Biology and clinical relevance of T-cell allo-HLA reactivity

Amir, A.

3

ALLO-HLA REACTIVITY OF VIRUS-SPECIFIC MEMORY T-CELLS IS COMMON

Avital L. Amir, Lloyd J.A. D'Orsogna, Dave L.Roelen, Marleen M. van Loenen, Renate S. Hagedoorn RH, Renate de Boer, Menno A.W.G. van der Hoorn, Michel G.D. Kester, Ilias I.N. Doxiadis, J.H. Frederik Falkenburg, Frans H.J. Claas and Mirjam H.M. Heemskerk

Blood. 2010 Dec 2;116(23):5079

ABSTRACT

Graft versus host disease and graft rejection are major complications of allogeneic HLA mismatched stem cell transplantations or organ transplantation that are caused by alloreactive T-cells. Since a range of acute viral infections have been linked to initiating these complications, we hypothesized that the cross-reactive potential of virus specific memory T-cells to allogeneic (allo) HLA molecules may be able to mediate these complications. To analyze the allo-HLA reactivity, T-cells specific for Epstein Barr virus, cytomegalovirus, varicella zoster virus and influenza virus were tested against a panel of HLA typed target cells, and target cells transduced with single HLA molecules. 80% of T-cell lines and 45% of virus specific T-cell clones were shown to cross-react against allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was primarily directed against HLA class I and II, respectively. However, a restricted number of CD8 T-cells exhibited cross-reactivity to HLA class II. T-cell receptor (TCR) gene transfer confirmed that allo-HLA reactivity and virus specificity were mediated via the same TCR. These results demonstrate that a substantial proportion of virus specific T-cells exert allo-HLA reactivity, which may have important clinical implications in transplantation settings as well as adoptive transfer of third party virus specific T-cells.

INTRODUCTION

HLA disparity between donor and recipient increases the risk and the severity of graft versus host disease (GVHD) after stem cell transplantation (SCT). The risk of graft rejection is also significantly increased in HLA mismatched as compared to HLA matched SCTs, and solid organ transplantations. The negative effect of HLA disparity on the clinical outcome of transplantations is the result of high frequencies of alloreactive T-cells. In HLA mismatched mixed lymphocyte reactions (MLR) the frequency of reactive T-cells was demonstrated to be a 1000 fold higher than the frequency of T-cells reactive in HLA identical MLRs.[1;2] By testing alloreactive T-cells against panels of third party target cells expressing different HLA molecules[1;3-5] and against target cells blocked with different HLA antibodies,[6-8] it was determined that the recognition exhibited by alloreactive T-cells is directed against non-self HLA (allo-HLA) molecules, and that the frequency of allo-HLA reactive T-cells ranged between 1-10%.

During thymic development T-cells undergo an instruction process of positive and negative selection which results in the composition of a mature T-cell repertoire that is selected on the basis of tolerance for self-HLA molecules presenting self peptides.[9;10] However, during thymic development T-cells never encounter allo-HLA molecules, and therefore no selection based on tolerance for allo-HLA molecules occurs. We therefore hypothesize that every antigen specific self-HLA restricted T-cell could potentially cross-react with non-self HLA molecules and exert allo-HLA reactivity.

Although it was shown that alloreactivity is equally presented in the naïve and memory T-cell populations,[11] the ability of T-cells to exhibit allo-HLA reactivity could especially have serious consequences when exerted by memory T-cells. Memory T-cells lack the requirement for co-stimulation,[12;13] and therefore allo-HLA reactivity of memory T-cells can be efficiently triggered by non-professional antigen presenting cells after HLA mismatched SCT or solid organ transplantation. Based on the restricted TCR repertoire of virus specific memory T-cells[14-17] the number of different virus specific T-cells will be limited, but the total number of virus specific T-cells with an identical TCR will be much higher in the memory pool as compared to the naïve compartment. T-cells directed against latent viruses, like EBV and CMV, are present at high frequencies in blood of healthy individuals and patients.[18-21] Therefore, if certain virus specific T-cells with cross-reactive potential against the mismatched allo-HLA molecule are triggered by viral activation and expanded in the memory pool, these T-cells will react against the mismatched HLA molecule, and may induce severe GVHD or graft rejection.

Studies of Burrows and colleagues have first illustrated that virus specific T-cells exert allo-HLA reactivity by demonstrating that EBV-EBNA3A specific HLA-B8 restricted T-cells cross-react with HLA-B44.[22;23] T-cell specific for HSV-VP13/14 presented in HLA-A2 were also found to cross-react with HLA-B44,[24] and CD4 T-cells specific for tetanus toxoid presented in HLA-DR3 were found to be cross-reactive against HLA-DR4.[25] In addition, the association between

reactivation of viral infections during organ transplantation and increased graft rejection[26] supports the hypothesis that virus specific T-cells exhibit allo-HLA reactive potential.
In this study we investigate the ability of a large panel of virus specific T-cells to exert allo-HLA reactivity. We determined the cross-reactive potential of virus specific T-cells to allo-HLA molecules by screening single viral antigen specific T-cell lines and clones against a panel of EBV transformed B-cells (EBV-LCLs), together covering almost all prevalent HLA class I and II molecules, as well as single HLA transduced target cells. The tested CD8 and CD4 virus specific memory T-cells were specific for Epstein Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV) and influenza virus (Flu). Most virus specific T-cell lines and 45% of the virus specific T-cell clones were demonstrated to be cross-reactive against allo-HLA molecules. TCR gene transfer demonstrated that the virus specificity as well as the cross-reactivity to allo-HLA molecules was mediated by the same TCR. These results demonstrate that T-cells specific for different viruses exert cross-reactivity to allo-HLA molecules, and illustrate the high frequency of T-cells able to exert allo-HLA reactivity.

MATERIAL AND METHODS

Cell collection and preparation

After informed consent, peripheral blood (PB) was obtained from different individuals. Mononuclear cells (MNC) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. EBV-LCLs and K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) and 10% fetal bovine serum (FBS, Lonza). PHA blasts were generated by stimulation of PB-MNCs with phytohemagglutinin (PHA, 0.8 ug/ml, Murex Biotec Limited, Dartford, UK) in IMDM, 5% FBS, 5% human serum (HS) and IL-2 (120 IU/ml). K562 expressing single allo-HLA molecules and HLA transduced EBV-LCLs were generated by transduction with retroviral vectors encoding for the allo-HLA molecules or by transfection of allo-HLA molecules.[27;28] For the isolation of T-cells, B-cells and monocytes PBMC of healthy donors were stained with either anti-CD3, anti-CD19 or anti-CD14 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively and isolated according to manufacturer's instructions. CD40L activated B-cells were generated by culturing the CD19+ fraction for 3 days on CD40L transduced murine fibroblasts[29] in medium containing CpG (10 µg/ml) and IL-4 (500 IU/ml) (Schering-Plough, Innishammon, Cork, Ireland). Monocyte derived DCs were generated by culturing the CD14+ fraction in medium containing activating cytokines as described previously.[30] Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle medium (DMEM, Lonza) with 1g/l glucose (BioWhittaker, Verviers, Belgium) and 10% FBS.

Generation of virus specific T-cell lines and clones

PB-MNCs from healthy individuals were stained with tetramer and anti-CD8 mAb for 1 h at 4°C and washed once. The tetramers used were constructed as described previously[31] and are shown in table 1. Tetramer positive, CD8 positive T-cells were sorted 50 cells per well for the generation of lines or single cell per well for the generation of clones into U-bottom microtiter plates containing 100 µl of feeder mixture. Sorting was performed at 4°C using the FACS Vantage (BD). The feeder mixture consisted of IMDM, 5% FBS, 5% HS, IL-2 (120 IU/ml), PHA and 30 Gy irradiated allogeneic third-party PB-MNCs ($0.5x10^6$/ml). Proliferating T-cell clones were selected and further expanded by nonspecific stimulation every 14 days using the previously mentioned feeder mixture. The viral specificity of the expanded lines and clones was confirmed by tetramer staining, cytotoxicity and cytokine production assays. Polyclonality or monoclonality of the T-cell lines and clones was analyzed by TCR Vβ analysis using the TCR Vβ kit (Beckman Coulter, Fullerton, CA, USA).

Table 1. Tetramers used for the generation of virus specific T-cell lines and clones

virus	viral antigen	HLA	epitope
CMV	pp50	HLA-A*0101	VTEHDTLLY
CMV	pp65	HLA-A*0101	YSEHPTFTSQY
CMV	pp65	HLA-A*0201	NLVPMVATV
CMV	pp65	HLA-B*0702	RPHERNGFTVL
CMV	pp65	HLA-B*03501	IPSINVHHY
CMV	pp65	HLA-DRB1*0101	KYQEFFWDANDIYRI
CMV	IE1	HLA-B*0801	ELRRKMMYM
EBV	EBNA3A	HLA-A*0301	RLRAEAQVK
EBV	EBNA3A	HLA-B*0702	RPPIFIRRL
EBV	EBNA3A	HLA-B*0801	FLRGRAYGL
EBV	BMLF1	HLA-A*0201	GLCTLVAML
EBV	BRLF1	HLA-A*0301	RVRAYTYSK
EBV	BZLF1	HLA-B*0801	RAKFKQLL
EBV	LMP2	HLA-A*0201	CLGGLLTMV
FLU	IMP	HLA-A*0201	GILGFVFTL
FLU	HA	HLA-DRB1*0401	PKYVKQNTLKLAT
VZV	IE62	HLA-A*0201	ALWALPHAA

Allo-HLA reactivity of the virus specific T-cell lines and clones

In the IFNγ production assays 5000 T-cells were co-cultured with 20.000 stimulator cells in a final volume of 150µl IMDM culture medium supplemented with 100 IU/ml IL-2. After 18 h of incubation, supernatants were harvested and IFNγ production was measured by standard ELISA (ELISA; CLB, Amsterdam, the Netherlands). In the cytotoxicity assays the virus specific T-cells clones were tested in a standard 6 hour ^{51}Cr-release assay[32] against EBV-LCLs in an effector to target ratio of 10:1.

TCR gene transfer

The TCRAV and TCRBV gene usage of the BRLF1/HLA-A3 clone 19 the and the VZV-IE62/HLA-A2 specific T-cell clone 7 was determined using reverse transcriptase (RT)–PCR and sequencing.[27] Retroviral vectors were constructed that encoded the TCRα chain in combination with GFP and the TCRβ chain in combination with the marker gene ΔNGF-R.[27] CMV-IE1/HLA-A1 specific T-cells derived from an HLA-A*0301 negative healthy individual were transduced with the TCR α and β of the BRLF1/HLA-A3 clone 19. CMV-pp50/HLA-A1 specific T-cells derived from peripheral blood of a healthy individual negative for HLA-A*0201 and HLA-A*0205 were transduced with the retroviral vectors encoding for the TCR α and β chain of the VZV specific T-cell clone.[27] The TCR transduced T-cells were sorted on basis of double positivity for GFP and ΔNGF-R, and after expansion the T-cells were tested for viral specificity and allo-HLA reactivity in stimulation assays.

RESULTS

Alloreactivity of virus specific T-cell lines

To investigate the ability of virus specific T-cells to exert alloreactivity, virus specific T-cell lines were tested against a panel of EBV-LCLs, together covering almost all frequently occurring HLA class I and II molecules. The HLA typing of the EBV-LCLs used in the panel is listed in table 2. The virus specific T-cell lines were generated by isolation of tetramer positive CD8 positive T-cells by FACS sort and subsequent expansion. Tetramer and CD8 staining confirmed the purity of the virus specific lines and showed that all T-cell lines were more than 98% tetramer positive (figure 1A-G). In total eleven virus specific lines were tested, derived from 9 different donors, specific for 7 different antigens of CMV, EBV and VZV and restricted to 3 different HLA molecules. In the figures in which EBV specific T-cell lines were tested, the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted are not shown in the figures, to present only the alloreactivity of the T-cells and not the virus specificity. All LCLs were tested for IFNγ production in the absence of T-cells and did not show production of IFNγ (data not shown). In figure 1(A-G) seven representative lines are shown. Nine of the eleven tested virus specific T-cell lines were shown to be alloreactive, since these lines produced IFNγ upon stimulation with at least one of the EBV-LCLs of the panel. Two of the eleven tested lines exerted no alloreactivity against the EBV-LCLs tested in our panel (including figure 1C). The recognition pattern of the virus specific lines that demonstrated alloreactivity ranged from recognition of almost all EBV-LCLs, as shown in figure 1A and 1B by the CMV-pp50/HLA-A1 specific lines of individuals MBX and UKL, to recognition of a limited number of EBV-LCLs, as shown by the IE1/HLA-B8, the BMLF1/HLA-A2, the VZV-IE62/HLA-A2 and the EBNA3A/HLA-B8 specific lines (figure 1D, 1E, 1F and 1G respectively). The pattern of allo-recognition of some of the T-cell lines suggested that the alloreactivity was directed against one or several

Table 2. HLA expression of the EBV-LCL panel

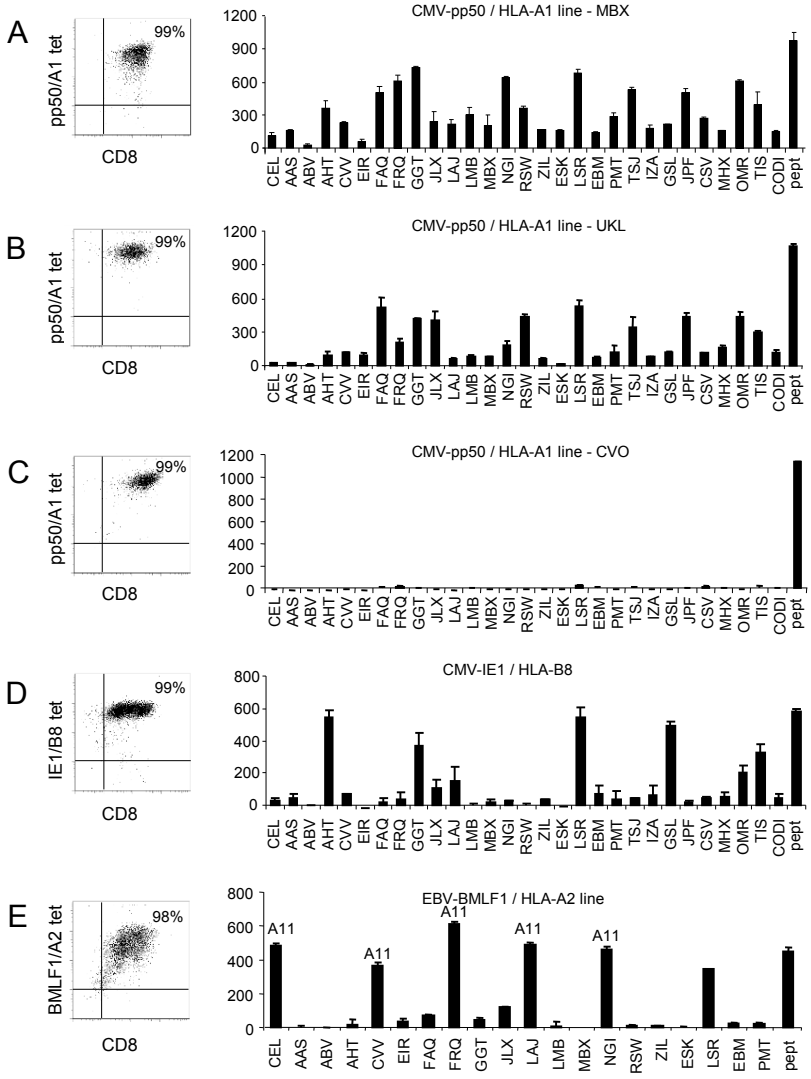
ID	HLA class I			HLA class II		
	A	B	C	DR	DQ	DP
CEL	1101	1502, 4001	0401, 0801	0901	0202, 0303	0501
AAS	3101, 6601	2705, 4102	0202, 1703	1101, 1303	0301	0401, 0402
ABV	0301, 2902	0702, 4403	0702, 1601	0701, 1454	0502, 0202	0401, 1101
AHT	2402, 2501	5501, 1501	0303	0806, 1501	0501, 0602	0401
CVV	11, 31*	57*, 1501	3*, 0602	0401, 7*	0301, 0303	0201, 0401
EIR	0101, 2402	3502, 3701	0401, 0602	0404, 1104	0301, 0402	0301, 0401
FAQ	2301, 6802	1402, 3801	0802, 1203	1301, 1303	0301, 0603	0201
FRQ	0101, 1101	0801, 4402	0501, 0701	0301, 0401	0201, 0301	0101, 0401
GGT	2601, 3101	1401, 4901	0701, 0802	0101, 0701	0202, 0504	0402, 1101
JLX	0101, 6802	0801, 5301	0401, 0701	0301, 1302	0201, 0604	0101, 0401
LAJ	1101, 2402	1302, 5501	0303, 0602	0701, 1601	0202, 0502	0401, 1701
LMB	2902	4402 , 5101	1402, 1601	0701, 0801	0202, 0402	0401, 1101
MBX	0101	0801, 1517	0701	1202, 0301	201,0301	0101, 0401
NGI	1101, 2402	0801, 3906	0701	0301, 0801	0201, 0402	0101, 1401
RSW	3001, 6802	4201	1701	0302	0402	0101, 0402
ZIL	2402, 2601	5601, 5801	0101, 0701	0101, 0804	0402, 0501	0201, 0301
ESK	23, 66*	51, 72*	2, 18*	12, 13*	1, 3*	ND
LSR	3201, 6801	3503, 5201	1202, 1203	1502, 1602	0502, 0601	0401, 1401
EBM	2301	1401	0802	0401	0302	0201
PMT	0301, 3301	1402	0802	0102	0501	0301, '0401
TSJ	2, 24	1501, 75	4	12, 15	6, 0301	ND
IZA	0201, 2402	0801, 4001	0304, 0701	0301, 1301	0603	0401, 1401
GSL	2,3*	47, 1501*	3, 6*	11, 13*	1*, 0301	ND
JPF	0201, 0205	4002, 1501	0202, 0304	0701, 1104	0301, 0303	0402, 1401
CSV	0201, 3101	5101, 5501	0303, 1402	0404, 1001	0501, 0301	0401, 0402
MHX	0101, 0205	1801, 5001	0602, 0701	0701, 0901	0201, 0303	0201, 0301
OMR	201	4501	1601	1301	0603	0101
TIS	0206, 0207	4601	0102, 0801	0901	0303	1301
CODI	2*, 8001	58, 70*	2, 6*	17, 11*	2, 7*	1, 4*
AKB	0101, 0201	3701, 3901	0602, 0702	0101, 1001	501	0201, 0401
PSJ	0201, 3001	1302, 4402	0501, 0602	0401, 0701	0202, 0301	0402, 1401
MWX	0101, 3401	1521, 3503	0403, 1203	0101, 1502	0501, 0601	0601, 1301
NGZ	1101, 2401	5201, 4002	0202, 1202	0101, 1101	0501, 0301	0201, 0301
RSB	0201, 0301	5701, 4402	0602, 0704	0701, 1101	0301, 0303	0201, 0401
WOH	1, 28	8, 27	2, 7	ND	ND	ND
FBV	0201, 1101	0702, 5501	0303, 0702	1454, 1501	0503, 0602	0401
RTN	0101, 1101	0801, 5701	0602, 0701	0301, 0701	0201, 0301	0401, 1401

The panel of EBV-LCLs was composed of HLA-typed EBV-LCLs which together covered almost all frequently occurring HLA molecules. The HLA typing was mainly determined molecularly; however some EBV-LCLs were only serologically typed.

*) indicates that the HLA expression was determined by serological typing

ND) indicates that the HLA expression was *not determined*

of the allo-HLA molecules presented by the EBV-LCL panel. The BMLF1/HLA-A2 specific line showed high reactivity against 6 EBV-LCLs, of which 5 EBV-LCLs expressed HLA-A*1101. All HLA-A*1101 expressing EBV-LCLs within this panel were highly recognized by this T-cell line. The EBV-LCLs recognized by the EBNA3A/HLA-B8 specific line expressed either HLA-B*4402 or HLA-B*5501. TCR Vβ analysis of the lines demonstrated that the complexity of the TCR composition was correlated with the broadness of the alloreactivity. T-cell lines that did not show alloreactivity expressed maximally two different TCR Vβ chains. T-cell lines with limited alloreactivity expressed one to four different TCR Vβs and lines that recognized almost all



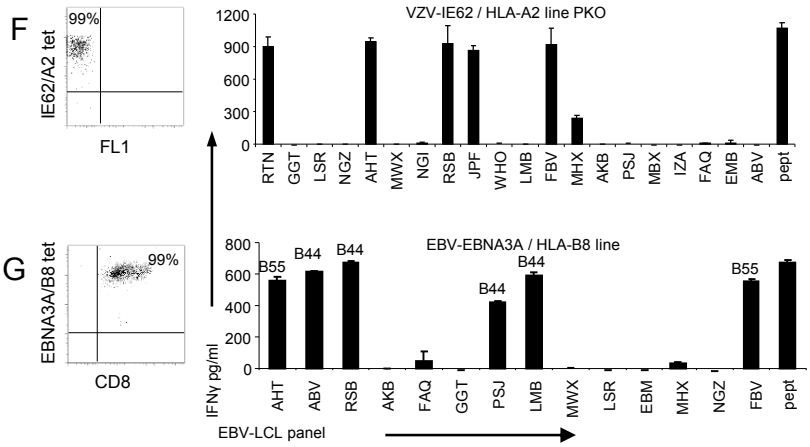


Figure 1. Alloreactivity of virus specific T-cell lines.
Eleven virus specific T-cell lines, of which seven are shown in this figure, were stimulated with a panel of EBV-LCLs for 18h and IFNγ production was measured by ELISA. In experiments in which EBV specific T-cell lines were tested, we excluded the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted. The purity of the virus specific lines was analyzed by tetramers and CD8 staining, and all T-cell lines proved to be more than 98% pure. As a positive control, the lines were tested against EBV-LCLs expressing the HLA restricting molecule of the viral epitope, loaded with the viral peptide recognized by the T-cell lines (pept). **(A)** The CMV-pp50/HLA-A1 specific lines of individual MBX recognized almost all EBV-LCLs. **(B)** The CMV-pp50/HLA-A1 specific line of individual UKL showed broad alloreactivity. **(C)** Two of the ten tested T-cell lines exerted no alloreactivity against the tested EBV-LCLs of which one, the pp50/HLA-A1 specific line, is shown. **(D)** The CMV-IE1/HLA-B8 recognized a limited number of EBV-LCLs. **(E)** The BMLF1/HLA-A2 specific line showed high reactivity against all HLA-A11 positive EBV-LCLs and one HLA-A11 negative EBV-LCL. **(F)** The VZV-IE62/HLA-A2 specific line of individual PKO recognized a limited number of EBV-LCLs. **(G)** EBNA3A/HLA-B8 specific line recognized EBV-LCLs expressing either HLA-B44 or HLA-B55.

EBV-LCLs expressed at least 8 different TCR Vβs. This relation between the clonal composition and the recognition of the lines suggests that the alloreactivity of the lines is the sum of the alloreactivity of the various clonal populations present within the lines.
The results demonstrate that 80% of the tested virus specific T-cell lines were able to exert alloreactivity. Some virus specific lines showed a pattern of alloreactivity suggestive for allo-HLA reactivity. However, for most of the virus specific cell lines allo-HLA reactivity could not be determined since the exerted alloreactivity was very broad.

Allo-HLA reactivity of virus specific T-cell clones

Since we were unable to determine allo-HLA reactivity with the oligoclonal virus specific T-cell lines, further characterization of the allo-HLA reactivity of virus specific T-cells was performed with T-cell clones. For this purpose tetramer positive T-cells were sorted single cell per well and expanded. The specificity of the T-cell clones was confirmed by tetramer staining and the

TCR Vβ usage of the clones was analyzed using the TCR Vβ kit. The T-cell clones as shown in table 3 were different based either on their different origin, TCR Vβ usage or their recognition pattern. In total, 41 virus specific CD8 and CD4 T-cell clones were tested against the EBV-LCL panel. These virus specific T-cell clones were derived from 16 individuals, specific for 13 different CMV, EBV, VZV and Flu antigens and restricted to 8 different HLA molecules. The results demonstrated that 18 of the 41 virus specific CD8 and CD4 T-cell clones were alloreactive, as shown by recognition of at least one of the EBV-LCLs from the panel. Most alloreactive T-cell clones exhibited cross-reactivity against EBV-LCLs that shared an HLA molecule, suggesting allo-HLA reactivity. To confirm that the reactivity of a T-cell clone was directed against a specific allo-HLA molecule, the virus specific T-cell clones were tested against HLA negative K562 cell line or EBV-LCLs negative for the recognized allo-HLA molecules, which were transduced with the particular allo-HLA molecule. The allo-HLA reactivity of seven representative T-cell clones is shown in figure 2(A-G). The EBV-EBNA3A/HLA-A3 specific CD8 T-cell clone exhibited alloreactivity against all HLA-A*3101 expressing EBV-LCLs within the panel. The reactivity directed against allo-HLA-A*3101 was confirmed by transfection of K562 with HLA-A*3101 and subsequent recognition by this T-cell clone (figure 2A). The EBV-EBNA3A/HLA-A3 clone also recognized the HLA-A*3101 negative EBV-LCL RSW, which however expressed HLA-A*3001. HLA-A*3101 and HLA-A*3001 are very similar in sequence and therefore we hypothesize that the molecules exhibit strong similarity in structure and peptide presentation, explaining recognition by this T-cell clone. To analyze whether the EBV-EBNA3A/HLA-A3 clone recognized HLA-A*3001, the clone was tested against three HLA-A*3001+ EBV-LCLs, of which one is shown in figure 2A, and one HLA-A*3101+ EBV-LCL with or without blocking mAbs directed against HLA class I, HLA-A30/A31 and HLA-A2. All HLA-A*3001+ EBV-LCLs and the HLA-A*3101+ EBV-LCL were recognized and this recognition was blocked by anti-HLA class I and anti-HLA-A30/A31 mAbs and not by anti-HLA-A2 mAb, indicating that the clone indeed also recognized HLA-A*3001. The CMV-pp50/HLA-A1 specific T-cell clone exhibited alloreactivity against all HLA-A*1101 expressing EBV-LCLs (figure 2B). The allo-HLA-A*1101 reactivity could be confirmed by specific recognition of K562 transduced with HLA-A*1101 by this T-cell clone. In addition to reactivity against HLA-A*1101, the T-cell clone also exhibited low IFNγ production upon stimulation with a few EBV-LCLs negative for HLA-A*1101. Since these lower recognized EBV-LCLs did not share one HLA molecule, we did not determine whether this alloreactivity was also based on allo-HLA cross-reactivity. The EBV-BRLF1 specific HLA-A3 restricted clone, shown in figure 2C, exerted alloreactivity against all HLA-A*0201+ EBV-LCLs. The CD8 T-cell clone did not recognize K562 transduced with HLA-A*0201, however showed recognition of EBV-LCLs transduced with HLA-A*0201, suggesting that the peptide recognized by the clone in the context of HLA-A*0201 is not presented by K562 cells. The clone also showed recognition of HLA-A*0201+ PHA stimulated T-cell blasts (figure 2C, right figure), excluding the possibility that the clone recognized an EBV derived peptide presented in HLA-A*0201. Next to allo-HLA-A*0201 reactivity, this T-cell clone also

Table 3. Allo-HLA reactivity of virus specific T-cell clones

specificity	donor	TCR Vβ	EBV panel	HLA trans/block	figure
pp50/A1	MBX	#	UD		
pp50/A1	MBX	1			
pp50/A1	MBX	5.1			
pp50/A1	MBX	3	A*1101	A*1101	2B
pp65/A2	MRJ	2			
pp65/A2	MRJ	13			
pp65/A2	HRN	8	UD		2G
pp65/A2	HRN	2			
pp65/A2	AMJ	3			
BMLF1/A2	GFS	#			
LMP2/A2	JVW	#			
FLU/A2	FKR	17	B*6401		
FLU/A2	FKR	17			
VZV/A2	PKN	14	B*5501	B*5501	3A
VZV/A2	PKN	#	B*5701	B*5701	3B
VZV/A2	PKN	21,3	A*0205	A*0205	3C
EBNA3A/A3	HRN	#	A*3101	A*3101	2A
BRLF1/A3	AKO	7.1			
BRLF1/A3	AKO	14			
BRLF1/A3	AKO	17			
BRLF1/A3	AKO	1			
BRLF1/A3	DVO	7.1	UD		
BRLF1/A3	DVO	8			
BRLF1/A3	DVO	14	UD		
BRLF1/A3	DVO	17	UD		
BRLF1/A3	DVO	#			
BRLF1/A3	DVO	7.2	A*0201	A*0201	2C
pp65/B7	BDV	7.2			
pp65/B7	BDV	7.2	DRB1*0801	DRB1*0801	2D
EBNA3A/B8	LDO	#	B*4402, B*5501	B*4402, B*5501	2E
BZLF/B8	AVK	7.1			
BZLF/B8	AVK	#			
BZLF/B8	AVK	5.1			
pp65/B35	MED	3			
pp65/B35	MED	5.1	DRB1*0401	DRB1*0401	
pp65/B35	MED	#			
pp65/DR1	CBH	2			
pp65/DR1	CBH	#			
pp65/DR1	MSV	8	DRB1*0901		
pp65/DR1	MSV	#	DRB3*0101	DRB3*0101	
FLU/DR4	VKY	3	DRB1*1301	DRB1*1301	2F

#) indicates that the TCR Vβ of the clone could not be determined with the TCR Vβ kit
UD) *undetermined*, indicates allo-HLA reactivity, however allo-HLA reactivity could not be characterized since the recognized EBV-LCLs did not share one particular allo-HLA molecule.

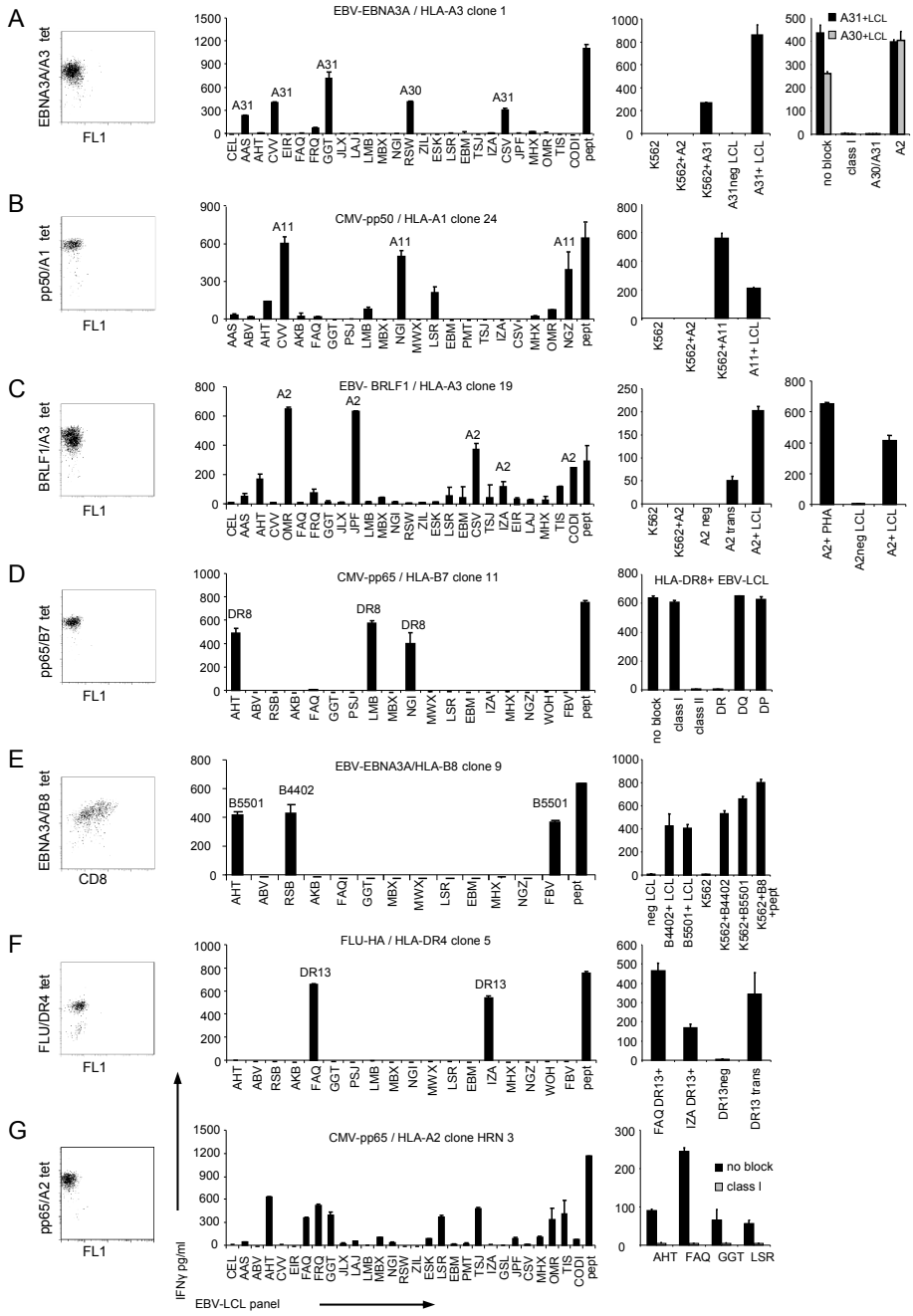

A
EBNA3A/A3 tet
FL1
EBV-EBNA3A / HLA-A3 clone 1
A31
A31
A31
A30
A31
CEL AAS AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM TSJ IZA CSV JPF MHX OMR TIS CODI pept
K562
K562+A2
K562+A31
A31neg LCL
A31+ LCL
A31+LCL
A30+LCL
no block
class I
A30/A31
A2
B
pp50/A1 tet
FL1
CMV-pp50 / HLA-A1 clone 24
A11
A11
A11
AAS ABV AHT CVV AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM PMT TSJ IZA CSV MHX OMR NGZ pept
K562
K562+A2
K562+A11
A11+ LCL
C
BRLF1/A3 tet
FL1
EBV- BRLF1 / HLA-A3 clone 19
A2
A2
A2
A2
A2
CEL AAS AHT CVV OMR FAQ FRQ GGT JLX JPF LMB MBX NGI RSW ZIL ESK LSR EBM CSV TSJ IZA EIR LAJ MHX TIS CODI pept
K562
K562+A2
A2 neg
A2 trans
A2+ LCL
A2+ PHA
A2neg LCL
A2+ LCL
D
pp65/B7 tet
FL1
CMV-pp65 / HLA-B7 clone 11
DR8
DR8
DR8
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
HLA-DR8+ EBV-LCL
no block
class I
class II
DR
DQ
DP
E
EBNA3A/B8 tet
CD8
EBV-EBNA3A/HLA-B8 clone 9
B5501
B4402
B5501
AHT ABV RSB AKB FAQ GGT MBX MWX LSR EBM MHX NGZ FBV pept
neg LCL
B4402+ LCL
B5501+ LCL
K562
K562+B4402
K562+B5501
K562+B8 pept
F
FLU/DR4 tet
FL1
FLU-HA / HLA-DR4 clone 5
DR13
DR13
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
FAQ DR13+
IZA DR13+
DR13neg
DR13 trans
G
pp65/A2 tet
FL1
CMV-pp65 / HLA-A2 clone HRN 3
IFNγ pg/ml
EBV-LCL panel
CEL AAS ABV AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM PMT TSJ IZA GSL JPF CSV MHX OMR TIS CODI pept
no block
class I
AHT FAQ GGT LSR

Figure 2. Allo-HLA reactivity of virus specific T-cell clones.
41 virus specific T-cell clones, of which seven are shown in this figure, were stimulated with a panel of EBV-LCLs for 18h and IFNγ production was measured by ELISA. **(A)** The EBV-EBNA3A/HLA-A3 specific T-cell clone 1 exhibited alloreactivity against all EBV-LCLs expressing HLA-A*3101 and one EBV-LCL expressing HLA-A*3001. To confirm allo-HLA-A*3101 reactivity, clone 1 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transfected with HLA-A*3101 (K562+A31), HLA-A*3101 negative (A31neg LCL) and HLA-A*3101 positive EBV-LCLs (A31+ LCL). To confirm the reactivity against HLA-A*3001 the clone was tested against three HLA-A*3001+ EBV-LCLs, of which one is shown, and one HLA-A*3101+ EBV-LCLs with or without blocking mAbs directed against HLA class I, HLA-A30/A31 and HLA-A2. **(B)** The CMV-pp50/HLA-A1 specific T-cell clone 24 exhibited alloreactivity against all HLA-A*1101 expressing EBV-LCLs. To confirm allo-HLA-A*1101 reactivity, clone 24 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-A*1101 (K562+A11) and HLA-A*1101 positive EBV-LCLs (A11+ LCL). **(C)** The EBV-BRLF1/ HLA-A3 specific clone 19 exerted alloreactivity against all HLA-A0201+ EBV-LCLs. This T-cell clone did not recognize K562 transduced with HLA-A*0201 (K562+A2). To confirm allo-HLA- A*0201 reactivity clone 19 was tested against untransduced HLA-A*0201 negative EBV-LCLs (HLA-A2neg LCL) or transduced with HLA-A*0201 (A2 trans), and HLA-A*0201 positive EBV-LCLs (A2+ LCL). To investigate whether this clone recognized an EBV derived peptide in the context of HLA-A*0201, the clone was tested against HLA-A*0201+ PHA blasts and HLA-A*0201 positive and negative EBV-LCLs as controls. **(D)** The CMV-pp65/HLA-B7 specific T-cell clone 11 exhibited reactivity against all three HLA-DRB1*0801+ EBV-LCLs. To confirm allo-HLA-DRB1*0801 reactivity, clone 11 was tested against the three HLA-DRB1*0801+ EBV-LCLs, of which one is shown, in the presence of either no (no block), anti-HLA class I (class I), anti-HLA-class II (class II), anti-HLA-DR (DR), anti-HLA-DQ (DQ), anti-HLA-DP (DP) blocking mAbs. **(E)** The EBV-EBNA3A/HLA-B8 specific T-cell clone 9 exhibited alloreactivity against all EBV-LCLs expressing either HLA-B*4402 or HLA-B*5501. To confirm HLA-B*4402 and HLA-B*5501 cross-reactivity, the clone was tested against K562 cells (K562), K562 cells transfected with HLA-B*4402 (K562+B4402) or HLA-B*5501 (K562+B5501). As controls, the clone was tested against HLA-B*4402 and HLA-B*5501 negative EBV-LCL (neg LCL), HLA-B*4402+ EBV-LCL (B4402+ LCL), HLA-B*5501+ EBV-LCL (B5501+ LCL) and HLA-B*0801+ K562 loaded with viral peptide (K562+B8+pept). **(F)** The Flu-HA/HLA-DR4 specific clone 5 recognized all HLA-DRB1*1301+ EBV-LCLs. To confirm allo-HLA-DRB1*1301 reactivity, clone 5 was tested against HLA-DRB1*1301+ EBV-LCLs (FAQ DR13+ and IZA DR13+) as well as HLA-DR13 negative EBV-LCLs non-transduced (DR13 neg) or transduced with HLA-DRB1*1301 (DR13 trans). **(G)** The CMV-pp65/HLA-A2 specific clone HRN 3 recognized EBV-LCLs which did not share one particular allo-HLA molecule. To investigate whether this reactivity was based on allo-HLA recognition, the clone was tested against four of the recognized EBV-LCLs with and without blocking mAb directed against HLA class I.

exhibited low cross-reactivity against EBV-LCLs negative for HLA-A*0201. Since these lower recognized EBV-LCLs did not share one HLA molecule, we did not determine the alloreactivity in detail. The CMV-pp65/HLA-B7 specific CD8 T-cell clone exhibited alloreactivity against two HLA-DRB1*0801 and one HLA-DRB1*0806 expressing EBV-LCLs present in the panel. The cross-reactivity exerted by this CD8 T-cell clone could be blocked with antibodies directed against HLA class II and HLA-DR and not by HLA class I, HLA-DQ or HLA-DP antibodies, confirming that the cross-reactivity of this virus specific CD8 T-cell clone was directed against HLA-DR8. The ability of CD8 T-cells to cross-react against allo-HLA class II molecules was also demonstrated by the CMV-pp65 specific HLA-B35 restricted CD8 T-cell clone that was shown to be cross-reactive against HLA-DRB1*0401 (table 3). As previously described[22], we also observed that EBV-EBNA3A/HLA-B8 specific T-cell clone exhibited alloreactivity against

all EBV-LCLs expressing HLA-B*4402. Furthermore, we observed alloreactivity against EBV-LCLs expressing HLA-B*5501 as was recently described by us.[33] Allo-HLA reactivity against HLA-B*4402 and HLA-B*5501 was confirmed by recognition of K562 transfected with either HLA-B*4402 or HLA-B*5501 by this T-cell clone (figure 2E).

In addition to CD8+ T-cell lines and clones we also analyzed the cross-reactive potential of CD4+ T-cell clones to allo-HLA molecules. The alloreactivity of a Flu-HA/HLA-DR4 specific T-cell clone demonstrated to be directed against HLA-DRB1*1301. As shown in figure 2F both HLA-DRB1*1301 positive EBV-LCLs present in the panel were efficiently recognized by this T-cell clone. Allo-HLA-DRB1*1301 reactivity of the T-cell clone could be confirmed since the T-cells recognized EBV-LCLs transduced with HLA-DRB1*1301 whereas non-transduced EBV-LCLs were not recognized. In addition, allo-HLA reactivity was demonstrated for two other CD4+ T-cell clones of the in total 5 CD4+ T-cell clones tested, indicating that virus specific CD4+ T-cells also exert allo-HLA reactivity (table 3).

For 5 of the 18 allo-HLA reactive virus specific T-cell clones the recognized HLA molecules could not be determined since the recognized EBV-LCLs did not share one particular allo-HLA molecule. The alloreactivity of one of these clones, CMV-pp65 / HLA-A2 specific clone HRN 3, is shown in figure 2G. We hypothesize that this recognition is mediated by the recognition of several HLA molecules since the reactivity could be blocked by mAb specific for HLA class I.

The results of the allo-HLA reactivity exerted by the virus specific T-cell clones are summarized in table 3, and demonstrate that approximately 45% of the virus specific memory CD4 and CD8 T-cell clones exhibit cross-reactivity to allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was primarily directed against HLA class I and II, respectively. However, cross-reactivity of CD8 T-cells directed against HLA class II was also observed.

Different allo-HLA recognition by T-cell clones with the same specificity but different TCR usage

Burrows et al. showed that EBV-EBNA3A specific HLA-B8 restricted T-cell clones, derived from different HLA-B44 negative individuals were all alloreactive against HLA-B44.[22;23] It could therefore be suggested that allo-HLA reactivity of virus specific T-cells can be predicted. The EBV-EBNA3A response in HLA-B8+, HLA-B44- individuals is, however, a very homogeneous response in which all T-cells express an almost identical public TCR.[34] This is in contrast with most anti-virus responses, which are usually oligoclonal and different between individuals[16;35] (table 3). To assess whether virus specific T-cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, we tested the alloreactivity of three T-cell clones derived from the same individual, all specific for a peptide of the IE62 protein of VZV presented in HLA-A*0201 but with different TCR usage. As demonstrated in figure 3 A, B and C all 3 VZV specific T-cell clones recognized different allo-HLA molecules. Clone 5 showed alloreactivity against HLA-B*5501, clone 6 was alloreactive against HLA-B*5701 and clone 7 exhibited allo-HLA-A*0205 and allo-HLA-A*0207 reactivity. The recognition of the

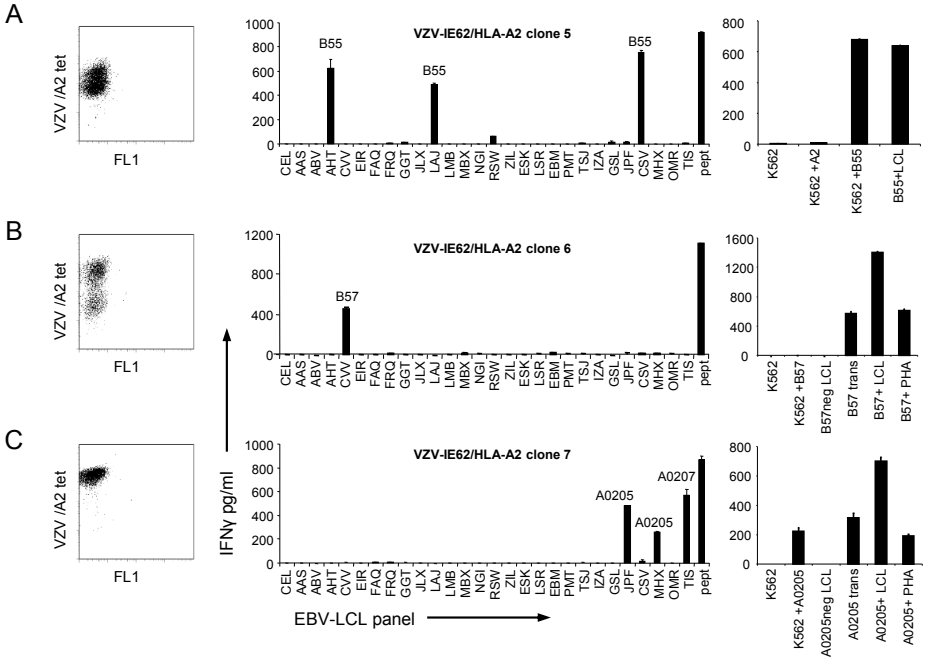


Figure 3. Variable allo-HLA recognition by T-cell clones with the same specificity but different TCR Vβ usage.

To investigate whether virus specific T-cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, three VZV-IE62/HLA-A2 specific T-cell clones expressing different TCRs were stimulated for 18h with a panel of EBV-LCLs and IFNγ production was measured by ELISA. **(A)** VZV clone 5 showed alloreactivity against all HLA-B*5501+ EBV-LCLs. To confirm allo-HLA-B*5501 reactivity, the clone was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-B*5501 (K562+B55) and HLA-B55+ EBV-LCLs (B55+ LCL). **(B)** VZV clone 6 was alloreactive against the HLA-B*5701+ EBV-LCL. Clone 6 did not show reactivity against K562 cells transduced with HLA-B*5701 (K562+B57). To confirm allo-HLA-B*5701 reactivity clone 6 was tested against HLA-B*5701 negative EBV-LCLs (HLA-B57neg LCL) or transduced with HLA-B*5701 (B57 trans), HLA-B*5701+ EBV-LCLs (B57+ LCL) and HLA-B*5701+ PHA blasts (B57+ PHA). **(C)** VZV clone 7 exhibited cross-reactivity against all HLA-A*0205+ and HLA-A*0207+ EBV-LCLs. Allo-HLA-A*0205 reactivity was confirmed by testing the clone against K562 cells (K562), K562 transduced with HLA-HLA-A*0205 (K562+ HLA-A0205), HLA-A*0205 negative EBV-LCLs (HLA-A0205neg LCL) or these EBV-LCLs transduced with HLA-A*0205 (A0205 trans), HLA-A*0205+ EBV-LCLs (A0205+ LCL) and HLA-A*0205+ PHA blasts (A0205+ PHA). The results demonstrate that virus specific T-cells with the same antigen specificity, but with different TCR usage exert alloreactivity against different HLA molecules.

three clones together was comparable to the recognition exerted by the VZV-IE62 specific line derived from the same individual. This confirms that alloreactivity exerted by the virus specific lines shown in figure 1 is the sum of the alloreactivity of the various clonal populations present within the lines. The allo-HLA reactivities of the T-cell clones were confirmed by transduction of the allo-HLA molecules in K562 or in non-recognized EBV-LCLs. Clone 5

and 7 recognized K562 transduced with HLA-B*5501 and HLA-*0205, respectively. Clone 6 was unable to recognize K562 transduced with HLA-B*5701, whereas EBV-LCLs transduced with HLA-B*5701 were recognized. Since this clone also recognized HLA-B*5701 expressing PHA stimulated T-cell blasts, specificity against EBV derived peptide in the context of allo-HLA-B*5701 is excluded. These results demonstrate that virus specific T-cells with the same antigen specificity, but with different TCR complexes can exert alloreactivity against different HLA molecules. Since T-cell responses against viruses are usually oligoclonal and different between individuals these results indicate that allo-HLA reactivity cannot be predicted.

The cytotoxic potential and affinity of the alloreactivity exerted by virus specific T-cells

Since cytotoxicity might be a relevant measure to predict the potency of the virus-specific T-cells to induce GVHD or graft rejection in vivo, we investigated the allo-HLA reactive cytotoxic capacity of the virus specific T-cells. Six virus specific T-cell clones were tested against a panel of EBV-LCLs positive and negative for the recognized allo-HLA molecules. As shown in figure 4A, all six T-cell clones tested showed cytotoxicity against the allo-HLA expressing EBV-LCLs. To investigate whether the affinity of the allo-HLA reactive response was comparable to the affinity of the virus specific response, the kinetics of recognition and antigen threshold were tested for both specificities. For this purpose the allo-HLA-A*3101/A*3001 reactive EBV-EBNA3A/HLA-A3 specific clone 19 was tested against HLA-A*0301+ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against two HLA-A*3101+ EBV-LCLs GGT and DSP at different effector : stimulator ratios. As is shown in figure 4B, the T-cell clone produced comparable amounts of IFNγ against the virus antigen expressing EBV-LCL as against the allo-HLA expressing EBV-LCLs in the different effector : stimulator ratios, indicating that the kinetics of recognition and antigen threshold of the alloreactive response and the virus-specific T-cell response are comparable.

Normal cell subsets are recognized by virus specific T-cells

To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus specific T-cell clones against allo-HLA-expressing B cells, CD40L activated B-cells, T-cells, PHA-blasts, monocytes, monocyte derived DCs and fibroblasts with and without IFNγ pre-treatment. The results shown in figure 4C demonstrate the reactivity of three virus specific T-cell clones directed against the different cell subsets. Pp65/HLA-B*0702 specific clone 11 showed high recognition of HLA-DRB1*0801 positive CD40L activated B-cells and low recognition of B-cells and PHA blasts. VZV-IE62/HLA-A*0201 specific clone 5 showed high recognition of HLA-B*5501 positive CD40L activated B-cells, DCs and PHA blast and low recognition of monocytes and T-cells. This clone could unfortunately not be tested against fibroblasts since HLA-B*5501 positive fibroblasts were not available. VZV-IE62/HLA-A*0201 specific clone 6 highly recognized HLA-B*5701 positive DCs and

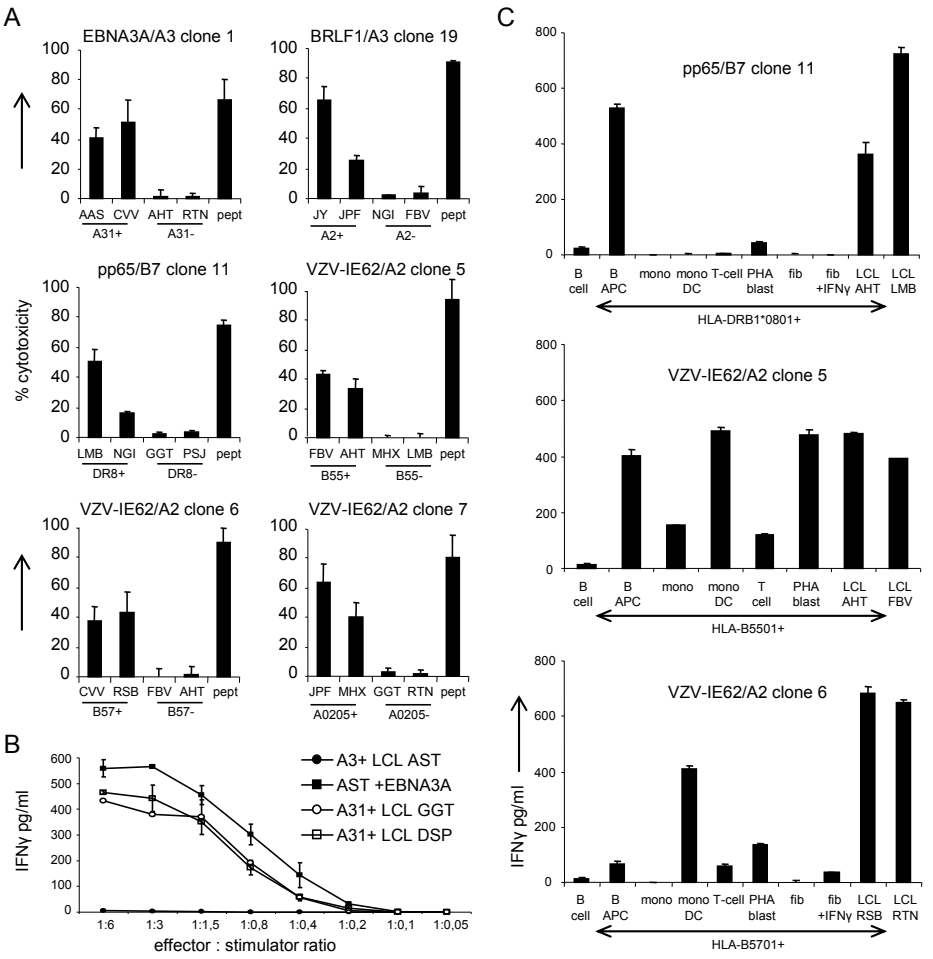


Figure 4. The potency of the alloreactivity exerted by virus specific T-cells.
(A) To investigate the allo-HLA reactive cytotoxic capacity of virus specific T-cells, 6 virus specific cell clones were tested in cytotoxicity assays against 2 EBV-LCLs expressing the recognized allo-HLA molecules and 2 EBV-LCLs negative for the allo-HLA molecules. EBV-LCLs expressing the virus specific restriction molecule were loaded with the viral peptide and used as positive control for cytotoxicity. **(B)** To compare the affinities of the allo-HLA reactive response and the virus specific response, the allo-HLA-A30/A31 reactive EBV-EBNA3A/HLA-A3 specific clone 19 was tested against the HLA-A*0301+ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against two HLA-A*3101+ EBV-LCLs GGT and DSP. To compare the kinetics of the two responses, the clone was tested against the EBV-LCLs in different effector : stimulator ratios. **(C)** To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus specific T-cell clones against allo-HLA-expressing B cells, CD40 ligand activated B-cells (B APC), monocytes, monocyte derived DCs, T-cells, PHA-blasts and fibroblasts with and without IFNγ pre-treatment.

showed low reactivity against CD40L activated B-cells, T-cells, PHA blasts and IFNγ stimulated fibroblasts. These results indicate that virus specific T-cells can also be reactive against in vivo relevant normal cell subsets.

One TCR complex mediates both virus specificity and allo-HLA reactivity

Since alloreactivity mediated by T-cells may be explained by T-cells expressing 2 TCR complexes at the cell surface,[36;37] we wanted to exclude that the allo-HLA reactivity was mediated via another TCR than the virus specific TCR. For this purpose we determined the TCR usage of two representative allo-HLA reactive virus specific clones, the allo-HLA-A*0201 reactive BRLF1/HLA-A*0301 specific clone 19 (figure 2C) and the allo-HLA-A*0205 reactive VZV-IE62/HLA-A*0201 specific clone 7 (figure 3C). By RT–PCR, we established that the BRLF1/HLA-A*0301 specific clone 19 expressed one TCRβ gene transcript, BV7S2, and two TCRα transcripts, AV12S1 and AV18S1. However, one of the TCRα chains, AV12S1, contained a stopcodon in the CDR3 region, indicating that this TCRα was not expressed. Flow cytometric analysis confirmed that 100% of the T-cells expressed TCR BV7S2 at the cell surface (data not shown). No antibodies were available for analysis of the specific TCRα chain expression at the cell surface. To investigate whether the BV7S2 and the AV18S1 mediated the dual recognition, IE1/HLA-A1 specific T-cells were transduced with retroviral vectors encoding for these TCR α

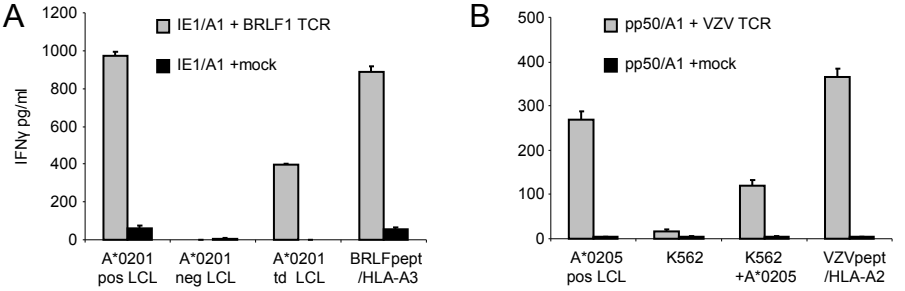


Figure 5. One TCR complex mediates both virus specificity and allo-HLA reactivity.
To exclude that allo-HLA reactivity was mediated via another TCR than the virus specific TCR, the TCR of two representative clones was transferred to T-cells with a different specificity. **(A)** IE1/A1 specific T-cells transduced with viral vectors encoding for the TCR of BRLF1/A3 specific clone 19 (IE1/A1 +BRLF1 TCR) and IE1/A1 specific T-cells transduced with a mock viral vector (IE1/A1 +mock) were tested for allo-HLA-A*0201 reactivity against HLA-A*0201 positive EBV-LCLs (A*0201 pos LCLs), HLA-A*0201 negative EBV-LCLs (A*0201 neg LCLs) and HLA-A*0201 negative EBV-LCLs transduced with HLA-A*0201 (A*0201 td LCLs) and for BRLF1 specificity against BRLF1 peptide loaded HLA-A*0301 positive EBV-LCLs (BRLF1pept /HLA-A3). **(B)** Pp50/A1 specific T-cells transduced with viral vectors encoding for the TCR of VZV clone 7 (pp50/A1 + VZV TCR) and pp50 specific T-cells transduced with a mock viral vector (pp50/A1 +mock) were tested for allo-HLA-A*0205 reactivity against HLA-A*0205 positive EBV-LCLs (A*0205 pos LCLs), K562 cells (K562), and K562 cells transduced with HLA-A*0205 (K562 +A*0205) and for VZV specificity against VZV-IE62 peptide loaded HLA-A*0201 positive EBV-LCLs (VZVpept /HLA-A2). The results demonstrate that virus specificity and allo-HLA reactivity exerted by virus specific T-cells were mediated by one TCR complex.

and β chains. The results shown in figure 5A demonstrate that the BRLF1-TCR transduced T-cells exerted reactivity against HLA-A*0201 expressing target cells cells as well as against EBV-BRFL1 peptide loaded HLA-A*0301+ target cells. No reactivity directed against peptide loaded or HLA-A*0201 expressing target cells was observed with mock transduced T-cells.
The VZV-IE62/HLA-A*0201 clone 7 expressed one TCRα transcript, AV6S1, and one TCRβ transcript, BV21S3. Pp50/A1 specific T-cells were transduced with retroviral vectors encoding for the VZV TCR chains. The results shown in figure 5B demonstrate that the VZV-TCR transduced T-cells exerted reactivity against HLA-A*0205 expressing target cells as well as against VZV-IE62 peptide loaded HLA-A*0201+ target cells. No reactivity directed against peptide loaded or HLA-A*0205 expressing target cells was observed with mock transduced T-cells. These results demonstrate that the virus specificity and the allo-HLA reactivity exerted by these virus specific T-cells were mediated via one TCR complex.

DISCUSSION

In this study, we demonstrated that a high percentage of virus specific memory T-cells exhibit cross-reactivity against allogeneic HLA molecules. CD8 as well as CD4 virus specific memory T-cells demonstrated to have allo-HLA reactive potential. In addition, we determined that the alloreactivity exerted by CD8 T-cells was directed against either HLA class I or HLA class II molecules, and that the alloreactivity of the T-cells was mediated by cytotoxicity and cytokine production. Furthermore, we demonstrate that virus specific T-cells can exert allo-HLA reactivity against normal cell subsets, indicating the potential clinical relevance of the response. By TCR transfer we confirmed that the allo-HLA reactivity and virus specificity were mediated via the same TCR.
Most virus specific T-cell lines and 45% of virus specific T-cell clones directed against EBV, CMV, VZV and FLU, exerted alloreactivity when tested against a panel of EBV-LCLs covering almost all common HLA molecules. The cross-reactivity exerted by the virus specific T-cells was confirmed to be based on allo-HLA recognition by testing the T-cell clones against K562 cells and EBV-LCLs transduced with single HLA molecules. Some of the alloreactive virus specific T-cell clones did not recognize K562 cells transduced with the specific allo-HLA molecules, but showed reactivity against EBV-LCLs transduced with the allo-HLA molecules. These data support the previous findings that allo-HLA reactivity is dependent on endogenous peptide.[38] Allo-HLA cross-reactivity was not only directed against EBV-LCLs, but also against PHA stimulated T-cells (figure 3B) indicating that the peptides responsible for allo-HLA reactivity were not EBV derived. Differential recognition of HLA transduced K562 cells and EBV-LCLs may indicate recognition of tissue specific peptides in allo-HLA molecules. Therefore it may be possible that we even underestimated the allo-HLA reactive repertoire of T-cells by initially screening only against an EBV-LCL panel.

Burrows et al. showed that EBV-EBNA3A specific HLA-B8 restricted T-cells derived from different HLA-B44 negative individuals all exert cross-reactivity against allo-HLA-B44[22;23]. Based on these finding it could be suggested that the allo-HLA reactivity of virus specific T-cells can be predicted. The EBV-EBNA3A specific T-cells however express an almost identical public TCR in all HLA-B8+ HLA-B44- individuals,[34] whereas most other virus responses are oligoclonal and the TCR usage of T-cells directed against the same viral epitope is variable between individuals[16;35] (table 3). We have demonstrated that virus specific T-cells with the same antigen specificity, but expressing different TCRs, exhibit cross-reactivity against different HLA molecules (figure 3). In addition, we have shown that three specific T-cell lines with the same specificity for CMV-pp50, but derived from different individuals, exerted a very variable pattern of allo-HLA reactivity ranging from no allo-HLA reactivity to very broad alloreactivity (figure 1). These results together illustrate that the cross-reactive potential of antigen specific T-cells against allo-HLA molecules is difficult to predict.

The alloreactivity exerted by the virus specific memory CD8 and CD4 T-cells was primarily directed against allo-HLA class I and II molecules, respectively, suggesting that the coreceptors expressed by the T-cells contributed to the affinity of the allo-HLA reactivity. However, we also demonstrated allo-HLA class II recognition by a small proportion of antigen specific CD8 T-cells, as was also shown recently by Rist et al.[39] HLA class II allorecognition by CD8+ T-cells could indicate an HLA class II-TCR interaction that is independent of CD8 co-receptor binding. It is however also possible that CD8 co-receptors bind to HLA class I molecules expressed on the target cell and thereby strengthen the TCR-HLA class II interaction, as was previously shown.[27] Although we did not observe HLA class I cross-reactive CD4 T-cells, only a limited number of CD4 T-cell clones were tested, and therefore we cannot exclude that CD4 T-cells may also cross-react with HLA class I complexes.

The results of our study illustrate that approximately 45% of all T-cells exert allo-HLA cross-reactivity. However, since T-cells were only analyzed for allo-HLA cross-reactivity against an EBV-LCL panel expressing most common HLA molecules, missing all infrequent HLA molecules as well as all tissue specific peptides presented in allo-HLA molecules, we speculate that virtually all T-cells may be allo-HLA reactive. Based on this assumption and the fact that the TCR repertoire of humans is highly diverse, after HLA mismatched transplantations sufficient allo-mismatched-HLA cross-reactive T-cells are likely to be present to induce acute GVHD or graft rejection. However, HLA mismatched stem cell transplantation or solid organ transplantations do not always lead to acute GVHD or graft rejection, indicating that other factors must be involved in these transplantation related complications. A range of acute viral infections have been linked to initiating GVHD and graft rejection following transplantation,[26] suggesting that virus specific T-cells may be mediators of GVHD and graft rejection. Since virus specific T-cell responses usually have a restricted TCR usage, high numbers of T-cells expressing an identical TCR can be found. In addition, herpes virus specific T-cell populations can remain present at high percentages for long periods of time in healthy individuals as

well as in patients,[18-21] and viral infections, leading to expansion of virus specific T-cells, are very common after HLA mismatched SCT or solid organ transplantation.[40-43] Therefore, given the high proportion of virus specific T-cells and the less stringent requirements for activation of memory T-cells,[12;13] it is tempting to speculate that if the HLA type of patient or the transplanted organ matches the cross-reactivity of the virus specific T-cells, these allo-HLA reactive virus specific memory T-cells may easily induce GVHD or graft rejection.

The ability of virus specific T-cells to exert allo-HLA reactivity might also have implications for the clinical applicability of virus specific T-cell lines. Because immune deficiency for viruses is a common complication after SCT, broad administration of virus specific T-cells lines over HLA barriers to SCT patients has been proposed.(44,45) The results of this study demonstrate that administration of virus specific T-cells over HLA barriers may increase risk of GVHD, and indicate that virus specific lines should be tested for alloreactivity against the patient before administration.

Based on our results we postulate that virtually all antigen specific T-cells will be cross-reactive against allo-HLA class I or II molecules. The high alloreactive potential of particularly virus specific memory T-cells might have important clinical implications in transplantation settings.

REFERENCE LIST

1. Lindahl KF, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J Exp Med* 1977; 145(3):508-522.
2. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol* 2001; 166(2):973-981.
3. Brondz BD, Snegirova AE. Interaction of immune lymphocytes with the mixtures of target cells possessing selected specificities of the H-2 immunizing allele. *Immunology* 1971; 20(4):457-468.
4. Brondz BD, Egorov IK, Drizlikh GI. Private specificities of H-2K and H-2D loci as possible selective targets for effector lymphocytes in cell-mediated immunity. *J Exp Med* 1975; 141(1):11-26.
5. Jones G. The number of reactive cells in mouse lymphocyte cultures stimulated by phytohemagglutinin, concanavalin A or histocompatibility antigen. *J Immunol* 1973; 111(3):914-920.
6. Hansen JA, Lee TD, Whitsett CF, Dupont B. Specific blocking of MLR-stimulating determinants (HLA-D) with B-cell alloantisera: preabsorption of antibody on stimulator cells. *Transplant Proc* 1977; 9(4):1721-1726.
7. Kalil J, Wollman EE. Role of class I and class II antigens in the allogenic stimulation: class I and class II recognition in allogenic stimulation; blocking of MLR by monoclonal antibodies and F(ab')2 fragments. *Cell Immunol* 1983; 79(2):367-373.
8. Nieda M, Juji T, Imao S. Allogeneic suppressive effects of pregnancy sera on monocytes of responding cells in human mixed lymphocyte reactions. *J Clin Invest* 1985; 76(4):1477-1484.
9. Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* 2001; 1(1):31-40.
10. Anderson G, Jenkinson WE, Jones T, Parnell SM, Kinsella FA, White AJ et al. Establishment and functioning of intrathymic microenvironments. *Immunol Rev* 2006; 209:10-27.
11. Macedo C, Orkis EA, Popescu I, Elinoff BD, Zeevi A, Shapiro R et al. Contribution of naive and memory T-cell populations to the human alloimmune response. *Am J Transplant* 2009; 9(9):2057-2066.
12. Chandok MR, Farber DL. Signaling control of memory T cell generation and function. *Semin Immunol* 2004; 16(5):285-293.
13. Farber DL, Ahmadzadeh M. Dissecting the complexity of the memory T cell response. *Immunol Res* 2002; 25(3):247-259.
14. Sourdive DJ, Murali-Krishna K, Altman JD, Zajac AJ, Whitmire JK, Pannetier C et al. Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. *J Exp Med* 1998; 188(1):71-82.
15. Trautmann L, Rimbert M, Echasserieau K, Saulquin X, Neveu B, Dechanet J et al. Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses. *J Immunol* 2005; 175(9):6123-6132.
16. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol* 2006; 6(12):883-894.
17. van Leeuwen EM, Remmerswaal EB, Heemskerk MH, ten Berge IJ, van Lier RA. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. *Blood* 2006; 108(9):3121-3127.
18. Hislop AD, Annels NE, Gudgeon NH, Leese AM, Rickinson AB. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med* 2002; 195(7):893-905.

19. Levitsky V, de Campos-Lima PO, Frisan T, Masucci MG. The clonal composition of a peptide-specific oligoclonal CTL repertoire selected in response to persistent EBV infection is stable over time. *J Immunol* 1998; 161(2):594-601.
20. Maecker HT, Maino VC. Analyzing T-cell responses to cytomegalovirus by cytokine flow cytometry. *Hum Immunol* 2004; 65(5):493-499.
21. Benninger-Doring G, Pepperl S, Deml L, Modrow S, Wolf H, Jilg W. Frequency of CD8(+) T lymphocytes specific for lytic and latent antigens of Epstein-Barr virus in healthy virus carriers. *Virology* 1999; 264(2):289-297.
22. Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J Exp Med* 1994; 179(4):1155-1161.
23. Burrows SR, Silins SL, Moss DJ, Khanna R, Misko IS, Argaet VP. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J Exp Med* 1995; 182(6):1703-1715.
24. Koelle DM, Chen HB, McClurkan CM, Petersdorf EW. Herpes simplex virus type 2-specific CD8 cytotoxic T lymphocyte cross-reactivity against prevalent HLA class I alleles. *Blood* 2002; 99(10):3844-3847.
25. Umetsu DT, Yunis EJ, Matsui Y, Jabara HH, Geha RS. HLA-DR-4-associated alloreactivity of an HLA-DR-3-restricted human tetanus toxoid-specific T cell clone: inhibition of both reactivities by an alloantiserum. *Eur J Immunol* 1985; 15(4):356-361.
26. Cainelli F, Vento S. Infections and solid organ transplant rejection: a cause-and-effect relationship? *Lancet Infect Dis* 2002; 2(9):539-549.
27. Heemskerk MH, de Paus RA, Lurvink EG, Koning F, Mulder A, Willemze R 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(12):6806-6811.
28. Zoet YM, Eijsink C, Kardol MJ, Franke-van Dijk ME, Wilson GL, de PR et al. The single antigen expressing lines (SALs) concept: an excellent tool for screening for HLA-specific antibodies. *Hum Immunol* 2005; 66(5):519-525.
29. Hoogendoorn M, Olde WJ, Smit WM, Schaafsma MR, Jedema I, Barge RM et al. Primary allogeneic T-cell responses against mantle cell lymphoma antigen-presenting cells for adoptive immunotherapy after stem cell transplantation. *Clin Cancer Res* 2005; 11(14):5310-5318.
30. Stumpf AN, van der Meijden ED, van Bergen CA, Willemze R, Falkenburg JH, Griffioen M. Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity. *Blood* 2009; 114(17):3684-3692.
31. Burrows SR, Kienzle N, Winterhalter A, Bharadwaj M, Altman JD, Brooks A. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. *J Immunol* 2000; 165(11):6229-6234.
32. van der Veken, Hagedoorn RS, van Loenen MM, Willemze R, Falkenburg JH, Heemskerk MH. Alphabeta T-cell receptor engineered gammadelta T cells mediate effective antileukemic reactivity. *Cancer Res* 2006; 66(6):3331-3337.
33. D'Orsogna LJ, Amir AL, Zoet YM, van der Meer-Prins PM, van der Slik AR, Kester MG et al. New tools to monitor the impact of viral infection on the alloreactive T-cell repertoire. *Tissue Antigens* 2009; 74(4):290-297.
34. Argaet VP, Schmidt CW, Burrows SR, Silins SL, Kurilla MG, Doolan DL et al. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. *J Exp Med* 1994; 180(6):2335-2340.

35. Wynn KK, Fulton Z, Cooper L, Silins SL, Gras S, Archbold JK et al. Impact of clonal competition for peptide-MHC complexes on the CD8+ T-cell repertoire selection in a persistent viral infection. *Blood* 2008; 111(8):4283-4292.
36. Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science* 1993; 262(5132):422-424.
37. Morris GP, Allen PM. Cutting edge: Highly alloreactive dual TCR T cells play a dominant role in graft-versus-host disease. *J Immunol* 2009; 182(11):6639-6643.
38. Felix NJ, Donermeyer DL, Horvath S, Walters JJ, Gross ML, Suri A et al. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat Immunol* 2007; 8(4):388-397.
39. Rist M, Smith C, Bell MJ, Burrows SR, Khanna R. Cross-recognition of HLA DR4 alloantigen by virus-specific CD8+ T cells: a new paradigm for self-/nonself-recognition. *Blood* 2009; 114(11):2244-2253.
40. Fischer SA. Emerging viruses in transplantation: there is more to infection after transplant than CMV and EBV. *Transplantation* 2008; 86(10):1327-1339.
41. Fisher RA. Cytomegalovirus infection and disease in the new era of immunosuppression following solid organ transplantation. *Transpl Infect Dis* 2009; 11(3):195-202.
42. Fishman JA. Infection in solid-organ transplant recipients. *N Engl J Med* 2007; 357(25):2601-2614.
43. Gratwohl A, Brand R, Apperley J, Biezen AA, Bandini G, Devergie A et al. Graft-versus-host disease and outcome in HLA-identical sibling transplantations for chronic myeloid leukemia. *Blood* 2002; 100(12):3877-3886.
44. Haque T, Wilkie GM, Taylor C, Amlot PL, Murad P, Iley A et al. Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells. *Lancet* 2002; 360(9331):436-442.
45. Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood* 2007; 110(4):1123-1131.