HLA alloreactivity by human viral specific memory T-cells

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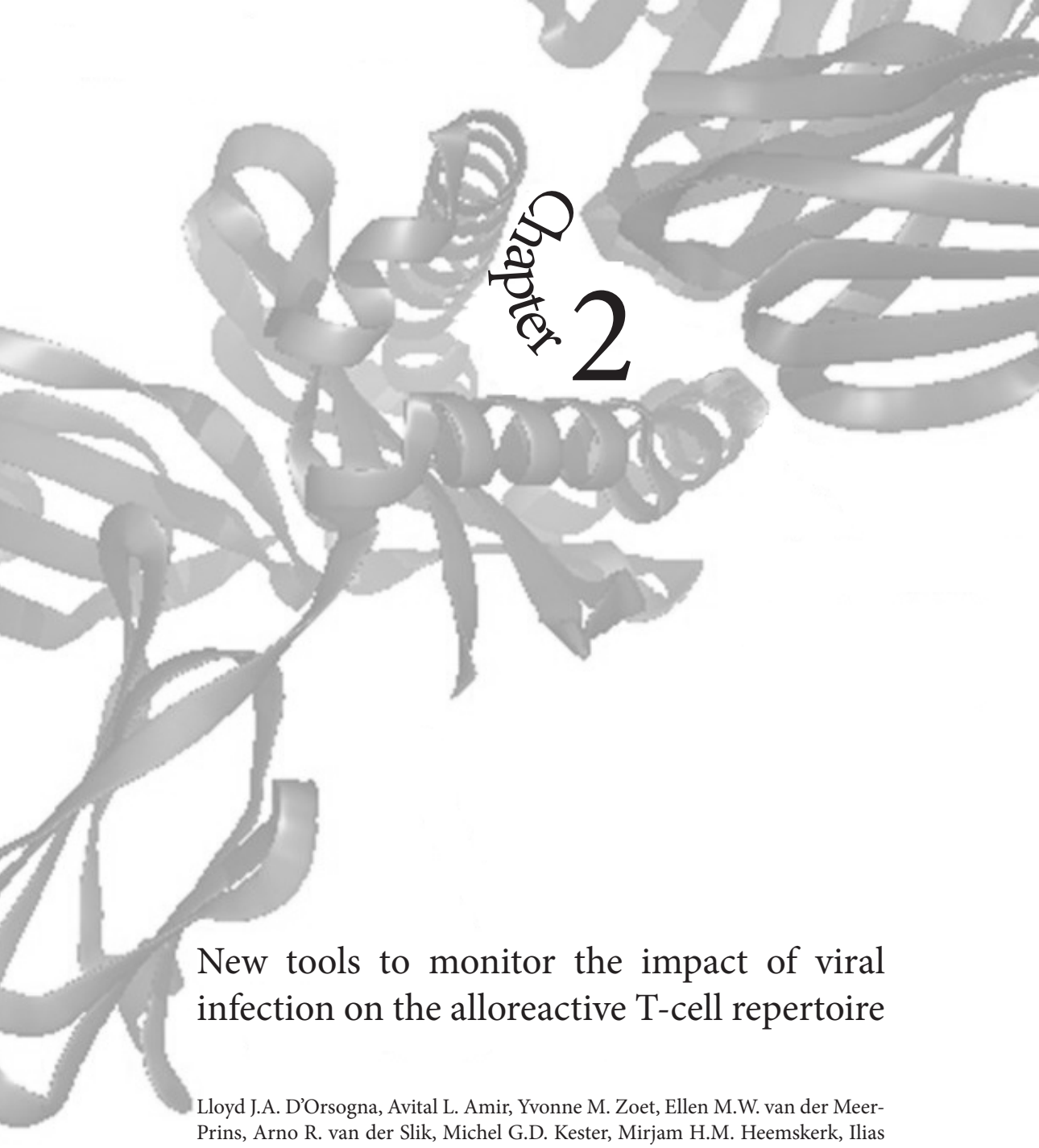

Chapter 2

New tools to monitor the impact of viral infection on the alloreactive T-cell repertoire

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Tissue Antigens 2009; 74: 290-297

ABSTRACT

Accumulating evidence suggests that alloreactive memory T-cells may be generated as a result of viral infection. So far a suitable tool to define the individual HLA cross-reactivity of virus-specific memory T-cells is not available. We therefore aimed to develop a novel system for the detection of cross-reactive alloresponses using single HLA antigen expressing cell lines (SALs) as stimulator. Herein we generated EBV EBNA-3A specific CD8 memory T-cell clones (HLA-B*08:01/FLRGRAYGL peptide restricted) and assayed for alloreactivity against a panel of SALs, using IFNγ Elispot as readout. Generation of the T-cell clones was performed by single cell sorting, based on staining with viral peptide/MHC complex specific tetramer. Monoclonality of the T-cell clones was confirmed by TCR PCR analysis. Firstly, we confirmed the previously described alloreactivity of the EBV EBNA3A specific T-cell clones against SAL expressing HLA B*44:02. Further screening against the entire panel of SALs also revealed additional cross-reactivity against SAL expressing HLA B*55:01. Functionality of the cross-reactive T cell clones was confirmed by chromium release assay using PHA blasts as targets. SALs are an effective tool to detect cross-reactivity of viral specific CD8 memory T-cell clones, against individual class I HLA molecules. This technique may have important implications for donor selection and monitoring of transplant recipients.

INTRODUCTION

Previous immunological exposures and resultant T cell memory can influence the course of future immune responses to unrelated pathogens (1,2). Less is known about the effect of an individuals immune history on the response to an allogeneic tissue transplant. However the presence of memory alloreactive T cells in humans who have never been exposed to alloantigens has been attributed to past viral infections (3-5). It is hypothesized that these viral specific memory T-cells are able to recognize cross-reactive allogeneic MHC with lower affinity because of lower activation thresholds (4). However, a reproducible in-vitro system for the detection of cross-reactive alloresponses from viral specific T-cells is currently not available.

Burrows et al have shown that the cytotoxic T-lymphocyte (CTL) response against the human HLA-B*44:02 alloantigen may actually be due to cross-reactivity against a previously primed viral antigen (3). Limiting dilution analysis of the alloresponse to HLA-B*44:02 in eight healthy individuals revealed that HLA-B*08:01, EBV seropositive donors had significantly higher CTL precursor frequencies for alloantigen HLA-B*44:02 than HLA-B8 positive, EBV seronegative control donors (3). The cytotoxic T-cell response against the immunodominant EBV peptide FLRGRAYGL presented on the HLA-B*0801 molecule also recognized the HLA-B*44:02 molecule (presumably presenting a self-peptide) to which the T-cells had never been exposed.

This study of Burrows demonstrates that the allospecific T-cell repertoire overlaps with the repertoire which recognizes a single viral epitope in the context of self-MHC. This theory is also supported by other groups that have reported similar cross-reactivity between environmental pathogens and allogeneic MHC molecules (6-9). Although the frequency of naïve T-cells available to respond to any given pathogen is relatively small (approx. 1:200,000), the proportion of memory T cells that can directly recognize foreign MHC represents a substantial fraction of the total T-cell repertoire (10,11). Analysis of cloned T-cell populations has demonstrated that between 20-60% of antigen specific, MHC restricted T-cell clones crossreact with alloantigens (12-13). It has also been shown that approximately half of a "primary" alloresponse is contributed by previously primed MHC-restricted T-cells (14-15).

Therefore accumulating evidence suggests that the CD8 T-cell alloresponse could, at least in part, result from molecular mimicry by an environmental antigen which induces an alloreactive memory T-cell response (3-5,8,16). It is therefore not surprising that increased alloreactivity is found following viral infection in experimental models (2, 17-19). These cross-reactive memory T-cell responses not only affect allograft survival but also prevent the induction of transplantation tolerance (4,20).

Human memory CD8 T-cells can be defined based on phenotypic and functional characteristics (21). Memory CD8 T-cells express CD8, CD45Ro, CD27, CD28, CD11a, CD49d, CD95 and can secrete IL-2, IL-4, IFNγ and TNFα. This memory subset contains virus-specific cytotoxic T lymphocyte (CTL) precursors that can have cytotoxic function including expression of perforin and granzyme B. Memory CD8 T-cells have less stringent requirements for activation, with a reduced requirement for co-stimulation, and have the potential to secrete a more

extensive array of cytokines (22-24). As primed (cross-reactive) memory T-cells may have lower activation thresholds than their naive counterparts, their presence before transplantation may increase the risk of a poor outcome of an allograft.

In our laboratory, cell lines have been established expressing a single MHC class I antigen on the cell surface. These cells, named single HLA antigen lines (SAL's), have originally been developed for humoral tests (25,26), as their expression of a single HLA antigen, instead of the 3-6 of usual peripheral blood lymphocytes, facilitates the definition of HLA antibody specificities in patients sera. Similarly the use of a SAL as target will allow the determination of the exact HLA specificity of the alloreactive T-cells.

The purpose of this study was to develop a reproducible in-vitro system for the detection of CD8 T-cell cross-reactive alloresponses by viral specific CD8 T-cell clones. We used EBNA3A specific CD8 memory T-cell clones to confirm the previously described cross-reactive alloresponse against HLA-B*44:02 and check whether additional crossreactivities can be observed using a panel of different SALs as stimulators. SALs proved to be the basis of an effective screening system for heterologous alloreactivity that lead to the definition of additional cross-reactive HLA-alloantigens.

METHODS

Generation of viral specific CD8 memory T-cell clones
EBNA3A-FLR/B8 CD8 T-cell clones were derived from healthy donor (x.x0116x) with HLA typing HLA-A*01:01,02:01; B*08:01,-; DRβ1*03:01,-. HLA-B8/FLR tetramer positive CD8 T-cells accounted for 1.7% of the peripheral blood CD8 T-cells (Figure 1a). The EBV-specific T cells were isolated from the peripheral blood as previously described (27). Briefly, PBMCs were harvested and labeled with HLA-B8/FLR tetrameric complexes for 30 minutes at 4 0C in RPMI without phenol, supplemented with 2% FCS, washed three times and single cell sorted at 4 0C using the FACS vantageTM (Becton Dickinson). Tetramer+ CD8 T-cells were non-specifically stimulated every 2 weeks with feeder cell mixture containing irradiated allogeneic PBMCs (3500 Rad), irradiated EBV transformed B-cells (5000 Rad), 800ng/ml phytohaemagglutinin (PHA), 100 IU/ml IL-2 in IMDM medium supplemented with glutamine, human serum (5%) and fetal calf serum (5%). Multiple clones for testing were generated from the same healthy donor.

Confirmation of T-cell clonality
TCRα and TCRβ rearrangements were analyzed on 4 separate EBNA-3A T-cell clones.
Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Oligo dT primed first-strand cDNA was synthesized from 1 μg RNA template using AMV reverse transcriptase (Promega, Madison, WI, USA). First RT-PCR was performed to determine the TCR AV and BV usage, using primers that cover the complete TCR repertoire. Sequencing templates were obtained performing high fidelity PCR using Pfx50 DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Each reaction contained forward primers targeting the

Va4S1 or Vb6S2 variable region and reverse primers specific for the alpha and beta chain constant region. Amplicons spanning the variable, CDR3 and joining regions were purified using illustra S-400 HR microspin columns (GE Healthcare, Buckinghamshire, UK) according to manufacturers' protocol. Thermo sequenase primer cycle sequencing (GE healthcare) reactions were performed using a CY5 labeled m13 sequencing primer (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturers' protocol. Sequencing reactions were run on an ALFexpress DNA sequencer (GE healthcare), and analyzed with sequence analyser 2.10 software (GE healthcare).

Generation of single HLA antigen expressing cell lines (SALs)
Plasmid constructs (pLNCX, ampicillin and neomycin resistant) containing various MHC class I heavy chain genes were obtained from the 13th International Histocompatability Working Group and were transfected in K562 cells, obtained from the American Type Culture Collection (Manassas, VA, order number CLL-243) (28) by electroporation using the Genepulser (Biorad, Hercules, CA) with instrument settings of 230V and 960μF. Electroporation was performed with 107 cells and 10μg of plasmid DNA. On day 2 after transfection, selection was started with G418 (neomycin derivative, final concentration: 200μg/ml; Invitrogen, Groningen, the Netherlands). The antibiotic-resistant transfectants were expanded for at least two weeks. Major histocompatability complex class I positive cells were enriched by cell sorting using w6/32 coated antimouse immunoglobulin (Ig) magnetic beads (Dynal, Oslo, Norway). Sorted cells were expanded using G418, tested for class I expression with HLA specific monoclonal antibodies (25,26) and cryopreserved in multiple aliquots. The full list of available transfected SAL cells is available in reference 25.

Elispot
Ninety six well ELISPOT plates (NUNC) were coated with capture antibody for IFNγ (Mab 1-D1K – Mabtech) in PBS overnight at 4 0C. The plates were then washed with PBS three times. 10000 responder EBNA-3A T-cell clone were added to each well in 100μL of IMDM supplemented with 10% FCS (without IL-2), together with 1.105 stimulator SALs (non-irradiated). Control wells contained responder EBNA-3A specific T-cell clone with medium, non-transfected K562 cells or FLRGRAYGL peptide (10μg/ml positive control). The plates were washed after 24 hours and biotinylated detection antibody (Mab 7-B6-biotin – Mabtech) was added to the wells for 2 hours at room temperature, followed by further washing step. Extravidin Alkaline Phosphatase conjugate (E2636 - Sigma) was then added for 1 hour at room temperature and plates were washed again. The spots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT plus B-5655 – Mabtech) and counted using a computer assisted ELISPOT image analyzer Immunospot.

Chromium release assay and generation of PHA blasts
EBNA-3A specific CD8 T-cell clones were evaluated for cytotoxicity by incubating 5000 PHA blast target cells with serial dilutions of the T-cell clone for 4 hours in a chromium release assay. PHA blasts were generated by stimulating PBMC with PHA (800ng/ml) and IL-2 (150IU/ml) for 7 days (Growth medium 15% human serum/RPMI), and were incubated with chromium for 60 minutes. Supernatants were harvested for gamma counting: *percent specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.* An

inhibition assay was also performed with and without the presence of HLA-B8/FLR tetramer or control HLA-B35/IPS tetramer (1μg/ml). Results are expressed as the mean of triplicate samples.

Statistics
Values for Elispot and specific lysis are presented as the mean of triplicate wells, with standard deviation. Comparative analyses are non-parametric (unpaired) t-tests, $p<0.05$ is considered to be significant. Statistics are derived using Graph Pad Prism 4 for Windows (Version 4.02, 2004).

RESULTS

Confirmation of monoclonality and TCR repertoire analyses of the EBNA3A-FLR/B8 specific CD8 T-cell clones
EBNA3A-FLR/B8 CD8 T-cell clones were all confirmed to bind viral peptide/HLA-B8 tetramer complexes (Figure 1b). Burrows et al have reported that persistent EBV infection in a HLA B*08:01 positive, B*44 negative individual gives rise to a public AV4S1, BV6S2 TCR (29). We therefore performed RT-PCR and sequencing to determine the TCR usage of the EBNA3A specific CD8 T-cell clones we have isolated. As shown in table 1, all clones analyzed

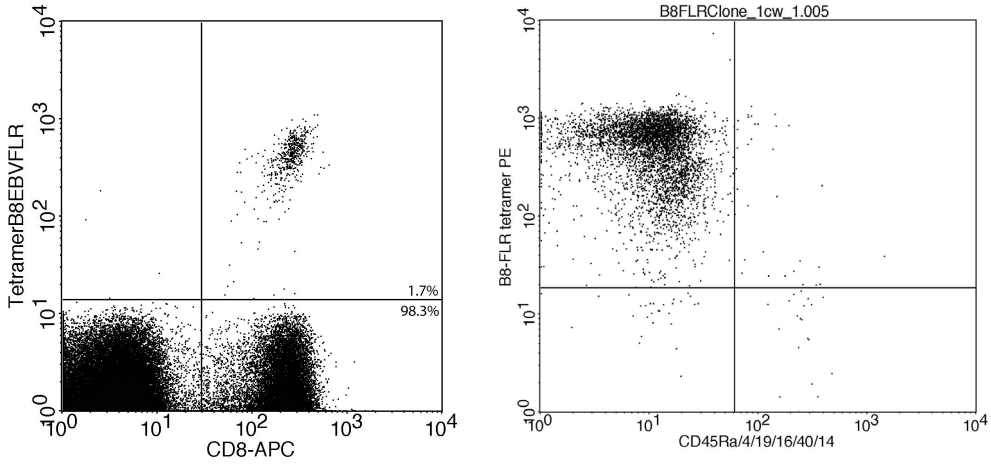

Figure 1. EBNA-3A CD8 memory T-cell clone.
Generation of the T-cell clone was performed by single cell sorting based on HLA-B*0801/FLRGRAYGL specific tetramer staining. (a): HLA-B8/FLR specific T-cells amounted to 1.7% of peripheral CD8 T-cells in the healthy donor from whom the EBNA3A-FLR/B8 T-cell clone was sorted. (b): T-cell clone is >99% HLA-B8/FLR tetramer binding and clonality was confirmed with TCR PCR (table 1). T-cell clone is of memory immunophenotype (CD45Ra-ve) and did not stain with markers specific for CD4 T-cells, B-cells, NK cells nor monocytes.

expressed the AV4S1 and the BV6S2 TCR. Three clones (#1,#8,#19) were identical, however differed from the clones described by Burrows et al at one amino acid located in the CDR3 region of the AV4S1 chain (29). Clone #2 was identical to the LC13 clone described by Burrows (29) (Table 1). Monoclonality of the T-cell clones was confirmed using TCR PCR analysis. The DNA and amino acid sequences of the TCR gene segments is given in table1, with comparison to clones reported by Burrows (29).

SAL cell lines are a suitable tool to detect "cross-reactive"alloresponses of viral specific memory CD8 T-cells

To confirm that SALs are an effective tool to detect cross-reactive alloresponses we tested the EBNA3A-FLR/B8 specific clones against SAL expressing HLA B*44:02. Strong IFNγ production was elicited, as measured by detection of the number of IFNγ producing cells ($p<0.0001$) (Figure 2). FLR peptide (positive control), medium, K562 cell and HLA matched SALs all gave appropriate control results. In addition to cross-reactivity against SAL expressing HLA-B*44:02, screening against the entire panel of SAL cells identified that our EBNA3A specific T-cell clones also cross-reacted with SAL expressing HLA B*55:01 ($p=0.0019$, Figure 2). Cross-reactivity was also confirmed with PHA blasts expressing HLA B*44:02 and HLA-B*55:01, in the same Elispot assay (data not shown).

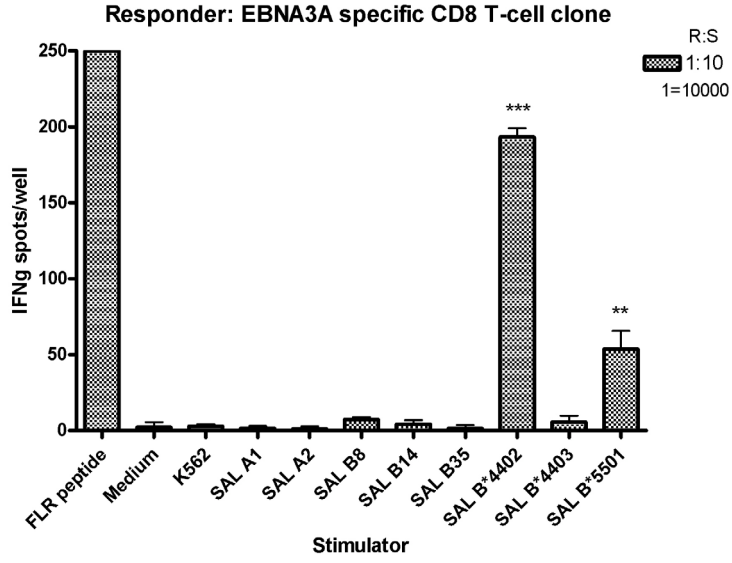

Figure 2. SALs are an effective tool to detect cross-reactive alloresponse(s) from a viral specific CD8 memory T-cell clone. EBNA3A-FLR/B8 T-cell clone recognized K562 cell transfected with either HLA-B*44:02 or HLA-B*55:01 (***$p<0.0001$ and **$p=0.0019$ respectively) (comparison to non-transfected K562). Remaining panel of SALs were not recognized, including SAL B14 and SAL B35. All available HLA-A and HLA-B SALs were tested, while HLA-C SALs were not tested (The full list of available transfected SALs is available in reference 25). HLA typing of donor from whom T-cell clone was sorted is HLA-A1:2; B*08:01,-; DR17,-.

*EBNA-3A viral specific CD8 T-cell clones exert cytolytic activity against HLA B*44:02 and HLA-B*55:01 expressing PHA blasts*

To confirm that the EBNA3A specific CD8 T-cell clones were cytolytic against allogeneic PBMCs expressing the cross-reactive HLA molecules, we performed a chromium release assay using PHA blasts as target cells. The EBNA-3A specific clones specifically lysed PHA blasts expressing either HLA B*44:02 or HLA-B*55:01 in proportion to the E/T ratio ($p<0.0001$ and $p=0.0054$ respectively), whereas HLA-B*44:03 expressing PHA blasts were not lysed (Figure 3).

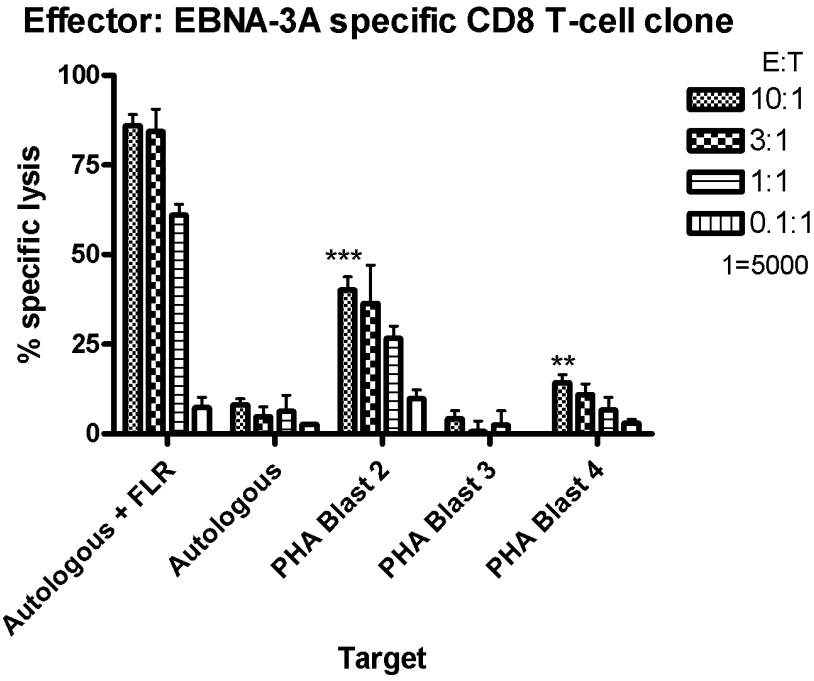

Figure 3. Cytolytic effector function of EBNA-3A clone against allogeneic target cells.
Chromium release cytotoxicity assay using effector EBNA-3A T-cell clone demonstrates functional activity against PHA blasts expressing either HLA B*44:02 or HLA-B*55:01 (***$p<0.0001$ and **$p=0.0054$ respectively) (comparison to PHA Blast 3 ratio 10:1). Autologous PHA blasts are from the same donor used to sort the EBNA3A-FLR/B8 T-cell clone.
Autologous: HLA-A1:2, B*08:01, DR17.
PHA Blast 2: A2,32; B7,**B*44:02**; DR9,11.
PHA Blast 3: A23,31; B39,**B*44:03**; DR4,7.
PHA Blast 4: A24,30; B41,**B*55:01**; DR7,13.

Cytotoxicity against the cross-reactive HLA molecules is specifically inhibited by the presence HLA-B8/FLR tetramer

To confirm that the crossreactive potential of the EBNA-3A specific T-cell clones was mediated by the same T-cell, a cytotoxicity assay was performed in the presence of HLA-B8/FLR tetramer or control tetramer. The results demonstrated that cytotoxicity against both HLA-B*44:02 and HLA-B*55:01 allogeneic molecules was specifically inhibited by the presence of HLA-B8/FLR tetrameric complexes, but not irrelevant tetrameric complexes ($p<0.0001$ and $p=0.0026$ respectively) (Figure 4). Thus confirming that a single viral specific memory T-cell can indeed simultaneously recognize autologous HLA molecules loaded with viral peptide, as well as allogeneic HLA molecule(s) to which it has never been primed.

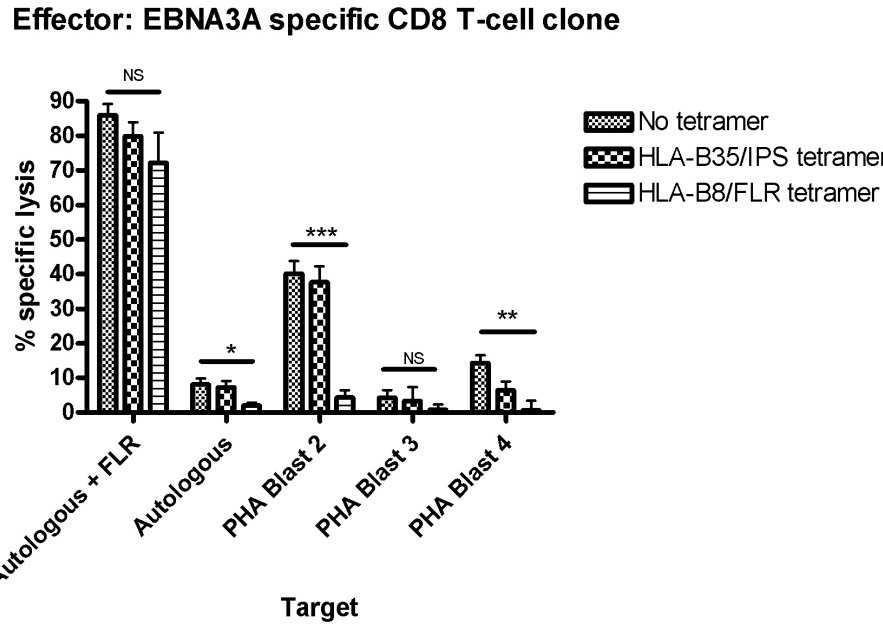

Figure 4. Alloreactivity and viral specificity are mediated by the same T-cell receptor.
Cytotoxicity of the EBNA3A-FLR/B8 CD8 T-cell clone against HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts is specifically inhibited by the presence of HLA-B8/FLR tetramer. An irrelevant tetramer does not suppress the cross-reactivity. Responder:target ratio 10:1, targets 5000. ***$p<0.0001$, **$p=0.0026$, *$p<0.05$. Note: Cytotoxicity against autologous PHA blast loaded with FLR peptide can be significantly inhibited with higher amounts of HLA-B8/FLR tetramer (data not shown).
Autologous: HLA-A1:2, B*08:01, DR17.
PHA Blast 2: A2,32; B7,**B*44:02**; DR9,11.
PHA Blast 3: A23,31; B39,**B*44:03**; DR4,7.
PHA Blast 4: A24,30; B41,**B*55:01**, DR7,13.

Table 1. Sequence analysis of TCR CDR3 regions

	AV4S1	N	Ja52	BV6S2	NDN	Jb2.7	Reported alloreactivity
EBNA3A(#2)	**C I** **tgc atc**	**L P L** **ctg ccc ct**	**A G G** **t gct ggt ggt**	**C A S S L** **tgt gcc agc agc tta g**	**G Q A** **gg cag g**	**Y E Q Y** **cc tac gag cag tac**	HLA-B*4402 & HLA-B*5501
EBNA3A(#1,8,19)	**C I** **tgc atc**	**L P L** **cta ccc tta ga**	**D G G** **t ggt ggt**	**C A S S L** **tgt gcc agc agc tta g**	**G Q A** **ga cag g**	**Y E Q Y** **cc tac gag cag tac**	HLA-B*4402 & HLA-B*5501
IM6 (29)	C I tgc atc	L P L cta ccc ct	A G G t gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
SC17 (29)	C I tgc atc	L P L ctt cct ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
AS1 (29)	C I tgc atc	L P L ctc cct ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
AS7 (29)	C I tgc atc	L P L ctt ccc ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A gc cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
LC13 (29)	C I tgc atc	L P L ctg ccc ct	A G G t gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A gg cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
DD1 (29)	C I tgc atc	L P L ctc cct ctt a	S G G ct ggt ggt	C A S S tgt gcc agc agc	I G Q A atc ggt caa g	Y E Q Y cc tac gag cag tac	HLA-B*4402

For each EBNA-3A clone (in bold) the α chain sequence, β chain sequence and alloreactivity is shown left to right. The one-letter amino acid code is shown above the first nucleotide of the codon. The borders between TCR V, N (DN) and J regions are displayed according to previously reported sequences (29). The EBNA-3A clones sequenced here are all single sorted from the same individual. Variable gene segments are depicted according to Arden nomenclature.

CONCLUSION AND DISCUSSION

We have shown for the first time that T-cell alloresponses from viral specific CD8 memory T-cell clones are reliably detectable in-vitro using transfected K562 cells expressing a single HLA molecule. Using this technique we have confirmed that EBNA3A-FLR/B8 CD8 memory T-cell clones exhibited an alloresponse against HLA-B*44:02, as reported by Burrows (3). In addition, our viral specific clones also exhibited an alloresponse against HLA B*55:01, demonstrating the power of our technique as a screening tool.

The EBNA-3A specific CD8 T-cell clones recognized SAL cells transfected with HLA-B*44:02 but not B*44:03. These two HLA molecules differ only by a single amino acid at position 156, a position critical for interaction with the TCR (30). However, HLA B*55:01 does not share this same amino acid and in fact has the same amino acid (L) at this position as does HLA B*44:03. Furthermore sequence alignment of these HLA molecules reveals there is no common amino acid between HLA B*08:01, B*44:02 and B*55:01 that is not present on HLA B*44:03 (31). Key amino acids within the MHC α2 helix may be critical for these cross-reactive alloresponses (32), however our work suggests that additional factors must also be necessary. In fact, alloreactivity between disparate cognate and allogeneic pMHC class I complexes is likely the result of highly focused, peptide dependent structural mimicry (33).

Our EBNA3A specific T-cell clones recognize HLA-B*55:01, in addition to the previously described HLA B*44:02. The EBV antigen FLRGRAYGL presented on HLA-B8 selects for a public TCR (29), a fact confirmed by sequencing of our own EBNA3A specific T-cell clones (Table 1). It is possible that the single amino acid difference within the CDR3 region of the TCR of our clones (EBNA3A #1,#8 and #19), as compared to the clones reported by others, retains alloreactivity against HLA B*44:02 but in addition enables alloreactivity against HLA B*55:01. Complex structural studies are required to determine if this is indeed the case.

However since clone #2 also exhibited alloreactivity against HLA-B*55:01 and this T-cell clone expressed an identical TCR compared to the EBNA3A specific T-cell clones of Burrows, the most likely explanation is that the EBNA3A specific clones reported by Burrows also exhibit alloreactivity against HLA-B*55:01, but this may not have been detectable without the use of single HLA expressing cell types. This demonstrates the sensitivity of our technique. EBV EBNA3A specific T-cell clones have never been reported not to recognize HLA-B*55:01.

Contrary to a previous report the EBNA3A specific T-cell clones described in this study did not cross-react with HLA-B14 nor HLA-B35 alloantigens (34). The HLA-B14 or HLA-B35 crossreactive T-cell clones however did not express an AV4S1, BV6S2 TCR, and did not recognize HLA-B*44:02 (34). Therefore we would indeed predict that our clones should not recognize HLA-B14 nor HLA-B35, thus demonstrating that our detection technique is not only sensitive but also specific. We propose that subtle amino acid differences of the α and/or β chains within the CDR3 accounts for the various patterns of cross-reactivity, even if these T-cell clones were all restricted by the same viral peptide presented on HLA-B8.

The possibility that the T-cell clones cross-reacted against a HLA class II molecule in this

assay (instead of the transfected HLA class I molecule) can be excluded. The single HLA molecule expression of the SALs has been confirmed against a panel of 84 human HLA-specific monoclonal antibodies (25,26), there is no surface expression of HLA class II. Furthermore, K562 cells lack IFNγ mediated induction of the class II transactivator (35).

The importance of our findings are reinforced by functional studies showing that our EBNA3A specific T-cell clones are able to specifically lyse both HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts, as determined by chromium release assay. Furthermore this dual alloreactivity was specifically inhibited by the HLA-B8/FLR tetramer complex, confirming that a single viral specific memory T-cell can indeed simultaneously recognize autologous HLA molecules presenting viral peptide, as well as allogeneic HLA molecule(s) to which it has never been primed.

The lower percentage of specific lysis of the HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts, versus the positive control (autologous PHA blast loaded with exogenous FLRGRAYGL peptide) is not unexpected. The HLA-B8 expressing PHA blast was exogenously loaded with excess amount of viral peptide, while the cross-reactive alloresponses are dependent on presentation of endogenous self-peptide. Furthermore, it has been suggested by others that cross-reactive alloresponses may be of lower affinity to the original viral specificity against which the T-cell was selected (2). Nevertheless, this cross-reactivity is clearly detectable using our novel technique.

The clinical relevance of our findings are re-enforced by the fact that a HLA-B*44:02 mismatch has been identified as higher risk amongst HLA-B*08:01 renal transplant recipients (36). A HLA-B*55:01 mismatch has not been identified as high risk within EBV positive, HLA-B*08:01 recipients, however further database studies may be warranted in light of our findings.

Results presented here support evidence that virally activated memory T-cells could play a major role in human alloresponses. EBNA3A specific T-cells amounted to 1.7% of peripheral CD8 T-cells in the healthy donor from whom the EBNA3A specific T-cell clone was derived. The frequency of memory CD8 T-cells is highest for the chronically persistent viruses such as human herpes viruses EBV and CMV, infections that are common and persistent in the general population. To our knowledge, this is the first report of a viral specific T-cell clone that can simultaneously cross-react with more than one allogeneic HLA class I molecule. This tool is not only useful for this particular clone but also for detecting cross-reactive alloresponses from other different viral specific clones (manuscript in preparation). The allo-MHC/self-peptide target antigen is presumably sufficiently similar to the MHC/viral-peptide complex involved in activating the T-cell, in three dimensions, to allow crossreactivity.

These findings may have important future implications for donor selection and monitoring. The immune response against the EBNA3A-FLR peptide presented on HLA-B8 selects for a public TCR, with alloreactivity against HLA-B*44:02. The ability to detect the viral specific memory T-cells giving rise to cross-reactive alloresponses may lead to better transplantation matching and/or monitoring. Assay of alloantigen specific T-cells in-vitro for renal trans-

plantation monitoring is not new (37-40), but does not have adequate sensitivity or specificity to enter routine clinical practice. However, the concept of producing tailor-made cells for analysis of cellular reactivity against individual HLA molecules is novel and may have advantages over these previous assays. If in vitro tools can more specifically predict which renal transplant patients are at risk for rejection and which patients are predisposed to tolerance, the immunosuppressive regimen could be adjusted accordingly (37). In theory, using our technique (un)acceptable mismatches might be partially determined based, not only on HLA typing, but also on immunological history.

If our technique can define pathogen driven clonal expansions of T-cells that are involved in initiating allograft rejection then it is possible that immunomodulating techniques could be used to inhibit these harmful T-cell clonotypes, as suggested by Burrows (41). We also hypothesize that anti-viral therapy or viral eradication may decrease the proportion of these cross-reactive alloresponsive T-cells. For example, it has already been shown that CMV prophylaxis post-transplantation is associated with less acute rejection episodes and better one year graft survival (42).

The significance and characteristics of memory CD8 T-cells in viral infections have been extensively studied. In many studies of T-cell memory and transplantation tolerance many experimental immunologists go to great lengths to ensure their animal colonies are pathogen free. Although these studies can be enlightening, humans are not immunologically naive. We have shown here that a single EBV specific memory T-cell can indeed cross react with two alloantigens, thereby possibly influencing the success of tissue transplantation. The presence of Tm correlates with both acute and chronic rejection and may be responsible for the failure to induce tolerance in human clinical settings (4-5). Our results support the conclusion that transplantation/tolerance studies using pathogen free models could be flawed, as has also been suggested by others (4-5,16).

In conclusion, we have shown that SAL cell lines are an effective tool to detect functional immune responses from CD8 T-cell clones, specifically; SALs are an effective tool to screen for cross-reactive alloresponses from viral specific memory T-cells. This technique to define cross-reactive T-cells may also lead to important future improvements in donor selection and monitoring. Cross-reactive alloresponses should be further defined (in-vitro) using CD8 T-cell clones directed against other common persistent viral infections.

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Appendix to chapter 2

Detection of allo-HLA crossreactivity by viral specific memory T-cell clones using single HLA transfected K562 cells

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Methods in Molecular Biology 2010 (In press)

SUMMARY

The ability to directly measure viral specific lymphocytes using fluorochrome labeled tetrameric complexes has proven a great advancement for the transplantation field. Viral peptide/HLA tetrameric complexes allow the rapid generation of viral specific clones using single cell sorting apparatus, permitting the determination of alloreactivity from a single TCR with known specificity. When combined with new target "detector" cells called single HLA antigen transfected K562 cells (SALs) the human alloresponse can for the first time be examined specifically and reliably. Here we describe a method for detection of "heterologous immunity" from viral specific memory T-cells using single HLA expressing cell lines as allogeneic targets.

1. INTRODUCTION

The mechanisms by which alloreactive memory T-cells are generated in non-sensitized individuals have begun to be elucidated. It is generally accepted that a very high level of crossreactivity is an essential feature of the T-cell receptor, for example, memory T-cells that have been generated as a result of a previous viral infection can subsequently respond to a second unrelated infection. However, it has only recently been shown that alloreactivity from viral specific memory T-cells is far more common than predicted, 45% of viral specific T-cell clones were found to be allo-HLA crossreactive (1).

Detection of alloreactivity from viral specific memory T-cells should first be performed by screening against a panel of cells expressing most common HLA molecules, such as a panel of EBV LCLs as described elsewhere (1). However crossreactivity should be confirmed using a single HLA transfected cell type. This will not only confirm the individual HLA molecule recognized but also exclude the possibility of the clone recognizing viral peptides presented on allo-HLA.

Single HLA transfected K562 cells (SALs) are a new sensitive and specific tool to detect alloresponses from viral specific T-cells (2). SALs express only the transfected HLA class I molecule (3), unlike C1R cells that may have low expression of other HLA molecules. For example, the previously described alloreactivity of the EBV EBNA3A specific T-cell against HLA-B*4402 has been confirmed using SALs (2). Furthermore, the same T-cell clone was also found to recognize HLA-B*5501 expressing SALs (2). Suggesting that SALs may be a more sensitive target than other cells expressing multiple HLA molecules.

Therefore detection of HLA-specific alloresponses from viral specific T-cells in-vitro is now feasible in the routine laboratory.

This technique to define cross-reactive T-cells may lead to important future improvements in donor selection and monitoring. The ability to define public TCR responses that give specific allo-HLA crossreactivity may assist in the definition of (un)acceptable mismatches. If in vitro tools can more specifically predict which transplant recipients are at risk for rejection and which patients are predisposed to tolerance, the immunosuppressive regimen could be adjusted accordingly. We also hypothesize that anti-viral therapy may decrease the proportion and activation status of these alloreactive T-cells.

2. MATERIALS

2.1 Viral specific T-cell cloning using single cell sorting
1. Viral peptide/HLA tetrameric complex of interest
2. PBMCs from donor known to have viral peptide/HLA complex binding T-cells in the peripheral blood (or at least known to be serologically positive for virus of interest)
3. Iscoves Modified Dulbecco Medium (IMDM) with L-glutamine
4. Fetal Calf Serum (FCS)
5. Human Serum (HS)
6. Phytohaemagglutinin (PHA) 800ng/mL
7. Interleukin-2 (IL-2)
8. FACS sorting apparatus e.g. FACSAriaII

2.2 Generation of single HLA transfected K562 cells lines (SALs)
1. K562 cell line
2. plasmid (pLNCX, pCDNA3.0, resistant for neomycin (G418), pEAK10 resistant for puromycin, with HLA-cDNA construct (10 µg per transfection)
3. IMDM supplemented with L-glutamine
4. Fetal Calf Serum (FCS)
5. G418 (200 µg/ml)
6. Pen/strep
7. Gene pulser (Biorad)
8. Dynabeads Sheep anti Mouse Ig
9. w6/32 (anti MHC class I antibody), w6/32-PE, w6/32-FITC
10. Goat anti Mouse FITC
11. Puromycin (0.5 µg/ml)
12. Hygromycin (50 µg/ml)
13. Sterile Gen pulser 0.4 cm cuvettes (Biorad)
14. FACS calibur flow cytometer
15. FACS tubes
16. Solution of 0.1% BSA in PBS
17. HLA-specific Human Monoclonal Antibodies
18. Rabbit anti Human IgG FITC
19. Rabbit anti Human IgM FITC
20. DMSO
21. Incubator
22. PBS/1% paraformaldehyde
23. 15 ml tubes
24. Dynal magnet

2.3 IFNγELISA
1. Viral specific T-cell clone
2. SALs transfected with the HLA molecule of interest

3. Non transfected K562 cells
4. Viral peptide for which the clone is specific (positive control), and a control viral peptide
5. IMDM supplemented with L-glutamine
6. FCS
7. HS
8. PHA 800ng/mL
9. Interleukin-2 (IL-2)
10. Sterile 96 well round bottom plates
11. Human IFN-γ Elisa kit.

2.4 Cytotoxicity assay

1. Viral specific T-cell clone
2. SALs transfected with the HLA molecule of interest
3. Non transfected K562 cells
4. Viral peptide for which the clone is specific (positive control), and a control viral peptide
5. IMDM supplemented with L-glutamine
6. FCS
7. HS
8. Phytohaemagglutinin (PHA) 800ng/mL
9. Interleukin-2 (IL-2)
10. Sodium Chromate (1 mCi/ml (370mBq))
11. 1% Triton X-100
12. 96-well round bottom plates

3. METHODS

3.1 Viral Specific T-cell Cloning using single cell sorting

1. Perform all steps at 4 degrees
2. Ensure person for single cell sorting is serologically positive for virus and has a population of the relevant tetramer binding T-cells
3. Wash PBMCs twice in medium containing 1% FCS
4. Add tetramer-PE at high concentration e.g. 1:100
5. Add negative markers CD45Ra/CD4/CD14-FITC
6. Incubate 30 minutes
7. Wash PBMCs twice in medium containing 1% FCS
8. Place 5 million cells for sorting in 1mL 10% FCS/IMDM without phenol red
9. Prepare T-cell Medium - 5% FCS/5% Human serum in IMDM medium with 1% Penicillin/Streptamycin, 3mg L-Glutamine and IL-2 100 IU/mL
10. Prepare T-cell feeder mix – To the T-cell medium add 0.5 million irradiated feeder cells, 0.05 million EBV LCLs and 2μL PHA per mL of required feeder mix.
11. Prepare 96 well round bottom plate(s) with 100μL feeder mix per well.
12. Perform single cell sorting based on tetramer positive gate using single cell sorting ap-

paratus
13. Add further 100µL T-cell medium per well on day 7
14. At day 14 select expanding wells and restimulate in 24 well plate with 1mL feeder mix.
15. After day 6 confirm tetramer positivity of T-cell clones
16. Determine Vβ TCR usage using Vβ kit or TCR PCR.
17. Freeze interesting clones after expansion.
18. 1 million defrosted T-cell clone can be restimulated in T-cell medium with 5 million feeder cells, 0.5 million EBV LCLs and 2uL/mL PHA

3.2 Generation of single HLA transfected K562 cells lines (SALs)
1. Culture K562 until you have 10*106 cells per transfection in IMDM supplemented with 10% FCS and pen/strep
2. Spin down the cells and wash two times in IMDM/10%FCS
3. Count the cells and resuspend the cells in IMDM/10%FCS at a concentration of 10*106 cells per ml
4. Mix 1 ml of the cells with 10µg of the plasmid DNA
5. Put 1 ml of the cell/DNA mixture in a 0.4 cm cuvette and store on ice for a minimum of 5 minutes
6. Mix and pulse (960 µF, 230 V)
7. Store on ice for 15 minutes
8. Add a few drops of medium to the cuvette and transfer the cells to a culture flask containing 9 ml medium (IMDM/10%FCS/pen/strep)
9. Culture the cells for two days
10. Add selection antibiotic at the correct final concentration
11. Culture for another week
12. Perform a flow cytometry test to see whether there are HLA-positive cells (incubate 105 cells with w6/32-PE for 30 minutes on ice, wash twice, take up in 100 µl PBS/1% paraformaldehyde, read in FACS calibur and analyze)
13. If positive cells are present, separate positive cells from negative cells
14. Take 20-25 ml of your cultured cells (leave some in the flask and continue culturing them)
15. Spin down, remove supernatant and resuspend the cell pellet
16. Add 0.5 ml un-labeled-w6/32 and mix
17. Incubate on ice for 30 minutes
18. Wash 3 times with ice cold medium (IMDM/10%FCS) in a 15 ml tube
19. Resuspend the cells and add 30 µl Sheep anti Mouse dynabeads
20. Incubate on a rollerbench in 4°C room for 30 minutes
21. Put the tube in the magnet, add 10 ml icecold medium
22. Wait for 5 minutes
23. Remove the medium with the non bound cells
24. Repeat from step 21 twice
25. Add 10 ml ice cold medium and resuspend the cells bound to the beads, put them in a small culture flask
26. Wait for the cells to grow
27. When the cells expanded well, remove the beads as follows

28. Put the cells in a 15 ml tube in the magnet
29. Wait for five minutes
30. Collect the medium with the cells in a culture flask, leave the beads in the tube
31. Add 10 ml fresh medium
32. Repeat twice from step 28
33. After a few days growing test the class I expression of the cells (step 12)
34. If there still are cells with no expression at all, repeat the bead sorting
35. If the cells all have a good expression, freeze several samples
36. Test the cells with a flow cytometry test against a panel of Human monoclonal antibodies to confirm the HLA-type of the cell

3.3 IFNγ ELISA using responder viral specific T-cell clones and SAL stimulator cells

1. Harvest the T-cell clone
2. Make a T-cell clone solution of 0.1*106/ml in T-cell medium
3. Dilute the SAL's and the K562 line in T-cell medium to a concentration of 0,25.106/ml.
4. Add 50µl /well of the T-cel clone in a sterile 96 well plate
5. Add 100µl /well of the different SAL's in duplicate or triplicate to the clone
6. Use a SAL transfected with the restricting HLA molecule loaded with the viral peptide that is recognized by the clone as a positive control
7. Use a SAL transfected with the restricting HLA molecule loaded with the control peptide that is not recognized by the clone as a negative control
8. Use non transfected K562 as a negative control
9. As background control also add only T-cell medium to some of the wells without SALs
10. Incubate 24 hours at 37°C, 5% CO2
11. Transfer 120µl supernatant to a new 96 well plate and store the samples at -20°C till use in IFNγ ELISA
12. Thaw the supernatants and use in an IFN-γ ELISA according to the manufacturer's protocol.

3.4 Cytotoxicity assay using effector viral specific T-cell clones and SAL target cells

1. Harvest the SALs and the K562 line and spin down the cells
2. Use a SAL transfected with the restricting HLA molecule loaded with the viral peptide that is recognized by the clone as a positive control
3. Use a SAL transfected with the restricting HLA molecule loaded with the control peptide that is not recognized by the clone as a negative control
4. Use non transfected K562 as a negative control
5. Add the appropriate amount of sodium chromate to the cell pellets
6. Incubate the cells in a 37°C water bath for one hour
7. Meanwhile harvest the T-cell clone and make the necessary dilutions of the clone. Add 100µl/well in a 96 well plate.
8. Wash the chromium labeled cells 3 times with 4ml IMDM+1%FCS
9. Add the labeled target cells to the T-cell clone in a concentration of 5000cells/100µl per well.

10. Make a control plate for spontaneous and maximum release. For each target make 3 wells with 100 µl TCM and 3 wells 100 µl 1% Triton X-100. Add 100ul target suspension per well.
11. Spin the plates 1 minute 2000 rpm
12. Incubate the plates 4 hrs at 37°C, 5%CO2
13. Spin the plates 1 minute 2000 rpm
14. Harvest the supernatants
15. Measure the chromium release and calculate the specific lysis.

4. NOTES

4.1 Viral Specific T-cell cloning using single cell sorting
1. We recommend using PE-labelled tetrameric complexes
2. Prior to attempting sorting we recommend screening first for the presence of viral peptide/HLA tetramer complex binding T-cells within the peripheral blood of the donor. Ensuring the donor has a high proportion of the viral specific T-cells of interest in the peripheral blood increases the probability of successful sorting and cloning. Viral seropositivity does not guarantee the donor blood will contain T-cells with the viral specificity of interest.
3. Do not forgot control tubes for set up of single cell sorting apparatus
4. Viral specific T-cell clones are used to confirm that the alloreactivity and viral specificity are mediated by the same TCR. Viral specific T-cell lines can also be generated by sorting multiple tetramer complex binding T-cells per well (e.g. 25 cells/well). T-cell lines are useful for screening purposes and may contain T-cells with the same viral specificity but different TVR Vb usage to that of the single cell sorted clones.
5. Freshly defrosted PBMCs are suitable for single cell sorting.
6. Only freshly collected PBMCs are suitable as feeder cells. Previously frozen cells are not suitable.
7. EBV LCLs can be used to provide additional non-specific stimulation to the sorted T-cells however are not definitely required for successful stimulation.
8. We recommend using fresh T-cell medium for every T-cell sorting or stimulation (Maximum two weeks old).
9. Perform all steps prior to sorting at 4 degrees. When tetramers are used at room temperature they are capable of activating the viral specific T-cells for sorting.
10. For single cell sorted wells expect growth at day 12-14.
11. The TCR will often be absent from the cell surface for the first 5 days after sorting/stimulation.

4.2 Generation of single HLA transfected K562 cells lines (SALs)
1. Leaving SALs at 26°C for two nights increases HLA expression
2. Culturing SALs with HLA-leader peptides increases the HLA expression of HLA-A and HLA-C SALs
3. Flow cytometry tests for HLA should always be performed on ice to avoid capping
4. When you have a low number of HLA-positive cells (before sorting) use PE-labeled w6/32,

it is more sensitive than FITC-labeled w6/32
5. IFNγ stimulation probably does increase HLA expression.
6. K562 cells do not have functional CIITA gene product (4).

4.3 IFNγ production from viral specific T-cell clones using SAL stimulator cells
1. Viral specific T-cell clones should be used in IFNγ ELISA assays at day 9-10 after stimulation. Earlier use of the T-cell clone may be associated with ongoing IFNγ production from the T-cells.
2. For easy transfer of your supernatants, make the layout of your culture plate the same as the layout of the ELISA plate and leave wells open for your standard dilutions and blank.
3. When the supernatants have to be stored for a longer period (> 1 month) it is better to store them in small siliconized vials.

4.4 Cytotoxicity assay using SAL target cells
1. Cytotoxicity assays are best performed at day 7-8 after stimulation.
2. The effector target ratio's used are commonly 30:1, 10:1, 1:1 and 0.1:1.
3. The half life off sodium chromate is short, be sure to use the right amount.
4. The cells survive better if the wash medium contains protein (e.g. 1% FCS). Instead of IMDM you can also use other media for washing e.g. RPMI
5. % specific lysis= ((test release - spontaneous release) /(maximum release - spontaneous release))x100%.

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