4

ALLO-HLA REACTIVE T-CELLS INDUCING GRAFT VERSUS HOST DISEASE ARE SINGLE PEPTIDE SPECIFIC

Avital L. Amir, Dirk M. van der Steen, Renate S. Hagedoorn, Michel G.D. Kester, Cornelis A.M. van Bergen, Jan W. Drijfhout, Arnoud H. de Ru, J.H. Frederik Falkenburg, Peter A. van Veelen, Mirjam H.M. Heemskerk

Blood. 2011 Dec 22;118(26):6733-42.

ABSTRACT

T-cell alloreactivity directed against non-self HLA molecules has been assumed to be less peptide specific than conventional T-cell reactivity. A large variation in degree of peptide specificity has previously been reported, including single peptide specificity, polyspecificity, and peptide degeneracy. Peptide polyspecificity was illustrated using synthetic peptides loaded target cells, but in the absence of confirmation against endogenously processed peptides this may represent low avidity T-cell reactivity. Peptide degeneracy was concluded based on recognition of antigen processing defective cells. In addition, since most investigated alloreactive T-cells were in vitro activated and expanded, the previously determined specificities may have not been representative for alloreactivity *in vivo*. To study the biologically relevant peptide specificity and avidity of alloreactivity, we investigated the degree of peptide specificity of 50 different allo-HLA reactive T-cell clones which were activated and expanded *in vivo* during graft versus host disease. All but one of the alloreactive T-cell clones, including those reactive against antigen processing defective T2 cells, recognized a single peptide allo-HLA complex, unique for each clone. Downregulation of the expression of the recognized antigens using silencing shRNAs confirmed single peptide specificity. Based on these results we conclude that biological relevant alloreactivity selected during *in vivo* immune response is peptide specific.

INTRODUCTION

Alloreactive T-cells directed against allogeneic HLA (allo-HLA) are involved in the development of graft versus host disease (GVHD) and graft rejection after HLA mismatched transplantation. In conventional T-cell reactivity directed against pathogens, each T-cell is specific for a single foreign peptide presented in a self HLA molecule.[1] During thymic development, T-cells that are reactive against self peptides presented in self HLA molecules undergo negative selection. Both T-cells reactive against a single peptide as well as T-cells exerting polyreactivity against more than one peptide presented in self HLA molecules will be deleted.[2;3] T-cells leaving the thymus are therefore tolerant for self-peptide-HLA complexes. Since T-cells never encounter allo-HLA molecules during thymic development, T-cells polyspecific for peptides presented in allogeneic HLA are not removed, and, therefore T-cell allo-HLA reactivity has been assumed to be less peptide specific than conventional T-cell reactivity.

The degree of peptide specificity of alloreactive T-cells has been extensively studied. Most investigators used cells defective in antigen processing, like Transporter Associated with antigen Processing (TAP) deficient human T2 cells or murine RMA-S cells to address the role of peptides in allorecognition.[4-11] In these studies, groups of alloreactive T-cell clones or lines were tested against these antigen processing deficient cells, unloaded or loaded with peptides. Some T-cells only recognized a single peptide and were therefore categorized as peptide specific. T-cells recognizing more than one peptide, without sequence homology, were considered polyspecific. In addition, some alloreactive T-cells were reactive against antigen processing deficient cells in the absence of exogenously loaded peptide, which was initially interpreted as peptide independent recognition, because it was assumed that these cells expressed empty MHC molecules on the cell surface. However, since it was demonstrated that these cells do express a limited number of peptides, which are independent of TAP to be expressed in MHC molecules on the cell membrane,[12] reactivity against antigen processing deficient cells may also be based on peptide specific recognition. Reactivity against allo-HLA molecules irrespective of the sequence of the peptide presented has been termed peptide degenerate allorecognition, although it is unclear whether this type of recognition occurs. Together these studies created the assumption that alloreactivity is a combination of different reactivities, ranging from peptide specific to peptide degenerate alloreactivity.

In several studies the degree of peptide specificity in allorecognition was determined by testing alloreactive T-cells against a limited number of defined peptides.[7;813-15] In these studies reactivity against target cells exogenously loaded with high concentrations of peptide was interpreted as biological relevant reactivity, and recognition of different peptides presented in allo-MHC by alloreactive T-cells was concluded to represent *bona fide* polyspecific allorecognition. However, since recognition of exogenously loaded peptides in the absence of reactivity against endogenously processed and presented antigen may represent low avidity T-cell recognition, biological relevance of reactivity against synthetic peptide loaded

target cells needs to be confirmed by investigating the T-cell reactivity against endogenously processed and presented antigen. In the absence of such confirmation, the polyspecific alloreactivity against synthetic peptides described in these studies might not be representative for alloreactivity that is exerted by T-cells during *in vivo* GVHD or graft rejection.

During *in vivo* immune responses T-cells with high avidity are selectively expanded, as indicated by the observation that the memory T-cell repertoire directed against microbial antigens comprises of T-cells with high avidity for the specific antigens, whereas the naïve T-cell repertoire specific for the same antigens contains a broad range of avidities.[16;17] In contrast, in vitro priming of T-cells results in a T-cell repertoire containing a large variety of avidities,[18;19] comparable to the repertoire of naïve T-cells, indicating that during in vitro activation and expansion no selection for high avidity occurs. Since under normal circumstances allo-HLA molecules are not encountered, alloreactive T-cells which can be present within the naïve as well as the memory T-cell pool,[20;21] will not have undergone selection for T-cells with high avidity for allo-HLA molecules. Therefore, the repertoires of alloreactive T-cells derived from individuals who have not been exposed to non-self HLA molecules, will contain a high variety of avidities. Most alloreactive T-cells used for the study of peptide specificity and avidity of allorecognition were activated and expanded *in vitro*, and, accordingly, many of these alloreactive T-cells have been reported to demonstrate a very broad range of avidities.[8;4;22] We hypothesize that only in circumstances in which *in vivo* selection does occur, like during *in vivo* allo-HLA directed immune responses including GVHD and graft rejection after HLA mismatched transplantations, T-cells with high avidity for allo-HLA molecules will be selected.

To study the biologically relevant degree of peptide specificity and avidity of T-cell alloreactivity, we investigated in detail the repertoire of allo-HLA reactive T-cells which were activated and expanded *in vivo*. From a patient experiencing severe GVHD after an HLA-A2 mismatched DLI, we isolated 50 different *in vivo* activated allo-HLA-A2 reactive CD8+ T-cell clones and investigated their peptide specific alloreactivity. All but one of the alloreactive T-cell clones showed reactivity against a single HPLC fraction of peptides eluted from HLA-A2, including 6 T-cell clones recognizing T2 cells without exogenously loaded peptides. Using multidimensional HPLC fractionation and mass spectrometry (MS), we identified the different peptides recognized by 7 alloreactive T-cell clones, including one T2 reactive T-cell clone, and demonstrated that the alloreactive T-cells exerted high avidity peptide specific alloreactivity. Downregulation of the expression of the recognized antigens using silencing shRNAs confirmed single peptide specificity of the alloreactive T-cell clones. These results demonstrate that T-cells exerting biological relevant alloreactivity *in vivo* exhibited peptide specificity. Based on these results we conclude that the biological relevant specificity which is selected during *in vivo* immune response is single peptide specific, as defined by recognition of endogenously processed and presented antigen.

MATERIALS AND METHODS

Cell collection and preparation

After informed consent, peripheral blood was obtained from different individuals, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. HLA-A2 negative donor EBV-LCLs and K562 cells were transduced with a retroviral vector encoding for HLA-A*0201 as previously described.[23] EBV-LCLs, T2 cells (obtained from the American Type Culture Collection (ATCC)), K562 cells, renal cell carcinoma cell line 1774 and melanoma cell line 1.14 were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) and 10% fetal bovine serum (FBS, Lonza). Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM; Lonza) and 10% FBS. Drosophila cells expressing HLA-A2, CD54 and CD80 were kindly provided by Dr. Hans Stauss (Department of Immunology and Molecular Pathology, Royal Free Hospital, University College London, London, UK) and cultured in Schneider's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS, at 24°C and atmospheric CO_2. 100 µM copper sulphate was added 48 hours prior to peptide-loading to induce expression of HLA-A2, CD54 and CD80.

Generation of allo-HLA-A2 reactive T-cell clones

Allo-HLA-A2 reactive T-cell clones were isolated from a patient experiencing acute GVHD after single HLA locus mismatch SCT and subsequent donor lymphocyte infusion (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. To isolate the allo-HLA-A2 reactive T-cells, PBMCs collected during GVHD were stained with anti-HLA-A2 FITC (Pharmingen, San Jose, CA, USA), anti-HLA-DR APC (Pharmingen) and anti-CD8 PE mAbs (Carlsbad, CA, USA) at 4°C for 30 min and washed once. Activated (HLA-DR+), donor derived (HLA-A*0201-) CD8+ T-cells were sorted single cell per well into U-bottom microtiter plates containing 100 µl of feeder mixture consisting of IMDM, 5% FBS, 5% human serum (HS), IL-2 (120 IU/ml, Chiron, Amsterdam, The Netherlands), phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK) and 50 Gy irradiated allogeneic PBMCs ($0.5x10^6$ /ml). Proliferating T-cell clones were selected and further expanded non-specifically with PHA, IL-2 and irradiated allogeneic PBMCs. For frequency analyses, HLA-DR+ HLA-A*0201- CD8+ T-cells were sorted in bulk and non-specifically expanded with irradiated autologous PBMCs, IL-2 and PHA.

TCR-Vβ chain analysis

For determination of TCR Vβ usage of T-cell clones, the TCR Vβ kit (Beckman Coulter, Fullerton, CA) was used. TCRβ chains which could not be stained with mAbs were determined using multiplex amplification as previously described.[24] By sequence analyses the variable region and CDR3 region of the TCR Vβ chains of T-cell clones was determined.[25]

Peptide identification by peptide elution, high performance liquid chromatography (HPLC) and mass spectrometry

$3x10^{10}$ EBV-LCLs or T2 cells were lysed with lysisbuffer, composed of 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 and protease inhibitor mix (Complete; Roche). Lysates were centrifuged for 30 min at 11,000 x g and precleared for 60 min with CL4B sepharose beads. HLA-A2 immunoaffinity chromatography was performed with anti-HLA-A2 purified BB7.2 mAb covalently coupled to protein-A beads. Subsequently, HLA/peptide complexes were eluted with 10% acetic acid. High molecular mass material (HLA heavy chain and light chain) was removed by size filtration through Centriprep filtration units with a cutoff value of 10 kDa. After freeze drying, the peptide mixture was subjected to multidimentional HPLC fractionation, followed by peptide identification using MS. In the first round of the multidimentional HPLC fractionation the freeze dryed peptide mixture was subjected to C18 reversed-phase HPLC (RP-HPLC) on an Agilent 1100 series HPLC system. This first dimension was performed on a Reprosil-Pur C18-AQ 3µm 100 x 2 mm column using a water/acetonitrile/TFA gradient and fractions of 50 µl were collected. The non-T2 reactive T-cell clones were tested against T2 target cells loaded with a small sample of each HPLC fraction of peptides eluted from the HLA-A2 of EBV-LCLs. The T2 reactive T cell clones were tested against HLA-A2 positive drosophila cells loaded with a small sample of each HPLC fraction of peptides eluted from the HLA-A2 of T2 cells. For this purpose the T2 cells or drosophila cells were pre-incubated with the HPLC fractions for 1 hour at 20°C, before addition of T-cells. The highly recognized fractions were selected for a second round of RP-HPLC fractionation, which was performed on an Eclipse XDB C18 3,5 µm 150 x 1 mm column using a water/isopropanol/TFA gradient. Fractions were collected and tested for recognition. The recognized fractions were selected for a third round of RP-HPLC fractionation, which was performed on a 200 µm x 100 mm Phenomenex Aqua 5 µm column using a water/acetonitrile/formic acid gradient and fractions were collected and tested for recognition. The peptide masses present in the recognized fractions and in the neighboring unrecognized fractions were determined by on-line nano-HPLC-MS, using a LTQ-FT Ultra. The LTQ-FT Ultra (Thermo, Bremen, Germany) was operating in data dependent mode, automatically switching between MS and MS/MS acquisition. Full scan mass spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 5,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. The selected ions were then fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post analysis process, raw data were converted to peak lists using Bioworks Browser software, Version 3.1. For peptide identification, MS/MS data were submitted to the human IPI database using Mascot Version 2.2.04 (Matrix Science) with the following settings: 2 ppm and 0.8-Da deviation for precursor and fragment masses, respectively; no enzyme was specified. Peptide masses of which the abundance correlated with the recognition pattern of the T-cell clone were selected for tandem MS and peptide

identification was performed. Candidate peptides were synthesized by Fmoc-chemistry. To confirm peptide specificity the allo-HLA reactive T-cell clones were tested against T2 or HLA-A2+ drosophila cells loaded with titrated concentrations of characterized peptides. In addition, tandem mass spectrum of the synthetic peptides was recorded and compared to its eluted counterpart as additional confirmation.

Functional assays and flow cytometry

HLA-A2 restriction of allo-HLA reactive T-cell clones was determined in cytotoxicity assays in which T-cells clones were tested in a 4h ^{51}Cr-release assay against patient EBV-LCLs, donor EBV-LCLs and donor EBV-LCLs transduced with HLA-A*0201 at effector to target ratio10:1. In addition, blocking studies were performed using BB7.2 (anti-HLA-A2), W6.32 (anti-HLA class I) and B1.23.2 (anti-HLA-B and C) mAbs. Patient EBV-LCLs were pre-incubated with saturating concentrations of mAbs for 1h at 20^{o}C before addition of T-cells.

Tetramer staining was performed at 37^{o}C for 20 min using tetramers composed of HLA-A*0201 and peptides CMV-IE1-VLE, CMV-pp65-NLV, EBV-BMLF1-GLC, EBV-BRLF1-YVL, EBV-LMP2-CLG, EBV-LMP2-FLY, and FLU-IMP-GIL. In addition, tetramers composed of HLA-A*0201 and newly identified epitopes HLA-HLA-DRA-FID, ATXN10-QVF, VPS13B-SLW, FDPS-YLD, USP11-FTW, and SHIP-GFP were generated as described previously.[26] Functionality of tetramers was controlled by staining of the specific T-cell clones. All tetramers were functional with the exception of ATXN10-QVF.

Allo-HLA reactive T-cell clones were tested against T2 cells loaded with a mixture of HLA-A2 binding peptides derived from CMV, EBV, and Flu at a final concentration of 1 µg/ml. In the stimulation assays 5,000 T-cells were stimulated with 20,000 EBV-LCLs, T2 cells, K562+A2 cells, melanoma 1.14 cells or RCC1774 cells or with 5,000 fibroblasts or with 100,000 HLA-A2+ drosophila cells. All functional assays were performed in IMDM containing 5% FBS, 5% HS and 100 IU/ml IL-2 at a total volume of 150µl in 96 well plates. After 18h of incubation, supernatant was harvested and IFNγ production was measured by standard ELISA.

Alanine substitution experiment

Of the two peptides recognized by clone HSS11 (FIDKFTPPV derived from HLA-DRA and FLLKLTPLL derived from THRAP4) modified peptides were generated with alanine substitutions at the amino acid positions identical between the two peptides (1, 4, 6 and 7). In addition, modified peptides were synthesized in which the anchor residues at position 2 and 9 between the two peptides were exchanged. To determine the peptide concentrations required for half maximum IFNγ production (EC50), clone HSS11 was incubated with T2 cells loaded with titrated concentrations of wild type (wt) and modified peptides. EC50 of the modified peptides was compared to the EC50 of wt peptides.

Inhibition of gene expression by silencing short hairpin RNA

Lentiviral vectors encoding for short hairpin (sh) RNA sequences specific for the genes coding for the proteins from which the identified peptides were derived in combination with the puromycin resistance gene were used from the Sigma-Aldrich shRNA library (Sigma-Aldrich, St. Louis, MO, USA). For USP11 shRNA TRCN0000007360, for FDPS TRCN0000036294, for VPS13B TRCN0000083955, for ATXN10 TRCN0000084093, for HLA-DRA TRCN0000057309, and for THRAP4 TRCN0000019649 were used. Fibroblasts were transduced with the lentiviral vectors and after 3 days puromycin was added. Lentiviral transduced fibroblasts were cultured for 2 weeks with 4 µg/ml puromycin before testing. The lentiviral vector encoding the shRNAs for USP11, FDPS, VPS13B and ATXN10 were transduced into HLA-A*0201 fibroblasts derived from a healthy donor. The lentiviral vectors encoding the shRNAs for HLA-DRA and THRAP4 were transduced into SV40 transformed HLA-A*0201 fibroblasts derived from a Bare Lymphocyte Syndrome patient (BLS) (EBA) and its HLA identical healthy sibling (CBA), that were kindly provided by Dr. P. van den Elsen (IHB, LUMC). The fibroblasts from BLS patients were previously demonstrated to be HLA-DRA deficient.[27] Cells were tested in 384 well plates with 3,000 fibroblasts/well and 1,000 T-cells/well. The fibroblasts transduced with shRNA for HLA-DRA and THRAP were stimulated for 72h with 100 IU IFN-γ/ml to increase HLA-DRA expression.
To confirm shRNA downregulation of expression of the different genes, quantitative real-time PCR (qRT-PCR) (TaqMan) for the different genes was performed. Total RNA was isolated from cells using the micro RNaqueous kit (Ambion). First strand cDNA synthesis was performed with oligo dT primers using M-MLV reverse transcriptase (Invitrogen). Samples were run with EvaGreen on an ABI 7500. Primers specific for the different genes are depicted in table 1.

Table 1. Primers used for qRT-PCR

gene	position	sequence
USP11	forward	GGGCCTGTATAACGTCCTGA
	reverse	CTCTGGCTCCCAGTCGATAG
FDPS	forward	TGAGATCTGTGGGGGTCTTC
	reverse	TCCCGGAATGCTACTACCAC
VPS13B	forward	ATGATTGTGTGTGCCTTGGA
	reverse	TTGGCACTGAATGTTCCAGA
ATXN10	forward	CAAGCCATGTTTCCCAAACT
	reverse	ACCCGCAAAAGATCAATCAC
HLA-DRA	forward	CATCCAGGCCGAGTTCTATC
	reverse	CTGGTGGGGTGAACTTGTCT
THRAP4	forward	CTGCACATCGCCAAACTAGA
	reverse	TACCCTCGGGAGACTCAATG

RESULTS

Isolation and characterization of allo-HLA-A2 reactive T-cells activated during acute GVHD

To be able to characterize the peptide-HLA ligand specificity of allo-HLA reactive T-cells exerting allo-immune responses *in vivo*, we isolated activated donor derived CD8 T-cells from a patient experiencing severe acute GVHD after single HLA-A2 locus mismatch SCT and subsequent DLI. The activated, HLA-DR positive CD8 T-cells of donor origin (HLA-A2 negative) were sorted single cell per well from peripheral blood collected at the time of GVHD and expanded. Fifty of 56 isolated CD8 T-cell clones were shown to be alloreactive, since these T-cells recognized patient but not donor EBV-LCLs. All 50 alloreactive T-cell clones were allo-HLA-A2 reactive, since clones were reactive against donor EBV-LCL retrovirally transduced with HLA-A2, as shown for 8 representative clones (figure 1A). In addition recognition of HLA-A2 positive EBV-LCLs could be blocked by anti-HLA class I as well as anti-HLA-A2 mAbs (data not shown). By flow cytometric analysis using Vβ mAbs and multiplex PCR followed by sequencing of the different TCR Vβ chains (data not shown), we demonstrated that all 50 allo-HLA-A2 reactive CD8 T-cell clones were of different clonal origin. In addition, a high variety in TCR Vβ chain usage was observed, and no sequence homology between the allo-HLA reactive clones was detected.

The allo-HLA reactivity of GVHD inducing T-cells is dependent on peptide specific recognition.

To determine whether allo-HLA-A2 reactive T-cells recognized HLA-A2 irrespective of the peptide presented in the peptide binding groove, T-cell clones were stained with a mix of tetramers composed of HLA-A2 and different virus-specific peptides derived from CMV, EBV and Flu. None of the T-cell clones stained with the viral HLA-A2 tetramers (data not shown). Next, the 50 allo-HLA-A2 reactive T-cell clones were tested against T2 cells and T2 cells loaded with the different CMV, EBV and Flu HLA-A2 binding peptides. 44 of the 50 T-cell clones showed no reactivity against peptide loaded or unloaded T2 cells (figure 1A). 6 of the 50 T-cell clones were reactive against unloaded T2 cells (reactivity of 3 T-cell clones is shown in figure 2A) and showed comparable reactivity against peptide loaded T2 cells (data not shown). Since T2 cells express HLA-A2 peptide complexes, although with restricted peptides variation, the ability of allo-HLA reactive T-cells to recognize allo-HLA-A2 could still be based on peptide specific recognition. To determine whether these T-cell clones recognized allo-HLA-A2 independent of the peptide presented in HLA binding groove, the clones were tested against HLA-A2 expressing drosophila cells loaded with the different HLA-A2 binding peptides. The T2 reactive T-cell clones showed no reactivity against unloaded (figure 2A) or peptide loaded HLA-A2+ drosophila cells (data not shown). These results indicate that for all 50 alloreactive

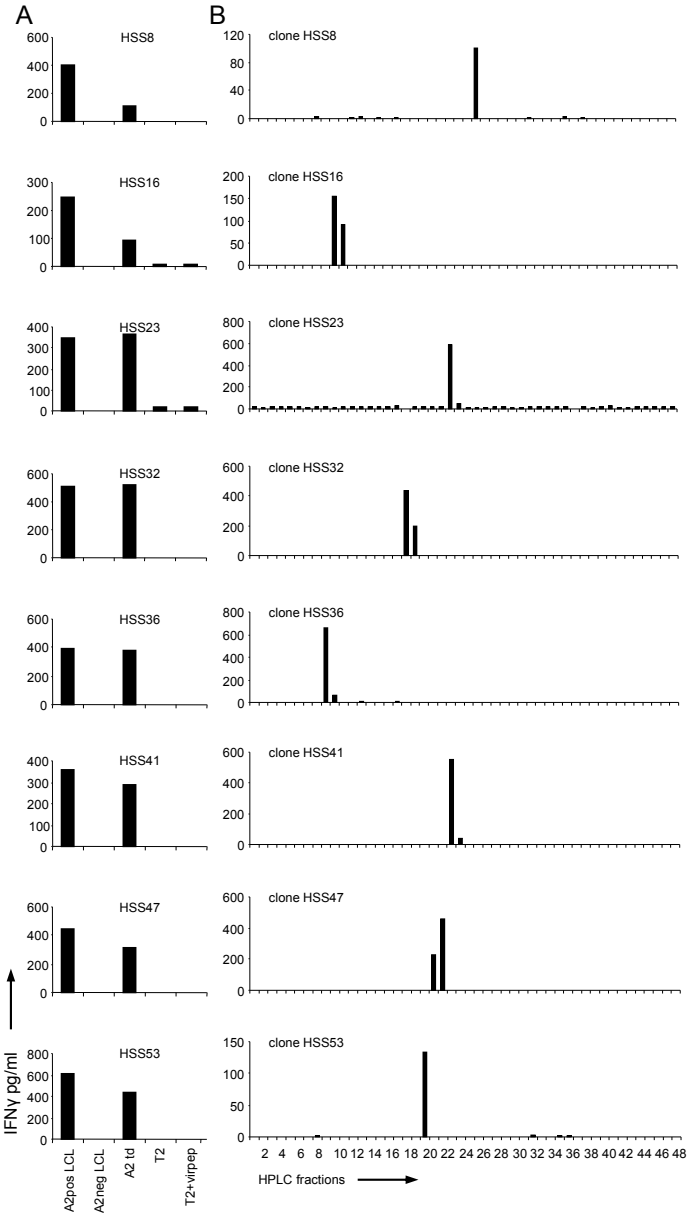

Figure 1. Recognition of HPLC fractions of HLA-A2 eluted peptides by the allo-HLA reactive T-cell clones.

(A) The allo-HLA reactive T-cell clones were stimulated with HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2 negative EBV-LCLs (A2neg LCL), HLA-A2 negative EBV-LCLs transduced with HLA-A2 (A2 td), T2 cells (T2) and T2 cells loaded with a mixture of different HLA-A2 binding peptides from CMV, EBV and Flu (T2+virpep). Supernatants were harvested after 18 hours of stimulation and IFNγ was measured by standard ELISA. **(B)** Peptides were eluted from the HLA-A2 molecules of EBV-LCLs and fractionated by RP-HPLC using a water/acetonitrile/TFA gradient. 26 of the 44 non T2 recognizing allo-HLA-A2 reactive T-cell clones, of which 8 are shown in this figure, were stimulated with T2 cells loaded with the HPLC fractions.

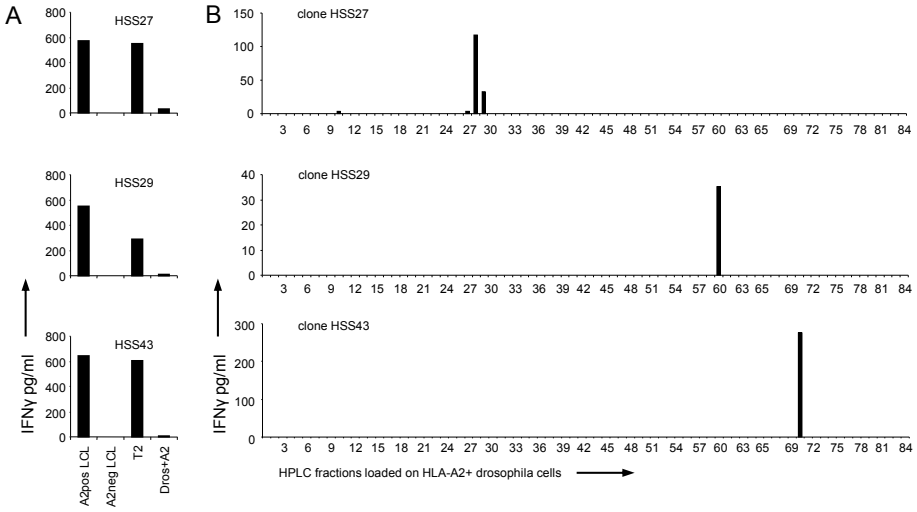

Figure 2. Investigation of the peptide specificity of T2 reactive T-cell clones.
(A) T2 reactive T-cell clones HSS27, HSS29 and HSS43 were stimulated with HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2 negative EBV-LCLs (A2neg LCL), T2 cells (T2) and drosophila cells expressing HLA-A2, CD80 and CD54 (Dros+A2). **(B)** Peptides were eluted from the HLA-A2 molecules of T2 cells and fractionated by RP-HPLC. The six allo-HLA reactive T-cell clones which recognized T2 cells were tested for IFNγ production against the HPLC fractionations loaded on drosophila cells expressing HLA-A2, CD80 and CD54. Three representative clones are shown.

T-cell clones activation was not only dependent on the interaction of the TCR with allo-HLA-A2 molecule but that specific peptides were required.

To investigate the degree of peptide specificity exerted by the *in-vivo* selected allo-HLA-A2 reactive T-cells, 26 T-cell clones, randomly selected from the 44 allo-HLA-A2 reactive T-cell clones not recognizing T2 cells, were tested against T2 cells loaded with HPLC fractions of HLA-A2 eluted peptides. For this purpose HLA-A2 positive EBV-LCLs were lysed and their peptide-HLA-A2 complexes were purified. Subsequently, the peptides were eluted from HLA-A2, and separated from the HLA-A2 heavy chain and light chain. The peptide mixture was then subjected to RP-HPLC and fractions were collected. The T-cell clones were tested against T2 cells loaded with a small sample of each fraction, cultured for 18 h, after which IFNγ was measured by ELISA. 21 of the 26 tested T-cell clones, of which 8 are shown in figure 1B, showed recognition of one or two subsequent HPLC fractions. Almost all clones recognized a different HPLC fraction, indicative that different peptides presented in allo-HLA-A2 were recognized. One of 26 tested clones, clone HSS11, showed recognition of two not adjacent HPLC fractions (figure 4A). Four of 26 tested clones showed no reactivity against the HPLC fractions (data not shown).

To investigate the degree of peptide specificity exerted by the 6 allo-HLA reactive T-cell clones recognizing T2 cells, the clones were tested against HLA-A2 expressing drosophila cells loaded with HPLC fractions of HLA-A2 eluted peptides derived from T2 cells. All 6 clones,

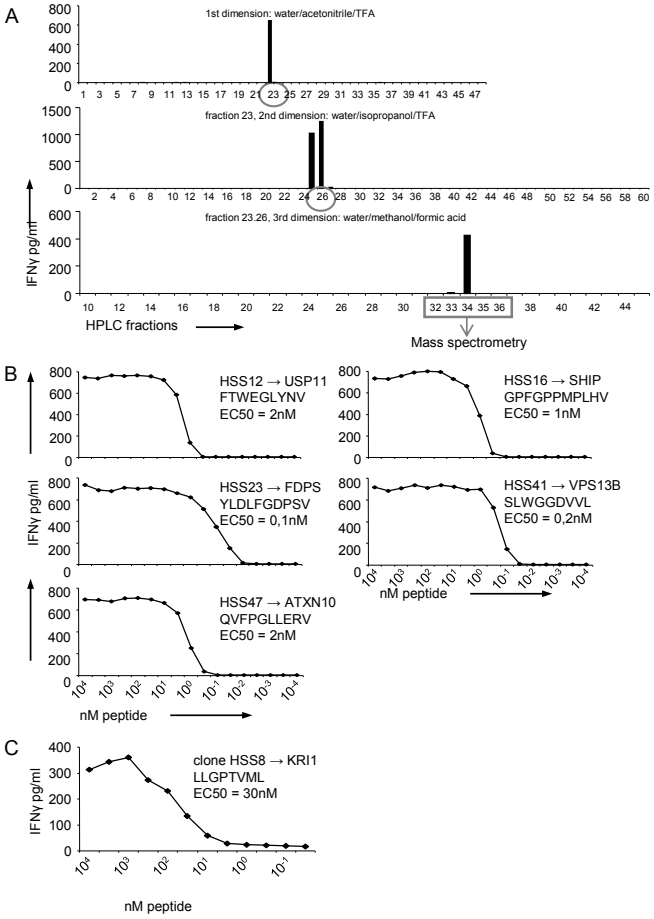

Figure 3. Identification of the peptides recognized by the allo-HLA reactive T-cell clones using multidimensional HPLC fractionation and MS.

(A) Five non-T2 recognizing allo-HLA reactive T-cell clones were stimulated for 18 h with T2 cells loaded with the first dimension HPLC fractions of peptides eluted from HLA-A2 derived from EBV-LCLs, and IFNγ was measured in the supernatant by standard ELISA. The recognized HPLC fractions were subjected to a second fractionation using a water/isopropanol/TFA gradient, loaded on T2 cells and subsequently tested for recognition by the T-cell clones. The recognized fractions were fractionated a third time, using a water/methanol/formic acid gradient and tested for recognition. After the third fractionation, the peptide masses present in the recognized and in the adjacent not recognized fractions were analyzed by MS and the sequences of the peptides were identified. The identification of the peptide recognized by clone HSS12, which is representative for the identification of the peptides recognized by the 4 other clones, is shown. **(B)** The 5 allo-HLA reactive T-cell clones, for which the recognized peptides were identified, were tested for their affinity for the respective peptides. The T-cell clones were stimulated with T2 cells loaded with titrated concentrations of the peptides for 18 h, and IFNγ was measured in the supernatant by standard ELISA. **(C)** By MS it was determined that the peptide recognized by the T2 reactive T cell clone HSS8 was derived from KRI1. To determine the affinity of the T-cell clone for this peptide, the clone was tested for IFNγ production against drosophila cells expressing HLA-A2, CD80 and CD54 loaded with titrated concentrations of the peptide. EC50 represents the peptide concentration needed for half of the maximum IFNγ production by the representative T-cell clone.

of which 3 are shown in figure 2B, showed recognition of one or two adjacent fractions, indicating that T2 reactive T-cell clones also exhibited peptide specific allo-HLA reactivity.

Identification of the peptides recognized in the context of allo-HLA-A2

To determine whether one or multiple peptides were recognized by the allo-HLA reactive T-cell clones, the peptides recognized by five randomly selected T-cell clones were identified using multidimensional RP-HPLC fractionation and MS. Identification of the peptide recognized by clone HSS12 is representative for the other 4 clones and is shown in figure 3A. All 5 clones recognized a single, but unique peptide (figure 3B and table 2).

Table 2. Identified peptides recognized by the alloreactive T-cell clones

Clone	Protein	Peptide	RefSeq ID
HSS8	KRI1	LLGPTVML	NM_023008
HSS11	HLA-DR1α	FIDKFTPPV	NM_019111
HSS11	THRAP4	FLLKLTPLL	NM_001079518
HSS12	USP11	FTWEGLYNV	NM_004651
HSS16	SHIP	GPFGPPMPLHV	NM_001017915
HSS23	FDPS	YLDLFGDPSV	NM_002004
HSS41	VPS13B	SLWGGDVVL	NM_181661
HSS47	ATXN10	QVFPGLLERV	NM_013236

For 7 T-cell clones (HSS8, 11, 12, 16, 23, 41 and 47), including one T2 reactive clone (HSS8) the recognized peptides were identified. Clone HSS11 showed recognition of peptides with sequence similarity.
The protein of which the peptides are derived from, the sequence of the peptides and the Reference Sequence identification number (http://srs.bioinformatics.nl/hspv/search.php) of the peptides are shown

To test the affinity of the 5 allo-HLA reactive T-cells for their respective recognized peptide presented in allo-HLA-A2, the allo-HLA reactive T-cell clones were tested against T2 cells loaded with titrated concentrations of the identified peptides. The concentration of peptide needed for half maximum IFNγ production (EC50) ranged between 3 nM to 100 pM for all T-cell clones.

In addition, for one of the T2 reactive T-cell clones, clone HSS8, peptide identification was performed with the HLA-A2 eluted peptides of T2 by multidimensional HPLC fractionation and MS (table 2). Peptide titration demonstrate that 30 nM of this peptide LLGPTVML derived from KRI-1 homolog, was needed for half maximum IFNγ production (EC50) by clone HSS8 (figure 3C). This illustrates that the recognition of TAP deficient T2 cells by allo-HLA reactive T-cells is also based on peptide specific allo-HLA recognition.

One of the allo-HLA reactive T-cell clones, clone HSS11, recognized two fractions after first HPLC fractionation (figure 4A). To determine whether this recognition pattern was based on recognition of two different peptides or based on recognition of different length variants of the same peptide, these two fractions were subsequently subjected to RP-HPLC for multidimensional fractionation, and peptides present in the positive fractions after three dimen-

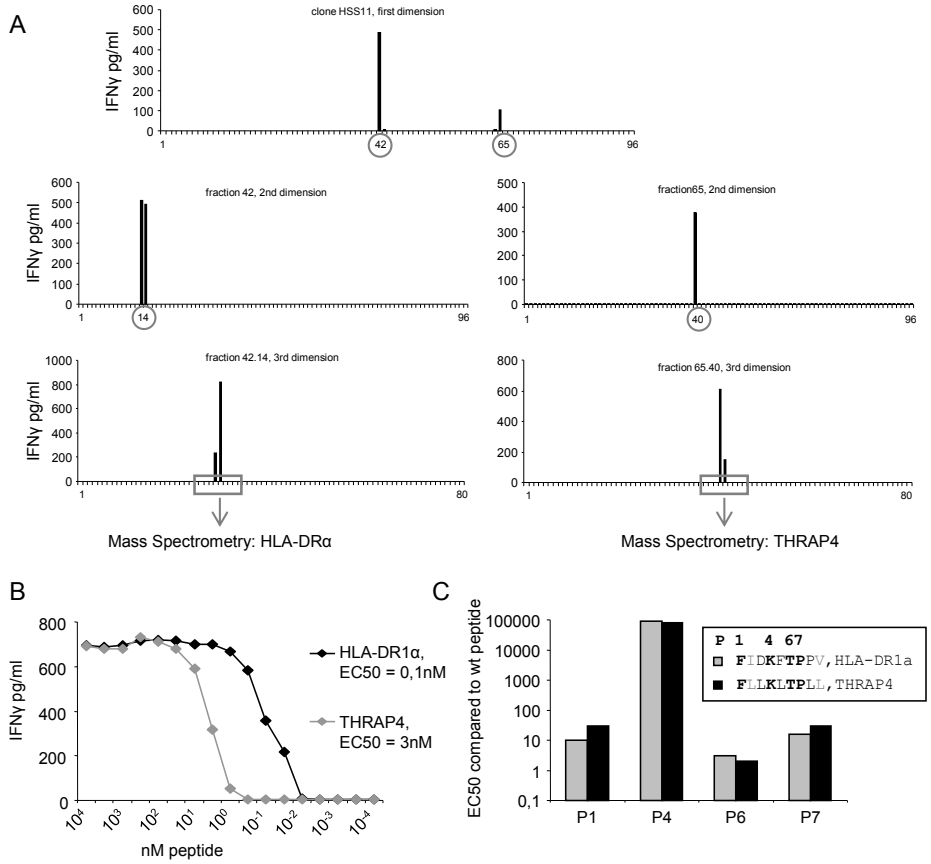

Figure 4. Identification of the peptides recognized by allo-HLA reactive T-cell clone HSS11.
(A) Clone HSS11 recognized two fractions after the first HPLC fractionation of peptides eluted from HLA-A2 derived from EBV-LCLs. These two recognized fractions were subjected to a second and a third fractionation. The peptide masses present in the fractions recognized by clone HSS11 after the third fractionation as well as in the adjacent not recognized fractions were determined by MS. Sequence analysis by tandem MS was performed on those peptide masses that correlated with the recognition pattern of the T cell clone. **(B)** To determine the affinity of clone HSS11 for the two identified peptides derived from HLA-DRA and THRAP4, the clone was stimulated with T2 cells loaded with titrated concentrations of the two peptides. EC50 represents the peptide concentration needed for half of the maximum IFNγ production by the T-cell clone. **(C)** To determine whether the 4 amino acids identical between the two peptides recognized by clone HSS11 at position P1, P4, P6 and P7, were involved in the TCR interaction, these amino acids were substituted by alanine residues. EC50 of the wt peptides and the modified peptides was determined by stimulating clone HSS11 with T2 cells loaded with titrated concentrations of the different peptides. The EC50 of the modified peptides was divided by the EC50 of the corresponding wt peptide.

sions were identified by MS (figure 4A, table 2). Testing the T-cell clone against the identified peptides loaded on T2 cells showed that clone HSS11 recognized epitope FIDKFTPPV derived from HLA-DRA, and epitope FLLKLTPLL derived from THRAP4. Peptide titration, shown in figure 4B, demonstrated that 100 pM of HLA-DRA peptide or 3 nM of THRAP4 peptides was needed for half maximum IFNγ production (EC50) of T-cell clone HSS11, illustrating that T-cell clone HSS11 was specific for two peptides.

The two recognized peptides share sequence homology. The amino acids at position 2 and 9 are different between the two peptides, however these amino acids form the anchor residues, which provide binding to HLA but do not interact with the TCR. Exchange of the amino acids at position 2 and 9 between the two peptides demonstrated no change in EC50 for both peptides (data not shown), indicating that the two anchor residues were similarly involved in HLA binding and did not influence the TCR interaction of the other amino acids. Of the 7 remaining amino acids 4 were identical. To investigate whether these identical amino acids at position 1, 4, 6 and 7 were involved in TCR interaction, these amino acids were substituted by an alanine residue. As illustrated in figure 4C all 4 amino acids were involved in TCR interaction, since alanine substitution at all positions increased EC50, demonstrating a lower binding affinity between the TCR and peptide/HLA-A2 complex. In addition, an identical change in EC50 for both peptides at all 4 positions was observed. Substitution at position 1 and 7 resulted in an intermediate 10-30 fold increase in EC50, substitution at position 4 resulted in a dramatic increase in EC50, and substitution at position 6 resulted only in a small change in EC50 of 3 fold. Together, these data suggest that the two peptide/HLA-A2 complexes recognized by clone HSS11 are conformational look-alikes. These results illustrate that allo-HLA reactive T-cells, including T2 reactive T-cells, derived from an *in vivo* GVH response recognize one or a restricted number of peptides with high affinity in the context of allo-HLA.

Confirmation of single peptide specificity using inhibition of gene expression by silencing shRNA

The allo-HLA-A2 reactive T-cell clones recognized EBV-LCLs as well as other HLA-A2 positive target cells including tumor cells and fibroblasts, as shown for one representative clone, clone HSS12 (figure 5A). To confirm the single peptide specificity of the allo-HLA reactive T-cells and biological relevance of the identified specificities, and to demonstrate that the reactivity directed against the other HLA-A2 positive target cells was also mediated by recognition of the same HLA-A2/peptide-complex, we transduced fibroblasts with silencing shRNAs specific for the respective recognized antigens of the characterized T-cell clones. By quantitative real-time PCR (qRT-PCR) we confirmed that all shRNAs downregulated the mRNA expression, varying between 5 to 31 fold (see legend figure 5B and 5C). The results in figure 5B demonstrate that silencing with the specific shRNA almost completely blocked the recognition of the corresponding allo-HLA-A2 reactive T-cell clones whereas the reactivity of other allo-HLA-A2 reactive T-cell clones was unaltered. In figure 5C we demonstrate

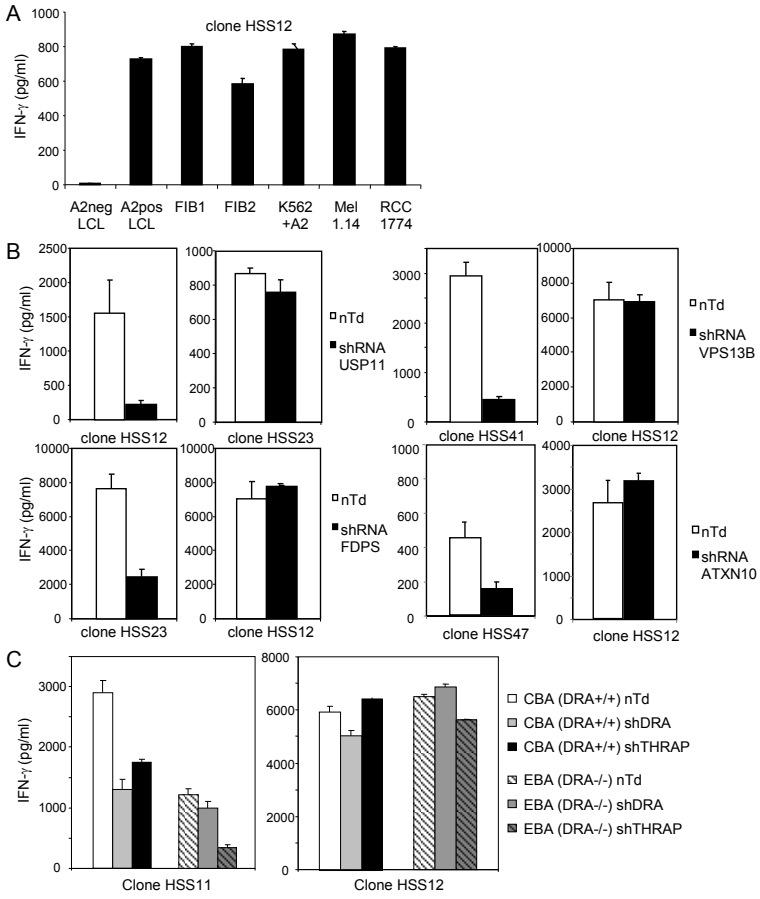

Figure 5. Recognition of different HLA-A2 positive target cells by allo-HLA reactive T-cell clones and confirmation of their single peptide specificity by transduction of shRNA specific for their respective recognized antigens.

(A) The allo-HLA reactive T-cell clones were stimulated with HLA-A2 negative EBV-LCLs (A2neg LCL), HLA-A2 positive EBV-LCLs (A2pos LCL), HLA-A2+ fibroblasts derived from two different individuals (FIB1 and FIB2), K562 cells transduced with HLA-A2 (K562+A2), HLA-A2 positive melanoma cell line 1.14 (Mel 1.14) and HLA-A2 positive renal cell carcinoma cell line 1774 (RCC 1774). Reactivity of one representative clone, clone HSS12, is shown. **(B)** Allo-HLA reactive T-cell clones HSS12, HSS23, HSS41 and HSS47 were tested against fibroblasts transduced with lentiviral vectors encoding shRNAs specific for USP11, FDPS, VPS13B or ATXN10 in combination with the puromycin resistance gene. shRNA transduced cells were cultured for 14 days with puromycin (4 ug/ml) and used as stimulator cells for the corresponding allo-reactive T cell clone and a control allo-reactive T cell clone. Non-transduced cells (nTd) were used as control stimulator cells. By qRT-PCR the downregulation of mRNA of the different genes compared to non-transduced cells was analyzed. The fold decrease for USP11= 5, FDPS=31, VPS13B= 5, ATXN10=10. **(C)** HSS11 and HSS12 were tested against HLA-DRA+/+ (CBA) and HLA-DRA-/- (EBA) fibroblasts transduced with lentiviral vectors encoding shRNAs specific for HLA-DRA or THRAP4 and subsequently selected for 14 days with puromycin and cultured for 72h with 100 IU IFNγ/ml. Downregulation of mRNA of HLA-DRA and THRAP4 was analyzed by qRT-PCR, the decrease for THRAP4 in CBA and EBA was 10 fold, the decrease for HLA-DRA in CBA was 4 fold. No expression of HLA-DRA was measured in EBA.

that the double peptide specificity of HSS11 directed against HLA-DRA and THRAP4 could be confirmed by transduction of fibroblast CBA with shRNA for either HLA-DRA or THRAP4. Both shRNAs were able to downregulate the recognition of clone HSS11, whereas the reactivity of control T-cell clone HSS12 was unaltered. To confirm that the reactivity of HSS11 was mediated by recognition of both HLA-DRA and THRAP4, HLA-DRA-/- fibroblasts (EBA) were transduced with shRNA for HLA-DRA or THRAP4. The reactivity of HSS11 directed against HLA-DRA-/- cells was reduced compared to HLA-DRA+/+ cells. Transduction with shRNA for THRAP4 almost completely blocked reactivity, and as expected, transduction with shRNA for HLA-DRA did not alter reactivity. These results clearly demonstrate that all allo-reactive T-cell clones recognized unique allo-HLA/peptide complexes.

Determination of the relative frequency of the identified allo-HLA/peptide specificities

To determine the frequency of the different allo-HLA-A2 specificities at the time of acute GVHD, the activated CD8+ donor T-cells were isolated, expanded and used for frequency analyses. Frequency analyses were performed by staining the pool of donor T-cells with tetramers composed of HLA-A2 in complex with the different newly identified allo-epitopes. As shown in figure 6 all allo-HLA reactive T-cell specificities could be observed, and the frequency of these different specificities varied between 0.07 and 1.16% of activated donor CD8+ T-cells.

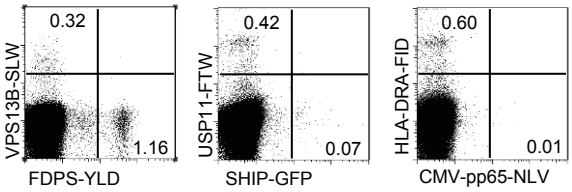

Figure 6. Variable frequencies of the different identified allo-HLA/peptide reactivities.
Activated CD8+ donor T cells were sorted from PBMCs at the time of GVHD, expanded non-specifically and stained with the different functional tetramers conjugated with either PE or APC, in combination with anti-CD8 Alexa700. As a control the CMV-pp65-NLV tetramer was included. In the dot-plots the CD8+ T cells are shown, and numbers in the different quadrants represent the percentage tetramer positive CD8+ T cells.

DISCUSSION

In this study we demonstrate that alloreactive T-cells isolated from a patient with GVHD after an HLA-A2 mismatched transplantation exerted peptide specific allo-HLA reactivity. The alloreactive T-cell clones derived from the *in vivo* allo-immune response, including those recognizing TAP deficient T2 cells, were reactive against one or two subsequent HPLC fractions

of peptides eluted from HLA-A2. Identification of the different peptides recognized in the context of the allo-HLA molecule followed by confirmation of the single peptide specificity by downregulation of the respective antigens using silencing shRNAs, demonstrated that the alloreactive T-cells exert high avidity recognition for a single endogenously processed and presented peptide.

Similar to other studies we observed that part of the allo-HLA reactive T-cells recognized TAP deficient T2 cells. Rammensee et al.[12] eluted and identified multiple peptides presented in the context of the HLA molecules expressed at the cell surfaces of TAP deficient T2 cells. Most of these peptides could also be found on TAP-expressing cells, indicating that many peptides can enter the ER and be presented in HLA molecules at the cell surface independent of TAP. It is therefore not surprising that part of the alloreactive T-cells recognized TAP independent peptides and were reactive against TAP deficient cells. Our results illustrate that high avidity reactivity against T2 cells is not based on peptide degenerate allorecognition, but on peptide specific allorecognition.

One of 26 T-cell clones tested against the HPLC fractions, clone HSS11, showed recognition of two different fractions, and peptide identification demonstrated that HSS11 recognized two different peptides. The two peptides share relevant sequence homology since 4 of 7 amino acids involved in interaction with the TCR are identical. Alanine substitutions of these 4 amino acids suggested that the two HLA-A2/peptide complexes are conformational look-alikes, most likely exhibiting a similar three dimensional structure. This degree of peptide cross-reactivity has also been described for T-cells specific for foreign peptides presented in self-HLA molecules.[28;29]

In most studies reporting polyspecific allorecognition, alloreactive T-cells were tested against a limited number of defined peptides loaded on allo-HLA expressing target cells.[2;8;13;14] Many of the alloreactive T-cells tested in this manner showed reactivity against several different peptides. However, since recognition of different peptides was not confirmed by testing the T-cells against endogenously processed and presented peptides, it is possible that the demonstrated T-cell reactivity directed against synthetic peptides loaded target cells represented low avidity T-cell reactivity. It was previously demonstrated that low avidity T-cell recognition does not lead to effective T-cell reactivity *in vivo*.[30] In our study, peptides recognized by the alloreactive T-cells were identified using multidimensional HPLC fractionations of peptides eluted from HLA-A2 of EBV-LCLs. In this method large numbers of peptides (10,000-20,000)[31] are tested at relatively low concentrations, especially after second and third fractionation. Therefore, by testing T-cells against HPLC fractions, only peptides recognized with high avidity were identified and low avidity T-cell recognition of other peptides was not determined. Downregulation of the identified antigens using silencing shRNAs almost completely blocked the reactivity of the corresponding allo-HLA reactive T-cells, demonstrating that the identified peptides, represented the actual biological relevant specificities of investigated alloreactive T-cells. During *in vivo* immune responses directed against pathogens, T-cells with high

avidity against a single antigenic peptide presented in the context of self-HLA molecules are selectively expanded.[16;17] Similarly, our results demonstrate that T-cells which are selectively activated and expanded during *in vivo* allo-HLA directed immune responses, apparently also exhibit high avidity recognition against single peptides presented in allo-HLA molecules.

Our results illustrate that high avidity allo-HLA recognition is as peptide specific as conventional T-cell recognition of foreign peptides presented in self-HLA molecules. These findings appear to be in conflict with the hypothesis that allorecognition is less peptide specific than conventional T-cell reactivity since allo-MHC molecules are not encountered during thymic development and therefore T-cells crossreactive against different peptides presented in allo-MHC molecules are not depleted.[2;3] This hypothesis is based on the assumption that the prethymic T-cell repertoire contains large numbers of MHC and peptide crossreactive T-cells and that thymic selection is responsible for removal of these T-cells. This assumption was already disputed by Zerrahn et al.,[32] who demonstrated that prethymic T-cells are as MHC crossreactive as T-cells after normal thymic selection, indicating that there may not be a large prethymic pool of highly MHC and peptide crossreactive T-cells which is removed during thymic selection. Our results demonstrating the peptide specific allo-HLA-A2 reactivity of 50 different *in vivo* activated and expanded alloreactive T-cells did also not provide evidence for the existence of T-cells crossreactive against multiple endogenously processed and presented peptides in the context of allo-HLA molecules not encountered during thymic development. We therefore speculate that highly crossreactive pre-thymic T-cells are either rare or non existent.

In summary, the results in this study demonstrate that T-cells exerting biological relevant alloreactivity *in vivo* exhibit high avidity recognition against single peptide-allo-HLA complexes. Based on the fact that downregulation of the identified antigens almost completely blocked the reactivity of the corresponding T-cells, we conclude that only these high avidity interactions, as defined by reactivity against endogenously processed and presented antigen, are biologically relevant *in vivo*. We hypothesize that during *in vivo* allo-HLA directed immune responses, only T-cells exhibiting high avidity recognition against single peptides presented in the context of allo-HLA molecules are selectively expanded.

ACKNOWLEDGEMENTS

We thank Guido de Roo and Menno van der Hoorn for expert technical assistance. We thank Martijn Rabelink and Prof. dr. Rob Hoeben for providing us the lentiviral constructs encoding the different shRNAs. Drosophila cells expressing HLA-A2, CD54 and CD80 were kindly provided by Prof. dr. Hans Stauss, and dr. Peter van den Elsen provided us with the HLA-DRA-/- fibroblasts derived from BLS patients.

REFERENCE LIST

1. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. Annu.Rev. Immunol. 2006;24:419-466.
2. Huseby ES, Crawford F, White J, Kappler J, Marrack P. Negative selection imparts peptide specificity to the mature T cell repertoire. Proc.Natl.Acad.Sci.U.S.A 2003;100:11565-11570.
3. Huseby ES, White J, Crawford F et al. How the T cell repertoire becomes peptide and MHC specific. Cell 2005;122:247-260.
4. Alexander-Miller MA, Burke K, Koszinowski UH, Hansen TH, Connolly JM. Alloreactive cytotoxic T lymphocytes generated in the presence of viral-derived peptides show exquisite peptide and MHC specificity. J.Immunol. 1993;151:1-10.
5. Aosai F, Ohlen C, Ljunggren HG et al. Different types of allospecific CTL clones identified by their ability to recognize peptide loading-defective target cells. Eur.J.Immunol. 1991;21:2767-2774.
6. Crumpacker DB, Alexander J, Cresswell P, Engelhard VH. Role of endogenous peptides in murine allogenic cytotoxic T cell responses assessed using transfectants of the antigen-processing mutant 174xCEM.T2. J.Immunol. 1992;148:3004-3011
7. Heath WR, Kane KP, Mescher MF, Sherman LA. Alloreactive T cells discriminate among a diverse set of endogenous peptides. Proc.Natl.Acad.Sci.U.S.A 1991;88:5101-5105.
8. Leisegang M, Wilde S, Spranger S et al. MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. J.Clin.Invest 2010;120:3869-3877.
9. Obst R, Munz C, Stevanovic S, Rammensee HG. Allo- and self-restricted cytotoxic T lymphocytes against a peptide library: evidence for a functionally diverse allorestricted T cell repertoire. Eur.J.Immunol. 1998;28:2432-2443.
10. Rotzschke O, Falk K, Faath S, Rammensee HG. On the nature of peptides involved in T cell alloreactivity. J.Exp.Med. 1991;174:1059-1071.
11. Weber DA, Terrell NK, Zhang Y et al. Requirement for peptide in alloreactive CD4+ T cell recognition of class II MHC molecules. J.Immunol. 1995;154:5153-5164.
12. Weinzierl AO, Rudolf D, Hillen N et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. Eur.J.Immunol. 2008;38:1503-1510.
13. de Koster HS, Vermeulen CJ, Koning F. The majority of HLA-DR3 alloreactive T cells is peptide specific, but does not recognize known DR3-bound sequences. Tissue Antigens 1998;51:88-95.
14. Felix NJ, Donermeyer DL, Horvath S et al. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. Nat.Immunol. 2007;8:388-397.
15. Hourigan CS, Harkiolaki M, Peterson NA, Bell JI, Jones EY, O'Callaghan CA. The structure of the human allo-ligand HLA-B*3501 in complex with a cytochrome p450 peptide: steric hindrance influences TCR allo-recognition. Eur J Immunol. 2006;36:3288-93.
16. Geiger R, Duhen T, Lanzavecchia A, Sallusto F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. J.Exp.Med. 2009;206:1525-1534.
17. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. Nat.Rev.Immunol. 2006;6:883-894.
18. Gorczynski RM, Rittenberg MB. Analysis of mixed leucocyte culture (MLC) reactive cells after in vitro priming. Changes in avidity of T cell receptors. Cell Immunol. 1975;16:171-181.
19. Walter S, Herrgen L, Schoor O et al. Cutting edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres. J.Immunol. 2003;171:4974-4978.

20. Amir AL, D'Orsogna LJ, Roelen DL et al. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010;115:3146-3157.
21. Macedo C, Orkis EA, Popescu I et al. Contribution of naive and memory T-cell populations to the human alloimmune response. Am.J.Transplant. 2009;9:2057-2066.
22. Wilde S, Sommermeyer D, Frankenberger B et al. Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood 2009;114:2131-2139.
23. Heemskerk MH, de Paus RA, Lurvink EG et al. Dual HLA class I and class II restricted recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex. Proc.Natl.Acad.Sci.U.S.A 2001;98:6806-6811.
24. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA et al. Direct cloning of leukemia-reactive T cells from patients treated with donor lymphocyte infusion shows a relative dominance of hematopoiesis-restricted minor histocompatibility antigen HA-1 and HA-2 specific T cells. Leukemia 2004;18:798-808.
25. Heemskerk MH, Hoogeboom M, de Paus RA et al. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. Blood 2003;102:3530-3540.
26. Burrows SR, Kienzle N, Winterhalter A et al. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. J.Immunol. 2000;165:6229-6234.
27. Nagarajan UM, Peijnenburg A, Gobin SJ, Boss JM, van den elsen PJ. Novel mutations within the RFX-B gene and partial rescue of MHC and related genes through exogenous class II transactivator in RFX-B-deficient cells. J Immunol. 2000;164:3666-74.
28. Borbulevych OY, Piepenbrink KH, Gloor BE et al. T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. Immunity. 2009;31:885-896.
29. Brehm MA, Pinto AK, Daniels KA et al. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nat.Immunol. 2002;3:627-634.
30. Offringa R. Antigen choice in adoptive T-cell therapy of cancer. Curr.Opin.Immunol. 2009;21:190-199.
31. Engelhard VH, Brickner AG, Zarling AL. Insights into antigen processing gained by direct analysis of the naturally processed class I MHC associated peptide repertoire. Mol.Immunol. 2002;39:127-137.
32. Zerrahn J, Held W, Raulet DH. The MHC reactivity of the T cell repertoire prior to positive and negative selection. Cell 1997;88:627-636.