500 IU/ml). CD34+ cells were isolated from peripheral blood stem cell grafts by MACS using anti-CD34 coated magnetic beads (Miltenyi Biotec). CD34 DCs were generated by culturing CD34 cells for 4 days in IMDM and 10% HS at a concentration of $0.25x10^6$ cells/ml in 24-well plates in

the presence of GM-CSF (100 ng/ml), SCF (20 ng/ml; kindly provided by Amgen), and TNFα (2 ng/ml), and subsequently for 3 days with additionally IL-4 (500 IU/ml). To maturate the CD34 DCs, the cells were cultured for another 48 h in IMDM and 10% HS supplemented with GM-CSF (100 ng /ml), SCF (20 ng/ml), TNFα (10 ng/ml), IL-1b (10 ng/ml), IL-6 (10 ng/ml), PGE-2 (1 ug/ml), and IFNγ (500 IU/ml). For the isolation of blood derived myeloid DCs (MDCs) and plasmacytoid DCs (PDCs), PBMCs were stained with anti-BDCA1-PE (Biolegend) or anti-BDCA2-PE mAbs (Miltenyi Biotec), respectively, and the BDCA1-PE or BDCA2-PE positive cells were isolated by MACS using anti-PE coated magnetic beads. The MACS isolated cells were stained with FITC conjugated anti-CD3, anti-CD14, anti-CD19 and anti-CD56 mAbs (BD) and the MDCs and PDCs were selected by cell sorting on bases of BDCA1 or BDCA2 positivity and the absence of lineage marker expression. To maturate the MDCs and PDCs, cells were cultured for 24 h in IMDM and 10% HS supplemented with either poly-IC (Amersham) or CpG (10 µg/ml) and IL-3 (50 ng/ml; kindly provided by Novartis), respectively. Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM; Lonza) with 1g/l glucose (BioWhittaker) and 10% FBS. Keratinocytes were cultured from skin biopsies in keratinocyte serum free medium supplemented with 30 µg/ml bovine pituitary extract and 2 ng/ml epithelial growth factor (EGF) (all components were purchased from Invitrogen). Fibroblasts and keratinocytes were cultured for 3 days in the presence or absence of IFNγ (200 IU/ml). Primary bronchial epithelial cells (PBEC) were derived and cultured as previously described[4]. Mesenchymal stromal cells (MSCs) were derived from bone marrow of healthy donors as previously described[5] and cultured in DMEM and 10% FBS. Colon epithelial cells were cultured in DMEM F12 (Lonza) and 10% FCS, and supplemented with EGF (10 ng/ml; Promega), T3 hormone (2nmol/l; Sigma), hydrocortisone (0,4ug/ml; Pharmacy LUMC), and insulin (5 ng/ml; Sigma). Hepatocytes and intrahepatic biliary epithelial cells (IHBEC) (both purchased from ScienCell) were cultured in RPMI (Lonza) and 10% FBS. Proximal tubular epithelial cells (PTEC) were isolated and cultured as previously described[20].

Isolation and analysis of PRAME specific T-cells

All studies were conducted with approval of the institutional review board at MACA. After informed consent, peripheral blood mononuclear cells (PBMCs) were collected from a patient suffering from AML that experienced acute GVHD after single HLA-A2 mismatched SCT and subsequent DLI. Based on a cross-over, the patient was HLA-A*0201 positive and the sibling donor was HLA-A*0201 negative, whereas all other HLA class I and II molecules were completely matched. Patient PBMCs collected during GVHD were stained with anti-HLA-A2-FITC (Pharmingen), anti-HLA-DR-APC (Pharmingen) and anti-CD8-PE (BD) for 30 min at 4°C, and activated (HLA-DRpos), donor derived (HLA-A2neg) CD8+ T-cells were isolated by cell sorting (FACSAria). Since PBMCs were limited, the sorted T-cells were first expanded with anti-CD3/CD28 and irradiated autologous PBMCs (0.5x106/ml) in T-cell medium. T-cell medium consist of Iscove's Modified Dulbecco's Medium (IMDM; Lonza) with 10% human serum (HS), IL-2 (120

IU/ml; Proleukin) and IL-15 (20 ng/ml; Peprotech). T-cells were stimulated non-specifically using irradiated allogeneic PBMCs ($0.5x10^6$ /ml), IL-2 (120 IU/ml), and phytohemagglutinin (PHA, 0.8 µg/ml; Murex Biotec Limited). After 14 days of culture, T-cells were labeled with anti-CD8-APC (BD Bioscience) and PE-conjugated HLA-A2 tetramers specific for the different TAA peptides[21-24]: for PRAME were tested: VLDGLDVLL (VLD), SLYSFPEPEA (SLY), ALYVDSLFFL (ALY), and SLLQHLIGL (SLL), for WT-1: RMFPNAPYL, for Pr-1: VLQELNVTV. For single cell sorting, T-cells were stained with APC conjugated tetramers in combination with TCR-Vβ repertoire kit staining (Beckman Coulter) for 1 h at 4°C, and SLL tetramer+ Vβ1+ and SLL tetramer+ Vβ3+ CD8+ T-cells were sorted and stimulated non-specifically.

Self-restricted PRAME specific T-cell clones were isolated from an HLA-A*0201 patient that was transplanted with a fully HLA-identical donor graft. PBMCs derived from the patient after SCT were labeled with PE-conjugated SLL tetramer for 1 h at 4 °C. Tetramer positive T-cells were isolated by MACS using anti-PE coated magnetic beads (Miltenyi Biotec) and were expanded for 10 days with anti-CD3/CD28 beads as described above. For subsequent sorting, T-cells were stained with PE-conjugated SLL tetramer and anti-CD8-APC for 1 h at 4°C, and tetramer positive CD8+ T-cells were sorted single cell per well and expanded. Three SLL tetramer positive T-cell clones were selected, and used for further analysis.

Functional reactivity of the PRAME specific T-cell clones

Stimulation assays were performed with 5,000 T-cells and 20,000 targets in 96-well plates in Iscoves Dulbecco Modified Medium (IMDM), supplemented with 10% human serum (HS) and 100 IU/ml interleukin 2 (IL-2). The different malignant and non-malignant cells were collected and prepared as described in cell collection and preparation. For peptide titrations, T2 cells were preincubated for 1 h with different concentrations of peptide, and washed. After 18 h of stimulation, supernatant was harvested and IFNγ production was measured by standard ELISA. In the cytotoxicity assays, T-cells were tested at different effector-target ratios against 1,000 51Cr labeled targets in 96-well plates in a standard 4h 51Cr-release assay. In these experiments a control HLA-A2 restricted T-cell clone HSS12 specific for a peptide derived from USP11 was included.

In the CD34 cell proliferation inhibition assay, CD34 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described, and resuspended in progenitor cell culture medium[25].

Peptide elution, reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS)

Peptide elution, RP-HPLC and MS were performed as previously described[26]. Briefly, $3x10^{10}$ Epstein Barr Virus transformed B-cells (EBV-LCLs) were lysed and the peptide-HLA-A2 complexes were purified by affinity chromatography using HLA-A2 specific BB7.2 monoclonal antibody (mAb). Subsequently, peptides were eluted from HLA-A2 molecules, and separated

from the HLA monomers and ß2-microglobulin by size filtration. After freeze drying, the peptide mixture was subjected to a first round of RP-C18-HPLC using a water/acetonitrile/TFA, and fractions were collected. A small sample of each fraction was loaded on T2 cells and tested for recognition by the T-cell clones. The recognized fraction was subjected to a second and a third round of RP-C18-HPLC fractionation. In the second fractionation a water/isopropanol/TFA gradient was used, and in the third fractionation a water/methanol/formic acid gradient was used. After the third fractionation, the peptide masses present in the recognized fractions and in the adjacent non-recognized fractions were determined by MS. Peptides of which the abundance correlated with the recognition pattern of the T-cell clone were selected for tandem mass spectrometry and their sequences determined.

PRAME expression by quantitative real-time PCR, and inhibition of PRAME expression by silencing RNA

PRAME expression was quantified by real-time PCR (TaqMan). Total RNA was isolated from cells using a RNeasy mini kit (Qiagen) or the micro RNaqueous kit (Ambion). First strand cDNA synthesis was performed with oligo dT primers using M-MLV reverse transcriptase (Invitrogen) or with the Transcriptor reverse transcriptase (Roche). Samples were run on a 7900HT RT-PCR System of Applied Biosystems. The following PRAME primers were used, sense 5' CGTTTGTGGGGTTCCATTC 3', anti-sense 5' GCTCCCTGGGCAGCAAC 3' and for the anti-sense probe 5' CCTGCCAGCTCCACAAGTCTCCGTG 3'. The Probe used VIC as dye and TAMRA as quencher, both primers were chosen over an intron/exon boundary. Each sample was run in duplo with cDNA from 50 ng total RNA. The Porphobilinogen Deaminase (PBGD) gene was measured as housekeeping gene to ensure good quality of the cDNA.
Inhibition of PRAME was performed using retroviral vectors encoding for short hairpin (sh) RNA sequences specific for RAME in combination with the puromycin resistance gene that were kindly provided by Dr. R. Bernards, NKI, Amsterdam, The Netherlands[27]. Retrovirally transduced cells were cultured with different concentrations of puromycin for at least 1 week before testing. Proximal tubular epithelial cells (PTECs) were cultured with 3 μg/ml, renal cell carcinoma cell line RCC1257 with 4 μg/ml, and CD34+ derived dendritic cells (CD34DCs) with 0.4 μg/ml puromycin. CD34DCs were generated as described in cell collection and preparation, and were transduced on day 1 of culture.

TCR gene transfer

The TCRAV and TCRBV gene usage of clone HSS1 was determined using reverse transcriptase (RT)–PCR and sequencing[17]. HSS1 expressed TCR-AV1S1 and TCR-BV1S1. A retroviral vector was constructed with a codon optimized and cysteine modified TCRα and TCRβ chain linked by the T2A sequence in combination with the truncated nerve growth factor receptor (ΔNGF-R)[28;29]. Cytomegalovirus (CMV)-IE1 specific HLA-B8 restricted T-cells were sorted using CMV-IE1 tetramers, stimulated for 2 days with PHA and irradiated allogeneic PBMCs and

transduced with PRAME-TCR or mock. Transduced T-cells were sorted based on positivity for ΔNGF-R, and tested for functional reactivity.

RESULTS

Isolation of high avidity PRAME specific allo-restricted T-cells clones from a patient experiencing GVHD after HLA-mismatched SCT

The presence of allo-HLA restricted TAA specific T-cells was analyzed in a patient transplanted with a single HLA-A2 mismatched SCT that experienced GVHD after treatment with donor lymphocyte infusion (DLI) for relapsed acute myeloid leukemia (AML). The activated donor derived CD8+ T-cells at the time of GVHD were selected by FACS sort and expanded using CD3/CD28 expansion beads to be able to perform multiple reactivity screenings. T-cells were labeled with tetramers specific for the HLA-A2 restricted epitopes of different TAAs. The T-cells did not stain with most TAA tetramers, however 0.6% of CD8+ T-cells specifically stained with the PRAME-SLL tetramer (figure 1A). TCR-Vβ usage analysis using TCR-Vβ specific mAbs illustrated that the SLL tetramer positive T-cells consisted of minimally three different clonal populations, one dominant TCR Vβ1+ population (74%), a TCR Vβ3+ population (5%), and a T-cell population with unknown TCR Vβ usage (20%) (figure 1B). To analyze the specificity and avidity of the PRAME specific T-cells, the SLL tetramer positive Vβ1+ and Vβ3+ CD8+ T-cells were single cell sorted and expanded. Of both cell subsets one T-cell clone was selected for further analysis. Both T-cell clones HSS1, expressing TCR-Vβ1, and HSS3, expressing TCR-Vβ3, efficiently stained with the SLL tetramer, but not with control CMV-pp65 tetramer (figure 1C left part). FACS analysis with Vβ specific mAbs confirmed their difference in clonal origin (data not shown).

To determine whether the PRAME specific T-cell clones effectively recognized endogenously processed and presented PRAME, clone HSS1 was tested against K562, previously demonstrated to highly express PRAME[14], and K562 transduced with HLA-A2. To demonstrate that recognition was actually directed against PRAME derived peptide presented in HLA-A2, clone HSS1 was tested against COS cells, either only expressing HLA-A2 or PRAME, or expressing both HLA-A2 and PRAME. In addition, clone HSS1 was tested against different HLA-A2+ and HLA-A2- EBV-LCLs. As shown in figure 1D, HSS1 efficiently recognized K562+A2 and COS cells expressing both PRAME and HLA-A2, whereas untreated K562 and COS cells as well as COS cells expressing only HLA-A2 or PRAME were not recognized. These results indicate that HSS1 recognized endogenously processed and presented PRAME peptide in the context of HLA-A2. Interestingly, HSS1 also recognized all five different HLA-A2+ EBV-LCLs, although with variable strength, whereas HLA-A2- EBV-LCLs were not recognized (reactivity against two representative HLA-A2+ and one HLA-A2- EBV-LCL are shown in figure 1D).

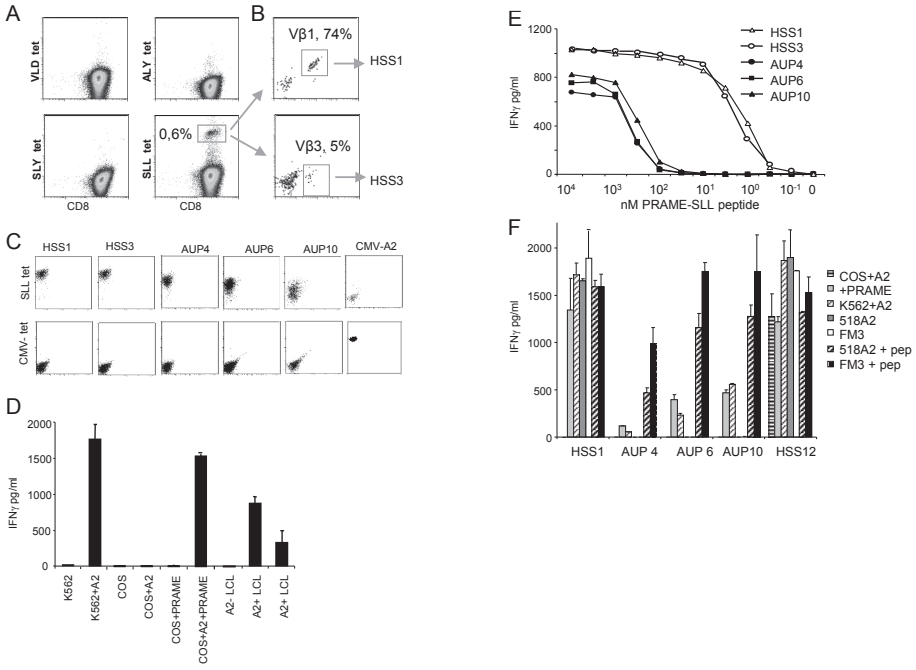


Figure 1. Isolation of allo-restricted and self-restricted PRAME specific T cell clones.
(A) Activated (HLA-DRpositive), donor derived (HLA-A2negative) CD8+ T cells were isolated from a patient experiencing GVHD after HLA-A2 mismatched SCT and subsequent DLI. The isolated T cells were expanded using CD3/CD28 beads and labeled with anti-CD8 and TAA tetramers, only the PRAME tetramers (VLD, SLY, ALY, and SLL) are shown. **(B)** To determine the TCR-Vβ usage, the T cells were labeled with anti-CD8, SLL tetramer and the Vβ repertoire kit. The Vβ1+ (74% of tet+) and Vβ3+ (5% of tet+) T cells were single cell sorted. **(C)** Allo-restricted PRAME-SLL clone HSS1, HSS3, CMV-A2 control clone, and the self-restricted PRAME-SLL clones AUP4, AUP6 and AUP10, were stained with SLL- and pp65-tetramer. **(D)** HSS1 was tested against K562, K562 transduced with HLA-A2 (K562+A2), untransduced COS cells (COS), COS transduced with HLA-A2 (COS+A2), COS+A2 transfected with PRAME (COS+PRAME), COS transduced with HLA-A2 and transfected with PRAME (COS+A2+PRAME), HLA-A2 negative EBV-LCL (A2- LCL), and 5 HLA-A2 positive EBV-LCLs of which two are shown (A2+ LCL). **(E)** HSS1, HSS3, AUP4, AUP6 and AUP10 were stimulated with T2 cells loaded with titrated concentrations of the PRAME-SLL peptide. **(F)** HSS1, AUP4, AUP6 and AUP10, and the USP11 specific T cell clone HSS12 used as a control, were tested for IFNγ production against COS+A2, COS+A2 transfected with PRAME (+PRAME), K562+A2, two HLA-A2+ melanomas (518A2 and FM3) unloaded, or loaded with PRAME peptide (+ pep).

As it has not yet been shown that EBV-LCLs express PRAME, and since these T-cell clones were allo-HLA reactive, we investigated whether clone HSS1 and HSS3, in addition to PRAME, also recognized other peptides presented in HLA-A2. For this purpose the HLA-A2+ EBV-LCLs recognized most prominently by the allo-restricted clones were expanded to 3 x 10^{10} cells, HLA-A2 was isolated by affinity chromatography, and peptides eluted from HLA-A2 were fractionated by multidimensional RP-HPLC, and loaded onto T2 cells. As shown in figure 2, HSS1 was reactive against T2 cells loaded with only one RP-HPLC fraction. This recognized

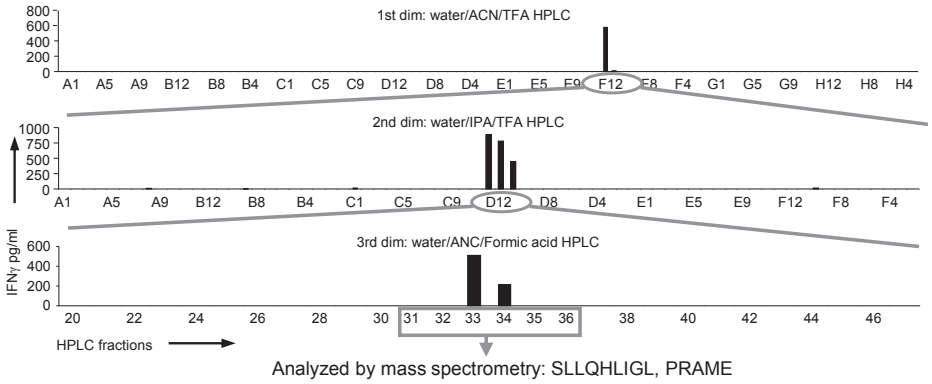


Figure 2. PRAME specific T cell clones are single peptide specific.
Peptides eluted from HLA-A2 were fractionated by RP-HPLC, loaded on T2, and used as stimulators for HSS1. The recognized fraction was fractionated a second and third time, using different organic solvents. After the third fractionation, MS analyses demonstrated that the recognized peptide was PRAME SLLQHLIGL.

fraction was subfractionated using a different HPLC gradient composition, and loaded on T2 cells. Again one fraction of the second RP-HPLC separation was efficiently recognized by HSS1, and this fraction was again further sub-fractionated. The peptide masses present in the recognized third dimension RP-HPLC fractions and in the adjacent not recognized fractions were determined by MS. Comparing the presence and abundance of these peptide masses to the recognition pattern of the T-cell clone resulted in one unique peptide candidate, which was analyzed by tandem MS to be the SLLQHLIGL peptide of PRAME, The synthetic PRAME peptide showed a fragmentation pattern identical to the fragmentation pattern of the natural eluted peptide. Similar results were obtained with clone HSS3 (data not shown). These results demonstrate that reactivity of the allo-reactive PRAME specific T-cell clones against EBV-LCLs was mediated by the recognition of PRAME presented in HLA-A2, and in addition demonstrate that the allo-reactive T-cell clones derived from GVHD exerted single peptide specific recognition.

Comparing the functional avidity of allo-HLA and self-HLA restricted PRAME specific T-cell clones

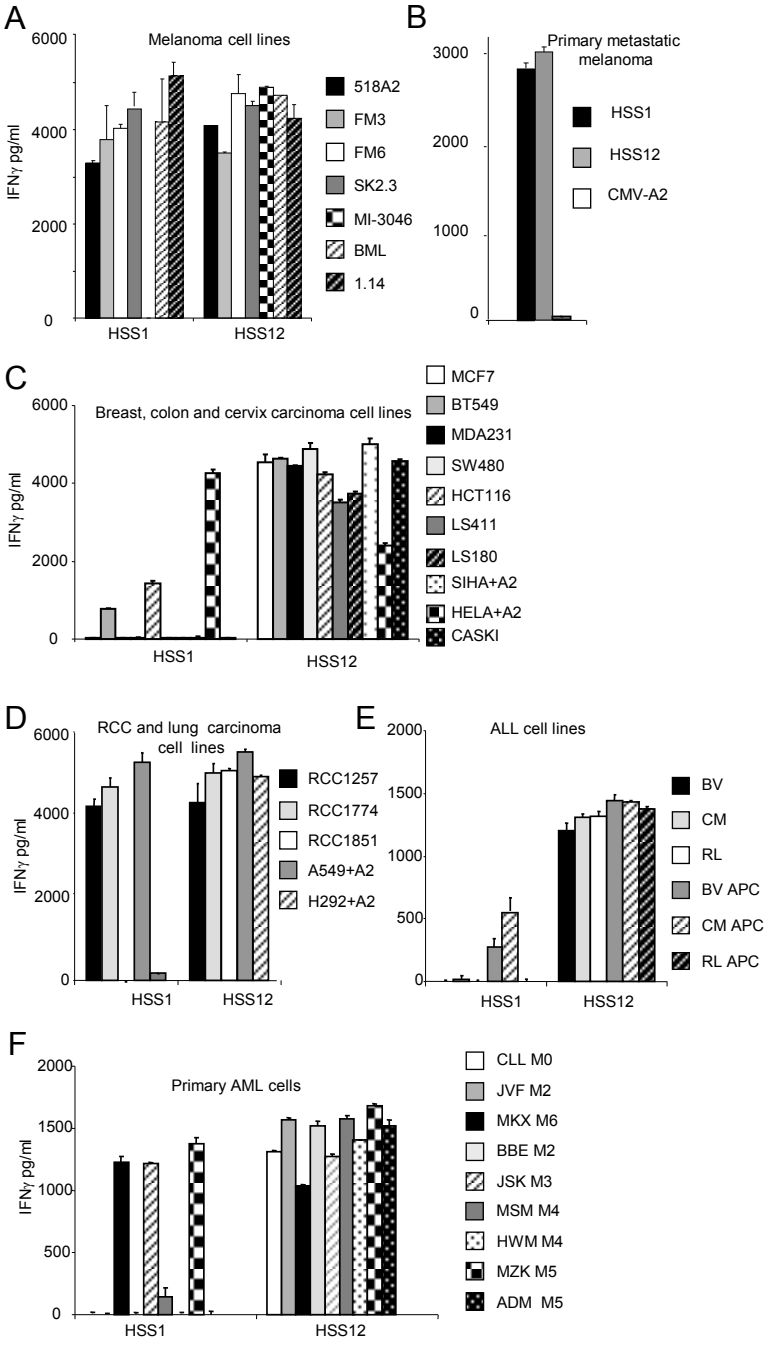
Since we speculated that the avidity of allo-restricted PRAME specific T-cells would be significantly higher compared to self-restricted PRAME specific T-cells, we isolated by flowcytometry using the SLL tetramer, three PRAME-SLL specific self-restricted T-cell clones (AUP4, AUP6 and AUP10) from an HLA-A2 positive patient after HLA-matched SCT. The self-restricted PRAME specific T-cell clones showed specific staining with the SLL tetramer, although with lower intensity compared to the allorestricted T-cell clones (figure 1C right part).

To compare the functional avidity of the self- and allo-restricted PRAME specific T-cell clones, AUP4, AUP6, AUP10, HSS1 and HSS3 were tested against T2 cells loaded with different concentrations of SLL peptide. Although all clones were able to recognize the SLL peptide, the peptide concentration needed for 50% of maximum IFN-γ production differed significantly between the self- and allo-restricted clones. As shown in figure 1E, HSS1 and HSS3 required only 1-2nM of peptide for half-maximal cytokine production, whereas AUP4, AUP6, and AUP10 needed approximately 300nM of peptide. These results demonstrate that in contrast to the allo-restricted PRAME specific T-cell clones, the self-restricted PRAME specific T-cell clones exerted low avidity recognition.

To investigate whether this large difference in avidity has consequences for reactivity of the clones against endogenously processed PRAME, the self- and allo-restricted clones were tested against COS+A2 transfected with PRAME, K562+A2, and 2 melanomas (figure 1F). As a positive control for the HLA-A2 expression and stimulatory capacity of the targets, the allo-HLA-A2 restricted control clone HSS12 specific for USP11 was also tested against all targets. Clone HSS1 efficiently recognized all PRAME expressing cell-lines. Although the self-restricted clones intermediately recognized PRAME transfected COS+A2 as well as K562+A2, the melanomas known to express approximately a 5-10 fold lower amount of PRAME compared to K562 were not recognized. The melanomas were strongly recognized by all four clones when exogenously loaded with SLL peptide. These results demonstrate that high avidity allo-restricted PRAME specific clones efficiently recognize melanoma, whereas the low avidity self-restricted PRAME clones exerted no reactivity against melanoma.

High avidity allo-HLA restricted PRAME specific T-cells effectively recognized a large proportion of tumors and a limited number of non-malignant cells

The PRAME gene is expressed at a high level in a large proportion of tumors, including melanomas, non-small-cell lung carcinomas, renal cell carcinoma (RCC), breast carcinoma, cervix carcinoma, colon carcinoma, as well as several types of leukemia[13;30-32]. We therefore analyzed whether clone HSS1 was able to recognize HLA-A2+ tumor cell-lines and primary tumor cells. Clone HSS12 was used as a control for the HLA-A2 expression of the targets and the ability of the targets to stimulate T-cells. Results shown in figure 3A demonstrate that in addition to the two previously tested melanomas, four different melanomas were also efficiently recognized by clone HSS1. In addition, from an HLA-A2 positive patient with lymph node metastatic melanoma we freshly isolated melanoma cells by ficolling the minced tumor cells and subsequently sorting the CD45, CD3, CD19, CD14, CD56 negative cells. HSS1 as well as the control HSS12 highly recognized the freshly isolated tumor cells, whereas a CMV-A2 control clone did not recognize the metastatic melanoma (figure 3B). As demonstrated in figure 3C and 3D clone HSS1 intermediately recognized 1 of 3 breast carcinomas, and 1 of 4 colon carcinomas, and efficiently recognized 1 of 3 cervix carcinomas, 2 of 3 RCCs, and 1 of 2 lung carcinomas. Three HLA-A2+ acute lymphoblastic leukemia's (ALLs) were not recognized, but two of these


A
Melanoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
518A2
FM3
FM6
SK2.3
MI-3046
BML
1.14
B
Primary metastatic melanoma
3000
2000
1000
0
HSS1
HSS12
CMV-A2
C
Breast, colon and cervix carcinoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
MCF7
BT549
MDA231
SW480
HCT116
LS411
LS180
SIHA+A2
HELA+A2
CASKI
D
RCC and lung carcinoma cell lines
IFNγ pg/ml
6000
4000
2000
0
HSS1
HSS12
RCC1257
RCC1774
RCC1851
A549+A2
H292+A2
E
ALL cell lines
2000
1500
1000
500
0
HSS1
HSS12
BV
CM
RL
BV APC
CM APC
RL APC
F
Primary AML cells
IFNγ pg/ml
2000
1500
1000
500
0
HSS1
HSS12
CLL M0
JVF M2
MKX M6
BBE M2
JSK M3
MSM M4
HWM M4
MZK M5
ADM M5

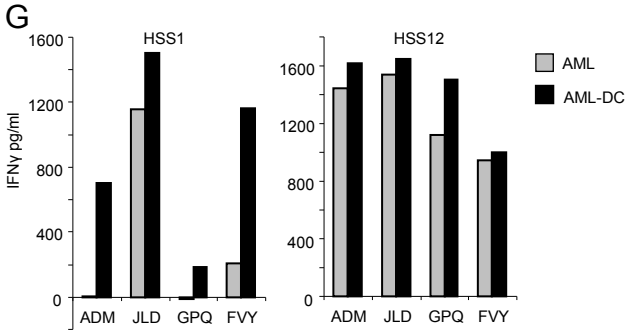
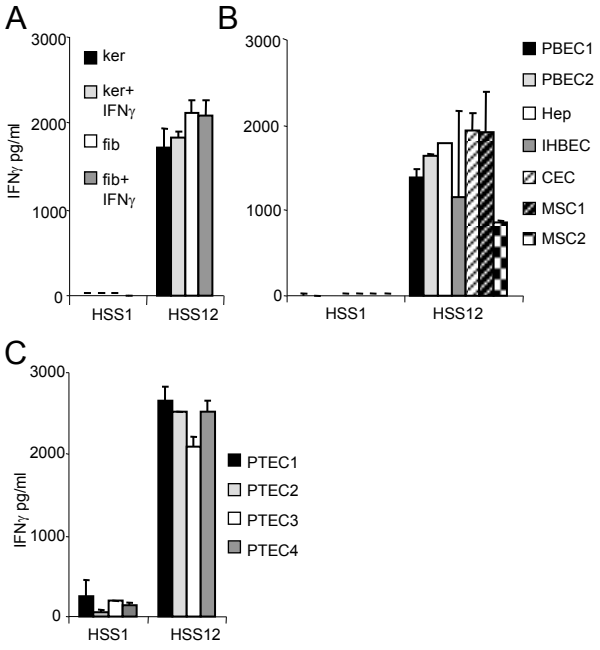


Figure 3. Allo-HLA restricted clone HSS1 specific for PRAME is able to recognize various different tumor cell-lines and primary malignancies.

HSS1 and control HSS12 were tested for IFNγ production against HLA-A2+ **(A)** melanoma cell lines: 518A2, FM3, FM6, SK2.3, MI-3046, BML, and 1.14, **(B)** primary metastasized melanoma (the CMV-A2 clone was included as negative control), **(C)** breast carcinoma cell lines: MCF-7, BT594 and MDA231, colon carcinoma cell lines: SW480, HCT116, LS411 and LS180 and cervix carcinoma cell lines: SIHA+A2, HELA+A2 and CASKI, **(D)** Renal cell carcinomas: RCC1257, RCC1774 and RCC1851, and lung carcinomas: A549+A2 and H292+A2, **(E)** 3 ALLs (BV, CM and RL) and , CD40L activated ALLs (APC), **(F)** 9 primary AMLs of different classification (as indicated), and **(G)** 4 primary AML samples not activated or activated (AML-DC) for 24h with GM-CSF, SCF, TNFα, IL-1b, IL-6, PGE-2, and IFNγ.



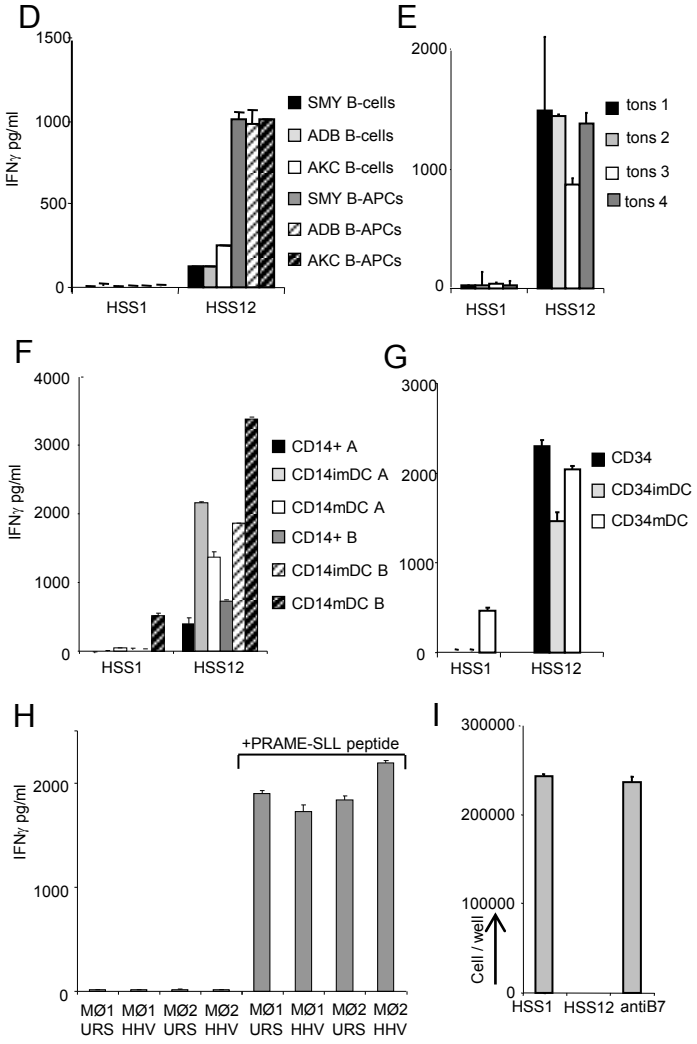


Figure 4. Recognition of non-malignant cells by the allo-HLA restricted PRAME T cells.
HSS1 and control HSS12 were tested for IFNγ production against HLA-A2+ **(A)** fibroblasts (fib) and keratinocytes (ker) untreated or treated with IFNγ (+IFNγ), **(B)** primary bronchus epithelial cells (PBEC1 and PBEC2), hepatocytes (Hep), intrahepatic biliary epithelial cells (IHBEC), colon epithelial cells (CEC) and mesenchymal stromal cells (MSC1 and MSC2), **(C)** 12 different PTECs, of which 4 are shown, **(D)** B cells and CD40L stimulated B cells (B-APC) from 3 individuals, **(E)** in vivo activated B cells derived from inflamed tonsils of 4 individuals (tons1-4), **(F)** CD14 cells, immature CD14 DCs (CD14imDCs) and mature CD14 DCs (CD14mDCs) derived from two individuals (A and B) and **(G)** CD34 cells, immature CD34 DCs (CD34imDC) and mature CD34 DCs (CD34mDC). **(H)** HSS1 was tested for IFNγ production against HLA-A2+ MØ1 and MØ2 macrophages (MØ1 and MØ2 from URS and HHV) unloaded or loaded with PRAME-SLL peptide. **(I)** HSS1, HSS12, and one HLA-B7 restricted T cell clone (negative control) were tested in a CFSE proliferation inhibition assay against HLA-A2+, HLA-B7- CD34 cells. The number of viable cells differentiated from CD34 cells was measured at 144h by FACS analysis.

3 ALLs were recognized after activation with CD40L and CpG (figure 3E). In addition, 4 of 9 primary AMLs were recognized by HSS1, of which 3 were strongly recognized (figure 3F). Also the patient AML was tested for recognition, however no recognition was seen (data not shown). Since no material of the patient was available anymore, we determined whether activated AML could be recognized using 4 primary AML samples from other patients with similar classification (M5). Results shown in figure 3G demonstrate that without activation 2 of 4 AML samples were recognized, whereas after activation all 4 tested AML samples were recognized.

Since PRAME has been described to be expressed at low and intermediate levels in certain normal tissues, we tested HSS1 against different HLA-A2+ non-malignant cell types. HSS1 showed no reactivity against fibroblasts and keratinocytes with or without pre-treatment with IFNγ (figure 4A), or against primary bronchus epithelial cells, hepatocytes, intrahepatic billiary epithelial cells, colon epithelial cells or mesenchymal stromal cells (figure 4B). In contrast, the clone showed low but significant recognition of all 12 tested HLA-A2+ proximal tubular epithelial cells (PTEC) derived from kidney tubules of which 4 are shown in figure 4C. Testing the clone against HLA-A2+ non-malignant cells of hematopoietic origin demonstrated that HSS1 did not recognize B cells, CD40L activated B cells or in vivo activated B cells derived from inflamed tonsils (figure 4D and 4E). In addition, T-cells and activated T-cell blasts were not recognized (data not shown). Monocytes or immature CD14 derived DCs (figure 4F), CD34 cells or immature CD34DCs (figure 4G), and MØ1 and MØ2 macrophages (figure 4H) were also not recognized by the PRAME specific T-cell clone. However, clone HSS1 showed low reactivity against mature DCs derived from CD14 and CD34 cells (figure 4F and 4G, respectively). To investigate whether the PRAME specific T-cells were also reactive against blood derived myeloid and plasmacytoid DCs (MDCs and PDCs, respectively), HSS1 was tested against MDCs and PDCs. No recognition of immature MDCs and PDCs and mature PDCs was observed. However, low reactivity against mature MDCs was observed (data not shown). To determine whether allo-restricted PRAME specific T-cells were also reactive against other myeloid cell-lineages, HSS1 was tested against proliferating and differentiating CD34 cells in a proliferation inhibition assay. As is shown in figure 4I, HSS1 was not able to inhibit the proliferation of CD34 cells, and progeny of CD34 cells.

To investigate whether the high avidity allo-restricted PRAME T-cells exerted antigen specific cytolytic activity, HSS1 and control HSS12 were tested at different E/T ratio against HLA-A2+ targets including 2 melanomas, 3 RCCs, CD14mDCs and 2 PTECs. Clone HSS1 exerted high cytolytic activity against the previously recognized HLA-A2+ malignant cell-lines, whereas only low reactivity against mature mDCs and PTECs was observed (figure 5A). The HSS12 control clone was highly reactive against all HLA-A2+ targets, including mDCs and PTECs (figure 5B).

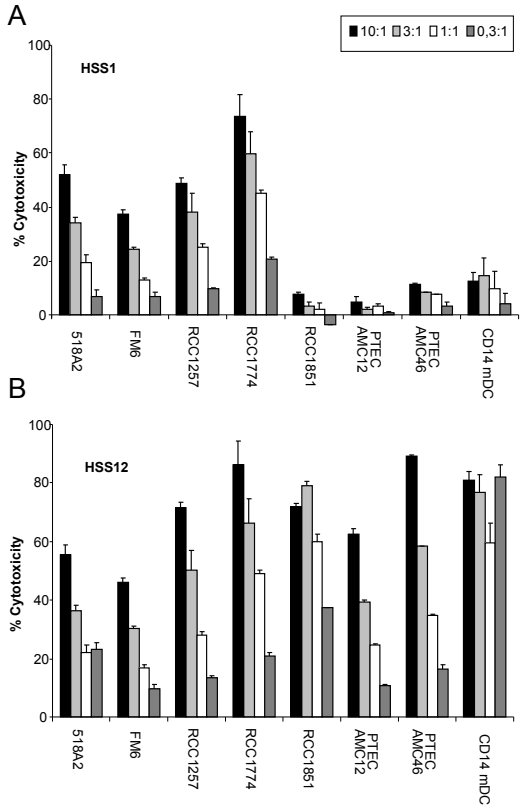


Figure 5. The allo-restricted PRAME specific T cell clone HSS1 exerts specific cytotoxic activity. HSS1 **(A)** and control clone HSS12 **(B)** were tested for cytotoxicity against HLA-A2+ melanoma's (518A2 and FM6), RCCs (RCC1257, RCC1774 and RCC1851), PTECs and CD14mDCs.

The results demonstrate that the allo-restricted PRAME T-cells are able to exert efficient reactivity against a large proportion of tumors. In addition, the results indicate that the PRAME T-cells exhibited low reactivity against non-malignant mDCs and PTECs.

Expression of PRAME gene, determined by quantitative RT-PCR, strictly correlates with recognition of PRAME specific T-cell clone

To establish whether the reactivity of the allo-HLA restricted PRAME T-cells correlated with expression of PRAME, we determined the mRNA expression level of PRAME by quantitative RT-PCR in the different cells. figure 6 illustrates that reactivity of HSS1 strictly correlated with PRAME expression. Cells with high or intermediate PRAME expression were effectively or intermediately recognized by the T-cells. Targets that did not express PRAME were not recognized. Targets expressing low levels of PRAME were also not recognized, with the exception of CD40L activated ALL, EBV-LCLs, and non-malignant PTECs and mDCs.

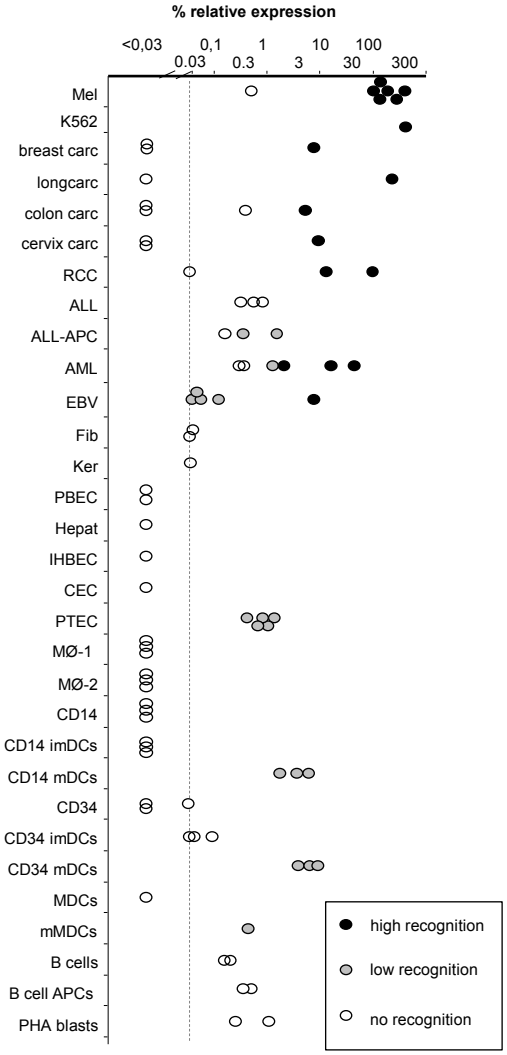


Figure 6. Correlation between functional reactivity of the PRAME specific T cell clone HSS1 and PRAME mRNA expression. The expression of PRAME mRNA in different cells was determined using quantitative RT-PCR, and referenced to the level of PRAME expression measured in Mel518. The limit of detection is indicated with the broken line. The black dots represent cells high or intermediate recognized by HSS1, gray dots represent cells low recognized by HSS1, and white dots represent cells not recognized by HSS1.

To confirm the strict correlation between PRAME expression and recognition, and to exclude that the reactivity was due to off-target toxicity mediated by crossreactivity against other peptide-HLA complexes, we transduced CD34mDCs, PTECs, and RCC1257 with PRAME silencing RNA (shRNA). The PRAME specific shRNA almost completely blocked the recognition of CD34mDCs, PTECs and RCC by HSS1, whereas the recognition of the targets by control HSS12

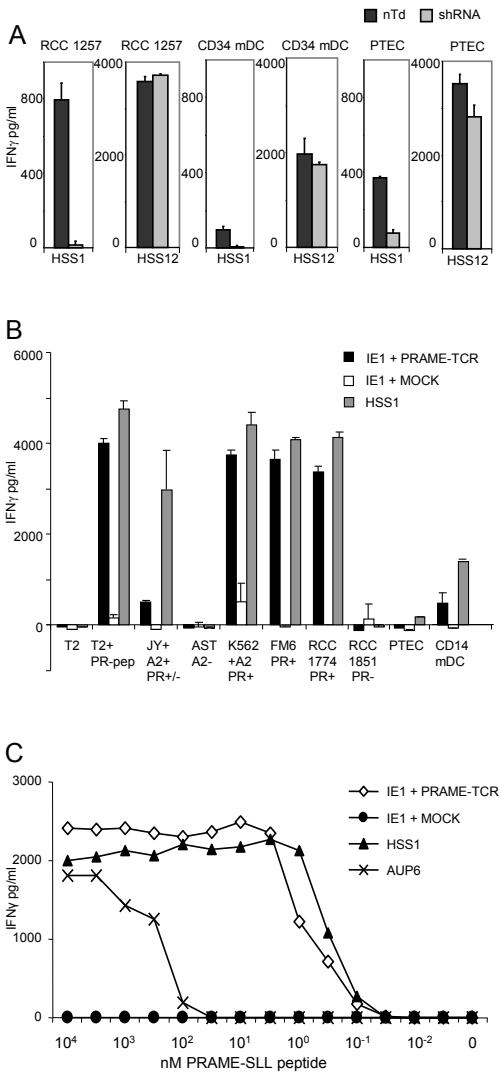


Figure 7. PRAME specific shRNA and PRAME-TCR transduction demonstrate that recognition of PTECs and mDCs is due to on-target toxicity, and tumor specific reactivity of PRAME-TCR can be efficiently transferred.

(A) HSS1 and control HSS12 were tested against HLA-A2+ RCC1257, CD34mDCs and PTECs transduced with a retroviral vector encoding for a PRAME specific shRNA and the puromycin selection gene. shRNA transduced cells were cultured for 7 days with puromycin (0.4-4 ug/ml) and used as stimulator cells. Non-transduced cells (nTd) were used as control stimulator cells. **(B)** CMV-IE1 specific T cells transduced with PRAME-TCR or mock transduced, and HSS1 were tested against T2 cells, T2 cells loaded with PRAME peptide, HLA-A2+ LCL-JY, K562+A2, FM6, RCC1774, RCC1851, PTECs, CD14mDCs, and HLA-A2 negative EBV-LCL (AST A2-) The expression of PRAME in the melanomas and RCCs is indicated (PR+ or PR-). **(C)** CMV-IE1 specific T cells transduced with the PRAME-TCR, mock transduced CMV-IE1 specific T cells, HSS1 and AUP6 were tested against T2 cells loaded with titrated concentrations of the PRAME-SLL peptide.

was unaltered (figure 7A). These results demonstrate that the reactivity against PTECs and mDCs by the allo-HLA restricted PRAME T-cells was based on PRAME recognition.

PRAME-TCR transduced T-cells exert clinically relevant PRAME specific reactivity

Based on the high reactivity against a large proportion of tumors and limited on-target toxicity exerted by the high avidity PRAME T-cells, their high affinity TCR may be effective tools for TCR gene therapeutic strategies[29;33]. Therefore, we investigated the functional activity of PRAME-TCR transduced T-cells. To prevent mixed TCR dimer formation and to optimize TCR expression the TCRα and β chain of HSS1 were codon optimized and cysteine modified[28;29]. Results shown in figure 7B demonstrate that the PRAME-TCR transduced T-cells exerted similar to the parental HSS1 high reactivity against PRAME peptide loaded T2 cells, and PRAME+ tumor cells, whereas PTECs and mDCs were only marginally recognized. To investigate the avidity of the PRAME-TCR transduced T-cells, these T-cells were compared to the parental high avidity PRAME specific T-cell clone HSS1 and to the low avidity self restricted T-cell clone AUP6 in a peptide titration experiment with the PRAME-SLL peptide loaded on T2 cells. As shown in figure 7C, PRAME-TCR transduced T-cells required comparable peptide concentration for 50% of maximum IFN-γ production as the parental clone HSS1, demonstrating that the avidity of PRAME-TCR transduced T-cells is similar to that of the parental high avidity PRAME specific T-cell clone. These results indicate that the PRAME-TCR of clone HSS1 can potentially be used for TCR gene therapy.

DISCUSSION

In this study, we succeeded in isolating beneficial GVT reactive T-cells restricted by allo-HLA-A2 and specific for PRAME from a detrimental allo-HLA directed immune response after HLA-A2 mismatched SCT. We demonstrated that the allo-restricted PRAME specific T-cells were single peptide specific and, in contrast to self-restricted T-cells, exerted high avidity reactivity against PRAME expressing tumor cells. The reactivity of the T-cells strictly correlated with PRAME expression, indicating that the allo-restricted PRAME T-cells were not cross-reactive against other peptides presented in the context of HLA-A2. The clones showed high reactivity against a large panel of tumor cell-lines, whereas a large panel of non-malignant cells was not recognized. The allo-restricted PRAME T-cells exerted limited on-target toxicity against mDCs and PTECs. Finally, we demonstrated high PRAME specific tumor reactivity of PRAME-TCR transduced T-cells, indicating that the PRAME-TCR can potentially be used in TCR gene therapeutic strategies.

In the patient at the time of severe GVHD approximately 85% of donor CD8 T-cells were highly activated. By isolating these activated polyclonal CD8 T-cells, we illustrated that 0.6%

of T-cells were PRAME-SLL specific, indicating that at the time of GVHD approximately 0.5% of the donor CD8 T-cells were directed against PRAME. Based on our results we speculate that the PRAME specific allo-restricted T-cells have likely been induced *in vivo* by activated patient derived HLA-A2+ mDCs or by the AML-DC. Although AML was not recognized in non-activated state, 4 primary AML samples with similar classification were recognized after activation (figure 3G), and may indicate that the AML-DC have induced the PRAME specific T-cell response. Based on the limited reactivity against PTECs even after IFN-γ activation, and the lack of expression of costimulatory molecules on these cells, we assume that it is unlikely that PTECs were the mediators of the PRAME specific allo-restricted T-cell response. Although the patient died of severe GVHD, at the time of the profound allo-HLA immune response in which a large variety of allo-HLA reactive T-cells including the PRAME specific T-cells were present no clinical signs of nephrotoxicity were observed.

The results demonstrating that mDCs express PRAME at levels able to activate high avidity PRAME specific T-cells, also explains why self-restricted T-cells exhibiting high avidity for the self-antigen PRAME can be deleted by negative selection during thymic development. The observation that we failed to isolate self-restricted PRAME specific T-cells exerting high avidity PRAME specific reactivity against tumor cells is in agreement with this hypothesis. In addition, although self-restricted PRAME specific T-cells were previously isolated that exhibited high peptide affinity, these T-cells exerted low anti-tumor reactivity[34-36]. Based on these findings we consider it unlikely that transfer of self-restricted PRAME specific T-cells[35;36] or vaccination with PRAME[35-39], even combined with optimal adjuvants, will induce high avidity PRAME specific T-cells exerting potent anti-tumor reactivity. In contrast, we isolated high avidity PRAME specific T-cells by circumventing immune tolerance, because negative selection is limited to self-HLA. The reactivity of the allo-restricted PRAME clones against multiple different tumor cell-lines and primary leukemia demonstrate that these PRAME-TCRs could potentially be useful in adoptive T-cell therapy with TCR engineered T-cells for the treatment of patients with many different malignancies.

The allo-restricted PRAME specific T-cell clones unfortunately also showed reactivity against mDCs and PTECs, due to low expression of PRAME. We hypothesize that the PRAME specific T-cells were induced in our patient after HLA-mismatched SCT and subsequent DLI by either the mDCs or the AML-DCs, and therefore we speculate that PRAME-TCR engineered T-cells may be reactive against autologous mDCs. The recognition and thereby eradication of mDCs by PRAME specific T-cells could lead to immune impairment, since mDCs play an important role in the initiation of new immune responses. In addition, the reactivity directed against PTECs could possibly lead to renal failure. It has indeed been previously demonstrated that recognition of specific non-malignant cells by administered high avidity TAA specific T-cells can result in toxicity. The infusion of high avidity T-cells directed against the RCC antigen carboxy anhydrase IX (CAIX) resulted in severe cholestasis, based on the CAIX expression by bile

duct epithelial cells[40]. In addition, patients who received high avidity gp100 T-cells developed uveitis and hearing loss due to expression of gp100 by melanocytes in eye and ear[15].
Based on the possible on-target toxicity induced by the PRAME-TCR a safety strategy may be necessary to co-transduce PRAME-TCR engineered T-cells with a suicide gene, which enables *in vivo* elimination of the engineered T-cells if serious adverse events occur[41;42]. Using this strategy a temporary loss of mDCs could be resolved, since imDCs, CD14+ and CD34+ progenitor cells as well as MDCs and PDCs from peripheral blood are not recognized by the PRAME T-cells. In fact, recognition of mDCs might also be beneficial since it could potentially boost anti-tumor responses and enhance persistence of the infused T-cells. In contrast, potential damage to the kidney powered by recognition of PTECs could be irreversible and therefore, co-transduction of a suicide gene might be necessary for protection against toxicity directed against the kidney. It is however also possible that the low reactivity observed against cultured PTECs even after IFN-γ activation *in vitro* does not necessarily indicate nephrotoxicity *in vivo,* as indicated by absence of renal dysfunction in our patient.
Based on these results we conclude that potentially beneficial high avidity TAAs specific T-cells can be isolated from detrimental allo-HLA immune responses after HLA mismatched SCT. The high affinity TCRs of these allo-restricted PRAME specific T-cells highly reactive against a large panel of tumors, may be effective tools to engineer T-cells with tumor-targeting TCRs that can be used for adoptive T-cell therapy of patients with metastasized cancer or relapsed leukemia.

ACKNOWLEDGEMENTS

The authors thank Menno van der Hoorn, Guido de Roo and Patrick van der Holst for expert technical assistance.

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