# Heterologous immunity in organ transplantation

Heuvel, H. van den

CHAPTER

# 4

# INFECTION WITH A VIRUS GENERATES A POLYCLONAL IMMUNE RESPONSE WITH BROAD ALLOREACTIVE POTENTIAL

Heleen van den Heuvel[a]

Ellen M.W. van der Meer-Prins[a]

Paula P.M.C. van Miert[a]

Xiaoqian Zhang[a]

Jacqueline D.H. Anholts[a]

Frans H.J. Claas[a]

[a] Department of Immunohematology and Blood Transfusion,

Leiden University Medical Center, Leiden, The Netherlands

*Hum Immunol 2019 Feb;80(2):97-102*

## ABSTRACT

Virus-specific T cells have been shown to cross-react with allogeneic HLA (allo-HLA) at a clonal level. However, the impact of a single virus on the allorepertoire has never been investigated at the polyclonal level. We made an inventory of the incidence and specificity of allo-HLA-cross-reactive-virus-specific $CD8^+$ T cells in 24 healthy individuals. T cells were stained for 25 virus-specific tetramers, and mixed lymphocyte reactions were performed against a panel of HLA-typed allostimulators. Allospecificity was confirmed by IFNγ-ELISA using T-cell clones against a panel of HLA-typed cell-lines. The polyclonal immune repertoire directed against CMV alone was associated with a memory response against six allo-HLA molecules. Besides, a single allostimulator activated memory T-cell responses with multiple viral specificities. Concluding, a single virus can substantially broaden the allo-HLA memory T-cell repertoire. This study only looked at CMV- and EBV-specific T cells, whereas the immune repertoire consists of T cells directed against many different viruses. Hence, transplant patients receiving an HLA-mismatched graft may already express a polyclonal repertoire of anti-donor-memory T cells before transplantation.

## INTRODUCTION

As a result of the inherent capacity of T-cell receptors (TCRs) to cross-react to multiple antigens, T cells can express memory phenotypes even for antigens they have never been exposed to. Virus-specific TCRs have been shown to commonly cross-react to allogeneic HLA (allo-HLA), and as a result, an alloreactive memory T-cell pool may exist without prior interaction with allogeneic HLA. This is of particular interest to the field of transplantation, where memory T-cell responses directed against donor cells pose a threat to transplant tolerance (60). Compared to naïve cells, memory T cells have a stronger effector potential, improved survival capacities and upregulated cell adhesion molecules that enable binding to and entering of inflammation sites. In addition, they have lower activation requirements as they do not rely on co-stimulation for their activation. Co-stimulation blockade is an important factor in routine immunosuppressive regimens and is very effective in preventing the activation of naïve T cells, but not of memory T cells. Calcineurin inhibitors (CNI) effectively suppress the activity of both phenotypes (137), but as they are extremely potent and non-specific, they come at the price of increased susceptibility to opportunistic infections (138). In addition, they have severe toxic side effects such as chronic nephrotoxicity and neuropathy (46, 47). In the quest for finding alternative immunosuppressive agents, a major focus lies on co-stimulation blockade, thereby leaving the memory compartment largely unaffected (59, 75-77). A recent report of a randomized clinical trial comparing the CNI tacrolimus to the CD28-CD80/86 co-stimulation inhibitor belatacept in kidney transplant recipients however shows that the acute rejection rate was significantly higher and more severe in the belatacept-treated versus the tacrolimus-treated group (139). Potentially, virus-specific memory T cells with cross-reactivity to donor HLA may have played a role in these rejections.



Several research groups have examined the potential cross-reactivity of virus-specific memory T cells toward allo-HLA. However, so far, studies primarily focused on the identification and characterization of individual allo-HLA-reactive virus-specific memory $CD8^+$ T-cell clones, whereas a viral infection generally induces a polyclonal immune response. The latter is comprised of T cells expressing a broad range of TCRs with different epitope specificities and large variation in TCR affinity and avidity for their epitopes. As TCR cross-reactivity of virus-specific T cells occurs in 45% of virus-specific T-cell clones and 80% of virus-specific T-cell lines (43), polyclonal immune responses that are generated in response to just a single virus are likely to induce many memory T cells that are able to cross-react to different allogeneic HLA molecules. The impact of such a broad polyclonal virus-induced immune response on the allorepertoire within an individual has not yet been determined. In this report, we made an

inventory of polyclonal anti-virus immune responses and their impact on the allorepertoire in healthy individuals.

## MATERIALS AND METHODS

### Collection of responder and target cells

Peripheral blood mononuclear cells (PBMCs) were derived from healthy individuals of both male and female origin with informed consent conform the Declaration of Helsinki. Standard density gradient centrifugation (Ficoll-Isopaque separation) was performed to isolate PBMCs from whole blood. PBMCs were cryopreserved prior to usage.

Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated from PBMCs by incubation with supernatant of the EBV-producing marmoset cell line B95.8 for 1.5 hours at 37°C. Culturing was done in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with penicillin/streptomycin (Gibco), glutamine and 10% fetal calf serum (FCS).

### Generation of virus-specific $CD8^+$ T-cell clones and lines

$CD8^+$ memory T-cell clones and lines were generated by fluorescence-activated cell sorting (FACS Aria; BD) (118). PBMCs were stained with phycoerythrin (PE)-labeled viral tetramers (Table 1) (Leiden University Medical Center Protein facility, Department of Immunohematology and Blood Transfusion, the Netherlands) and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) for CD4, CD19, CD45-RA, CD14, CD40, CD16 and CD56 (BD Pharmingen). FL1 was used as a dump channel to avoid TCR internalization as a result of simultaneous CD8 mAb and major histocompatibility complex (MHC)-tetramer staining. $CD8^+$ memory T-cell clones were generated by sorting 1 cell per well$^{96}$ and $CD8^+$ memory T-cell lines by sorting 10 cells per well$^{96}$. TCR usage was assessed by antibody staining against the TCR Vb (IO Test Vbeta TCR repertoire kit, Beckman Coulter, USA). $CD8^+$ memory T-cell clones and lines were cultured in the presence of irradiated allogeneic PBMCs (4000 Rad) from anonymous buffy coats (Sanquin, Leiden, the Netherlands) for 8 days prior to experimental testing to achieve optimal conditioning.

### HLA typing of responder and target cells

HLA typing was achieved by sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) genotyping, at the European Federation of Immunogenetics (EFI)-accredited national reference laboratory for histocompatibility testing at the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands.

### Mixed lymphocyte reactions

To assess proliferation of cross-reactive viral tetramer-positive $CD8^+$ T cells in response to the most commonly occurring HLA class I alleles in the Western population (>5%), PBMCs of healthy donors positive for multiple CMV and/or EBV tetramers were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with irradiated allogeneic PBMCs (3000 Gy) in mixed lymphocyte reactions (MLRs) against a panel of 16 HLA-typed stimulators. MLRs were performed in Roswell Park Memorial Institute medium (RPMI) supplemented with penicillin/streptomycin (Gibco), glutamine, 15% human serum (HS) and 10 CU/ml IL-2. Upon 8 days, proliferation of tetramer-positive cells was measured by flow cytometry as identified by the $tetramer^+CFSE^{low}CD8^+$ subset. MLRs were first performed against stimulator pools (4x4), and subsequently against individual stimulators of the pool(s) of interest.

### Cytokine production assays

Virus-specific $CD8^+$ T-cell clones and lines were stimulated with a panel of allogeneic EBV-LCLs (E:T 1:10; triplicate wells) for 24 hours at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% fetal calf serum (FCS; Lonza), 5% human serum (HS), and IL-2 (10 CU/mL). The panel was designed to cover the most commonly occurring HLA class I alleles in the Western population (>5%). Interferon γ (IFNγ) production was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, the Netherlands).

## RESULTS

For an overview of the experimental procedure, a flowchart is added in the supplementary material (Supplemental Figure 1).

### The polyclonal CD8$^+$ T-cell response directed against a single virus has the potential to recognize multiple allogeneic stimulators

First, an inventory was made of the incidence and specificity of allo-HLA cross-reactive virus-specific CD8$^+$ T cells in a cohort of 30 healthy individuals. PBMCs were stained with a panel of CMV (n = 13) and EBV (n = 12) tetramers (Table 1).

Healthy donors that stained positive for multiple tetramers directed against the same virus (n = 24) were screened for alloreactivity in mixed lymphocyte reactions (MLRs), which were performed against a panel of allogeneic cells (n = 16) designed to express the most common HLA class I antigens (>5%) in the Western population (Table 2).

**Table 1.** Panel of 25 CMV- and EBV-specific tetramers directed against public viral epitopes[a]

| CMV | | | EBV | | |
|---|---|---|---|---|---|
| HLA | Peptide | Origin | HLA | Peptide | Origin |
| A1 | VTEHDTLLY | pp65 | A2 | GLCTLVAML | BMLF1 |
| A1 | YSEHPTFTSQY | pp65 | A3 | RLRAEAQVK | EBNA3A |
| A2 | NLVPMVATV | pp65 | A3 | RVRAYTYSK | BRLF1 |
| A2 | VLEETSVML | IE-1 | A3 | KHSRVRAYTYSK | BRLF1 |
| A3 | TVYPPSSTAK | pp150 | B7 | RPPIFIRRL | EBNA3A |
| A11 | GPISGHVLK | pp65 | B8 | FLRGRAYGL | EBNA3A |
| A24 | QYDPVAALF | pp65 | B8 | RAKFKQLL | BZLF1 |
| B7 | RPHERNGFTVL | pp65 | B35 | EPLPQGQLTAY | BZLF1 |
| B7 | TPRVTGGGAM | pp65 | B35 | HPVGEADYFEY | EBNA-1 |
| B8 | ELRRKMMYM | IE-1 | B35 | MGSLEVMPM | LMP2A |
| B8 | ELKRKMIYM | IE-1 | B35 | YPLHEQHGM | EBNA3A |
| B8 | QIKVRVDMV | IE-1 | B35 | AVLLHEESM | EBNA3B |
| B35 | IPSINVHHY | pp65 | | | |

[a]All tetramers are phycoerythrin (PE)-labelled

**Table 2.** Panel of HLA-typed allogeneic stimulator PBMCs, designed to cover the most commonly occurring HLA-I antigens in the Western population (>5%)

| HLA ALLELES REPRESENTED IN THE STIMULATOR PANEL | | | | |
|---|---|---|---|---|
| HLA-A | HLA-B | HLA-C | HLA-DR | HLA-DQ |
| A1 | B7 | Cw1 | DR1 | DQ1 |
| A2 | B8 | Cw2 | DR4 | DQ2 |
| A3 | B13 | Cw3 | DR7 | DQ4 |
| A11 | B14 | Cw4 | DR8 | DQ5 |
| A24 | B18 | Cw5 | DR10 | DQ6 |
| A25 | B27 | Cw6 | DR11 | DQ7 |
| A26 | B35 | Cw7 | DR13 | DQ8 |
| A29 | B37 | Cw8 | DR15 | |
| A30 | B38 | Cw9 | DR16 | |
| A31 | B39 | Cw10 | DR17 | |
| A32 | B41 | C*12 | | |
| A33 | B44 | C*14 | | |
| A66 | B51 | C*15 | | |
| A68 | B55 | C*16 | | |
| | B57 | C*17 | | |
| | B58 | | | |
| | B60 | | | |
| | B61 | | | |
| | B62 | | | |

| HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF THE STIMULATOR PANEL | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Stimulator | HLA-A | | HLA-B | | HLA-C | | HLA-DR | | HLA-DQ | |
| 1 | A*02:01 | A*32:01 | B*35 | | C*04:01 | | DRB1*03:01 | DRB1*11 | DQB1*02 | DQB1*03:01 |
| 2 | A24(9) | A29(19) | B7 | B60(40) | Cw7 | | DR13(6) | DR8 | DQ6(1) | DQ4 |
| 3 | A*02:01 | A*11:01 | B*07:02 | B*13:02 | C*06:02 | C*07:02 | DRB1*15 | DRB1*07 | DQB1*02 | DQB1*06:02 |
| 4 | A*01 | A*02:01 | B*08:01 | B*44 | C*05 | C*07:01 | DRB1*03:01 | DRB1*15 | DQB1*02:01 | DQB1*06:02 |
| 5 | A*02:01 | A*30:01 | B*07:02 | B*13:02 | | | DRB1*04:03 | DRB1*15:01 | DQB1*03:02 | |
| 6 | A2 | A33 | B44 | B14 | Cw5 | Cw8 | DR1 | DR4 | DQ5 | DQ8 |
| 7 | A2 | A26 | B38 | B55 | Cw1 | | DR13 | | DQ1 | |
| 8 | A*26 | A*68 | B*51 | | C*15 | | DRB1*04:04 | DRB1*13:01 | DQB1*03:02 | DQB1*06:03 |
| 9 | A1 | A3 | B55 | B37 | Cw3 | Cw6 | DR16 | DR13 | DQ6 | |
| 10 | A1 | A31 | B62 | B57 | Cw3 | Cw6 | DR15 | DR11 | DQ1 | DQ7 |
| 11 | A*01:01 | A*25:01 | B*18:01 | B*58:01 | C*03:02 | C*12:03 | DRB1*08:01 | DRB1*13:01 | DQB1*04:02 | DQB1*06:09 |
| 12 | A1 | A11 | B8 | B35 | Cw4 | Cw7 | DRB1*01:03 | DRB1*03:01 | DQB1*02 | DQB1*05:01 |
| 13 | A*24:02 | A*29:01 | B*39:06 | B*44:03 | C*07:02 | C*16:01 | DRB1*07 | DRB1*08:01 | DQB1*02:02 | DQB1*04:02 |
| 14 | A*02:05 | A*66:01 | B*41:02 | B*58:01 | C*07:01 | C*17:01 | DRB1*13:03 | DRB1*07:01 | DQB1*02:01 | DQB1*03:01 |
| 15 | A*03:01 | A*31:01 | B*51:01 | B*18:01 | C*07:01 | C*14:02 | DRB1*10 | | DQB1*05:01 | |
| 16 | A*03:01 | A*31:01 | B*15:01 | B*40:02 | C*02:02 | C*03:03 | DRB1*04:01 | DRB1*13:01 | DQB1*03:02 | DQB1*06:03 |

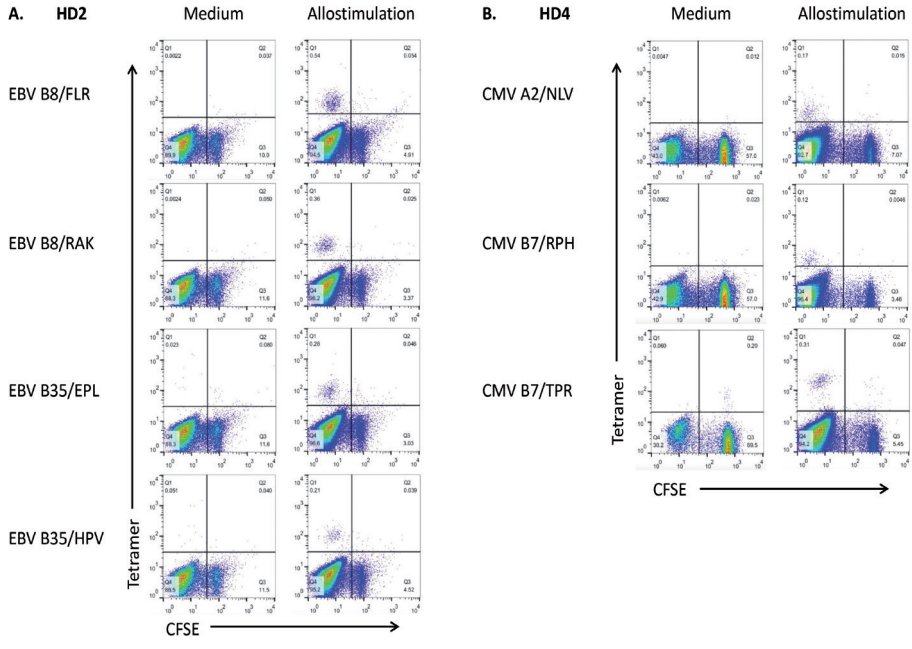



**Figure 1. Multiple virus-specific CD8⁺ T cells of the same individual proliferate in response to allostimulation.** A) Example of individual HD2 showing alloreactivity of the polyclonal immune response against EBV. Plots show: EBV B8/FLR x Pool 1 (stimulator 1-4); EBV B8/RAK x Pool 4 (stimulator 13-16); EBV B35/EPL x Pool 3 (stimulator 9-12); EBV B35/HPV x Pool 1 (stimulator 1-4). B) Example of individual HD4 showing alloreactivity of the polyclonal immune response against CMV. Plots show: CMV A2/NLV x Pool 1 (stimulator 1-4); CMV B7/RPH x Pool 4 (stimulator 13-16); CMV B7/TPR x Pool 3 (stimulator 9-12). All plots are gated on CD8⁺ lymphocytes.

Within polyclonal anti-viral immune responses, T cells with different viral epitope specificities were able to proliferate in response to allogeneic stimulation. This was observed for EBV and CMV responses, and also for both viruses within the same individual (Figure 1, Table 3). Interestingly, single allogeneic stimulators were able to induce multiple different virus-specific CD8⁺ T-cell responses in the same responder (Figure 2).

**Table 3.** Overview of all virus-specific T cells responding to allogeneic cells in MLR per healthy individual[ab]

| Virus | HD1 | HD2 | HD3 | HD4 | HD5 | HD6 | HD7 | HD8 | HD9 | HD10 | HD11 | HD12 | HD13 | HD14 | HD15 | HD16 | HD17 | HD18 | HD19 | HD20 | HD21 | HD22 | HD23 | HD24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CMV | A1/VTE | A2/NLV | A1/VTE | A2/NLV | B7/RPH | A2/NLV | A1/VTE | A1/VTE | B7/RPH | A1/VTE | A1/VTE | A1/VTE | A2/NLV | A1/VTE | A1/VTE | A2/NLV | A2/NLV | | A2/VLE | B7/RPH | | | A2/NLV | B7/RPH |
| | A1/YSE | B8/ELR | A2/NLV | B7/RPH | B7/TPR | B7/RPH | A1/YSE | B7/RPH | B7/TPR | A1/YSE | A1/YSE | A1/YSE | B7/TPR | B8/ELR | A1/YSE | B7/RPH | B7/RPH | | | B7/TPR | | | B35/IPS | B7/TPR |
| | A2/NLV | B8/QIK | B8/ELR | B7/TPR | | B7/TPR | B7/RPH | B7/TPR | | B8/ELR | A2/NLV | B8/ELR | | B8/ELK | B8/ELR | B7/TPR | B7/TPR | | | | | | | |
| | | B35/IPS | | | | | B7/TPR | | | B8/ELK | B8/ELR | B8/QIK | | | | | B35/IPS | | | | | | | |
| | | | | | | | B8/ELK | | | B8/QIK | B8/QIK | | | | | | | | | | | | | |
| | | | | | | | B8/QIK | | | B35/IPS | | | | | | | | | | | | | | |
| EBV | A2/GLC | B8/FLR | A2/GLC | | A2/GLC | A2/GLC | B8/FLR | B7/RPP | B7/RPP | n.d. | n.d. | n.d. | A2/GLC | n.d. | n.d. | n.d. | A2/GLC | A2/GLC | B7/RPP | A2/GLC | A2/GLC | A2/GLC | | A3/RLR |
| | B8/FLR | B8/RAK | B8/FLR | | B7/RPP | A3/RLR | B8/RAK | B8/RAK | B35/EPL | | | | | | | | B7/RPP | B7/RPP | B35/EPL | B7/RPP | B8/FLR | A3/RLR | | B7/RPP |
| | B8/RAK | B35/EPL | B8/RAK | | | B7/RPP | | | B35/HPV | | | | | | | | B35/EPL | B35/EPL | B35/HPV | B8/FLR | B8/RAK | B7/RPP | | |
| | | B35/HPV | B7/RPP | | | | | | | | | | | | | | B35/EPL | B35/HPV | | B8/RAK | | B35/HPV | | |
| | | B35/YPL | | | | | | | | | | | | | | | B35/HPV | B35/YPL | | | | | | |
| | | | | | | | | | | | | | | | | | B35/YPL | | | | | | | |

[a]Results are based on MLRs using the individual stimulators from the stimulator panel

[b]Red = proliferation; n.d. = not determined

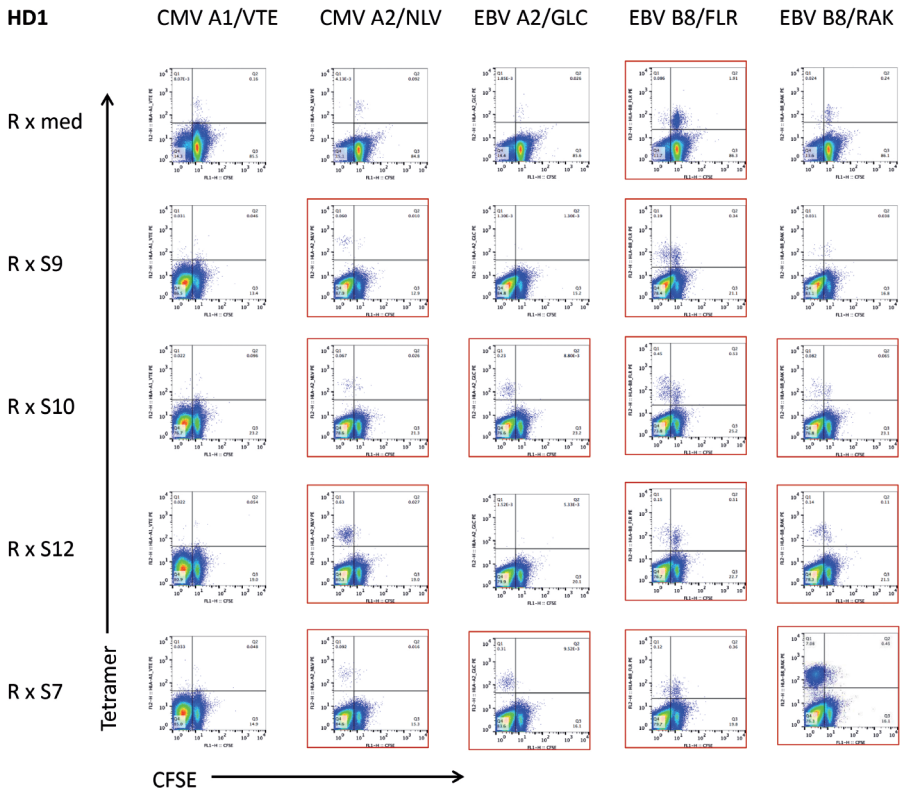



**Figure 2. Single allogeneic stimulators induced multiple virus-specific CD8⁺ T-cell responses in MLR in the same responder (HD1).** Although the EBV B8/FLR response should be interpreted with caution due to its proliferation background in media (% proliferated Tm-positive cells of total Tm-positive cells: 4.3%), its alloresponses were much more pronounced (% proliferated Tm-positive cells of total Tm-positive cells: respectively 35.8% (S9); 45.9% (S10); 22.7% (S12); and 25% (S7)). Plots are gated on CD8⁺ T cells. X-axis: CFSE. Y-axis: virus-specific tetramer.

## The polyclonal CD8⁺ T-cell response directed against a virus contains multiple allo-HLA specificities

Virus-specific T cells with different viral specificities exerted different patterns of alloreactivity against the stimulator panel in MLR, indicating that they had different allo-HLA specificities as well. To confirm, virus-specific CD8⁺ memory T-cell clones were generated as a proof of principle to determine their allospecificity in IFNγ ELISA against a panel of EBV-immortalized B-cell lines (EBV-LCLs) (Supplemental Table 1). For example, responder HD23 showed cross-reactivity of CMV A2/NLV- and CMV B35/IPS-specific T cells. The CMV B35/IPS response was directed against

HLA-B*51:01 and HLA-B*58:01/B*57:01, a public cross-reactivity that was recently identified by our group (140). The CMV A2/NLV alloresponse showed cross-reactivity in response to multiple allo-HLA molecules: a CMV A2/NLV T-cell line (1A2) showed cross-reactivity against HLA-B*39:01, and a CMV A2/NLV T-cell clone (#1) against the combination of HLA-A2 and HLA-B50 (Table 4, Supplemental Figure 2). TCR Vb usage analysis confirmed that the CMV A2/NLV T-cell line and clone expressed multiple TCR clonotypes, whereas the CMV B35/IPS T-cell lines and clones expressed a public TCR (140). Findings were confirmed in additional MLRs (data not shown). Infection with CMV in this individual therefore enabled alloreactivity towards (a minimum of) six different allogeneic HLA molecules.

**Table 4.** Virus-specific T cells derived from the same individual and directed against the same virus show multiple allo-HLA cross-reactivities[a]

| Viral specificity | Healthy Donor | T-cell clone / line | Reactivity against EBV-LCL | TCR Vβ usage | Allo-HLA cross-reactivity |
|---|---|---|---|---|---|
| CMV B35/IPS | HD23 | Clone 7C8 | 7, 9, 10, 12 | TRBV28 | HLA-B*51:01<br>HLA-B*57:01<br>HLA-B*58:01 |
| | HD23 | Clone 8C1 | 9, 12[b] | n.d.* | HLA-B*58:01[b] |
| | HD23 | Cell line 6A3 | 7, 9, 12 | TRBV28 + TRBV12 + TRBV6-2 | HLA-B*57:01<br>HLA-B*58:01 |
| | HD23 | Cell line 6A8 | 7, 9, 12 | TRBV28 + TRBV20-1 | HLA-B*57:01<br>HLA-B*58:01 |
| CMV A2/NLV | HD23 | Clone 1 | 23[c] | TRBV20-1 | HLA-A*02<br>HLA-B*50:01 |
| | HD23 | Cell line 1A2 | 15 | TRBV3-1 + TRBV18 + TRBV6 + TRBV20-1 | HLA-B*39:01 |

[a]Reactivity against EBV-LCLs expressing syngeneic HLA-B*35:01 and HLA-A*02:01 was disregarded for analyses of CMV B35/IPS and CMV A2/NLV responses respectively, as it potentially reflects reactivity towards the cognate epitope

[b]Potential minor reactivity towards EBV-LCL 7 (HLA-B*57:01), however the response was too small to include in analysis

[c]All T-cell lines and clones were tested against EBV-LCL panel 1, except CMV A2/NLV Clone 1 (EBV-LCL panel 2)

*n.d. = not determined

## DISCUSSION

As humans are exposed to a myriad of viruses throughout their life-time and TCR cross-reactivity is a common feature of T cells, it is not surprising that the majority of virus-specific T cells are able to cross-react to allo-HLA. Although our understanding of this cross-reactivity increases and even mechanisms underlying this cross-reactivity have been proposed (135, 141), the possible clinical relevance of these cross-reactive T cells remains under investigation (39-41, 142).

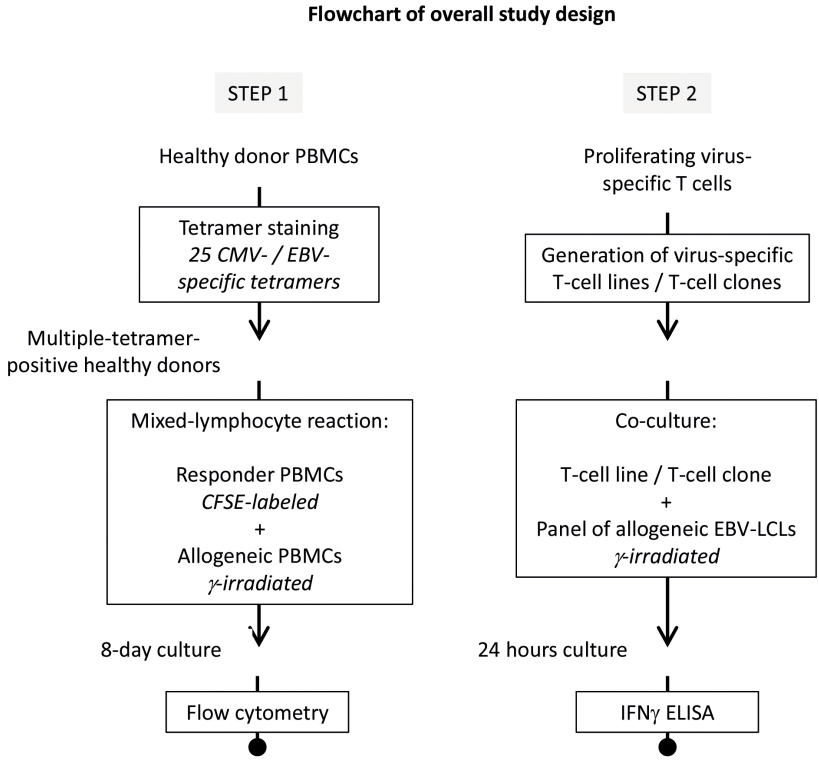
In this study, we aimed to determine the footprint of a single virus on the allorepertoire. We observed broad alloreactivity of virus-specific T cells on multiple levels: T cells with different viral epitope specificities, T cells with the same viral epitope specificities, and even T cells of the same clonotype were able to recognize multiple allogeneic HLA molecules. Polyclonal alloimmune responses of EBV and CMV T cells were identified in several individuals. This is particularly interesting given the fact that the experiments were restricted to known (dominant) viral epitopes for tetramer-staining. In total, 13 CMV- and 12 EBV-specific tetramers were available. It is thus remarkable that polyclonal alloresponses were found for both EBV and CMV, as the limited number of available tetramers inevitably leads to underestimation of the scope of the polyclonal alloresponse. Accordingly, a large population of tetramer-negative $CD8^+$ T cells responded to allostimulation (Figure 1, 2), possibly containing additional cross-reactive virus-specific T cells directed against unknown viral epitopes. In addition, alloreactivity screening was restricted to HLA-I alleles present in >5% of the Western population, and the allospecificity of polyclonal anti-virus responses will most likely be broader when taking into account less common HLA class I molecules as well.

Finally, we previously published that functional virus-specific T-cell responses can be induced by stimulation with allogeneic cells (143). We again observed that allostimulation was able to induce proliferation of virus-specific T cells, and in addition that a single allogeneic stimulator was able to stimulate T cells of multiple viral specificities (belonging to the same individual): further illustrating the impact of virus-specific immune responses on the allorepertoire.

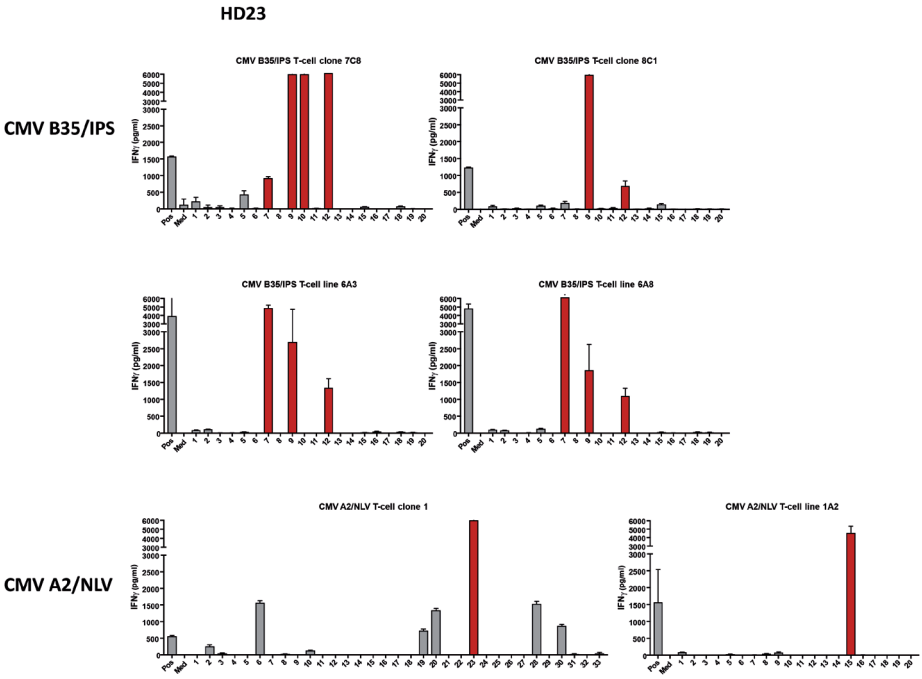
In conclusion, infection with a single virus can generate a diverse allorepertoire. Cross-reactive memory T-cells in the polyclonal anti-viral immune response can have broad alloreactive potential, as not only T cells with different viral epitope specificities, but also T cells sharing viral specificity and T cells of the same clonotype can be cross-reactive with multiple allo-HLA molecules. Thereby, the many viruses encountered throughout life could induce a

broad repertoire of (donor-specific) alloreactive memory T cells in transplant recipients already in place at the time of transplantation. This message is important to keep in mind, especially when seeking alternative immunosuppression strategies. Current standard-of-care immunosuppression covers suppression of the memory compartment, and it is still unclear what will happen to the alloresponse when the naïve compartment is selectively targeted instead. For example, based on the high prevalence of pre-existing allo-HLA cross-reactivity, one could argue that clinical rejection rates should be higher than is currently the case; potent immunosuppression is likely to play an important role here. In addition, the functional characteristics of the allo-HLA cross-reactive virus-specific T cells may not be sufficient to mount potent immune responses: for example due to low TCR avidity for the alloepitope (144). Yet, also low-avidity cross-reactive clonotypes could gain momentum when triggered upon viral infection or reactivation; and current standard-of-care anti-viral prophylaxis may also play an indirect role in preventing alloresponses (145, 146). Finally, continuous allostimulation, as is the case in a transplantation setting, may induce mechanisms of regulation or T-cell exhaustion (147). Answering these questions will make an invaluable contribution to unravel the clinical relevance of allo-HLA cross-reactive virus-specific memory T cells in transplantation.

## SUPPLEMENTARY MATERIAL




**Supplemental Figure 1.** Flowchart of the experimental procedure to determine alloreactivity of polyclonal T-cell immune responses.




**Supplemental Figure 2. Allospecificity of CMV-specific CD8⁺ memory T-cell clones was determined in IFNγ ELISA against a panel of HLA-typed EBV-LCLs.** All T-cell lines and clones were derived from HD23 and tested against EBV-LCL panel 1 or EBV-LCL panel 2 (CMV A2/NLV Clone 1) (Supplemental Table 1). Reactivity of CMV B35/IPS T-cell clones against EBV-LCLs expressing syngeneic HLA-B*35:01 (e.g. reactivity of CMV B35/IPS T-cell clone 7C8 versus EBV-LCL 5) and reactivity of CMV A2/NLV T-cell clones against EBV-LCLs expressing syngeneic HLA-A*02:01 (e.g. reactivity of CMV A2/NLV T-cell clone 1 versus EBV-LCL 30) were disregarded for analysis, as these potentially reflect reactivity towards the cognate epitope. X-axis: EBV-LCLs. Y-axis: IFNγ production in pg/ml. Positive control: EBV-LCL expressing syngeneic HLA + viral peptide (1000ng/ml). Red = reactivity against these EBV-LCLs was confirmed.

**Supplemental Table 1.** Panels of HLA-typed EBV-LCLs designed to cover the most commonly occurring HLA-I antigens in the Western population (>5%)[1]

| HLA ALLELES REPRESENTED IN EBV-LCL PANEL 1 | | | | |
|---|---|---|---|---|
| HLA-A | HLA-B | HLA-C | HLA-DR | HLA-DQ |
| A1 | B7 | Cw1 | DR1 | DQ1 |
| A2 | B8 | Cw2 | DR2 | DQ2 |
| A3 | B13 | Cw4 | DR3 | DQ4 |
| A11 | B14 | Cw5 | DR4 | DQ5 |
| A24 | B18 | Cw6 | DR5 | DQ7 |
| A25 | B35 | Cw7 | DR7 | DQ8 |
| A26 | B38 | Cw8 | DR8 | DQ9 |
| A29 | B39 | Cw9 | DR10 | |
| A30 | B41 | Cw10 | DR11 | |
| A31 | B42 | C*12 | DR12 | |
| A32 | B44 | C*15 | DR13 | |
| A33 | B49 | C*16 | DR14 | |
| A68 | B51 | C*17 | DR15 | |
| | B52 | | DR17 | |
| | B55 | | DR18 | |
| | B57 | | DR103 | |
| | B58 | | | |
| | B60 | | | |
| | B62 | | | |

| HLA ALLELES REPRESENTED IN EBV-LCL PANEL 2 | | | | |
|---|---|---|---|---|
| HLA-A | HLA-B | HLA-C | HLA-DR | HLA-DQ |
| A1 | B7 | Cw1 | DR1 | DQ1 |
| A2 | B8 | Cw2 | DR2 | DQ2 |
| A3 | B13 | Cw4 | DR3 | DQ4 |
| A11 | B14 | Cw5 | DR4 | DQ5 |
| A23 | B15 | Cw6 | DR7 | DQ6 |
| A24 | B18 | Cw7 | DR8 | DQ7 |
| A25 | B27 | Cw8 | DR9 | DQ8 |
| A26 | B35 | Cw9 | DR10 | DQ9 |
| A30 | B37 | Cw10 | DR11 | |
| A31 | B38 | C*12 | DR12 | |
| A32 | B39 | C*15 | DR13 | |
| A33 | B40 | C*16 | DR14 | |
| A66 | B42 | C*17 | DR15 | |
| A68 | B44 | | DR16 | |
| A80 | B45 | | DR17 | |
| | B46 | | DR18 | |
| | B49 | | | |
| | B50 | | | |
| | B51 | | | |
| | B52 | | | |
| | B53 | | | |
| | B55 | | | |
| | B57 | | | |
| | B58 | | | |
| | B60 | | | |
| | B62 | | | |
| | B63 | | | |
| | B70 | | | |

| HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 1 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| EBV-LCL | HLA-A | | HLA-B | | HLA-C | | HLA-DR | | HLA-DQ | |
| 1 | A*02:01:01 | A*32:01:01 | B*08:01:01 | B*44:05:01 | C*02:02:02 | C*07:01/07:06/07:18 | DR4 | DR7 | DQ2 | DQ7 |
| 2 | A*24:02 | A*33:01:01 | B*14:02:01 | | C*02:02/02:32 | C*08:02/08:29 | DR1 | | DQB1*05:01 | |
| 3 | A*03:01/03:22 | A*29:02/29:09 | B*07:02/07:61/07:114 | B*44:03/44:105 | C*07:02:01 | C*16:01:01 | DR2 | | | |
| 4 | A*11:01/11:43 | A*33:03/33:51 | B*18:01/18:17N | B*52:01:01 | C*07:02:01 | C*07:04:01 | DR15 | DRB1*14:10 | DQB1*05:01 | DQB1*05:03 |
| 5 | A*11:01/11:33 | A*30:01/30:54 | B*13:02:01 | B*35:01/35:42 | C*04:01:01 | C*06:02:01 | DR13 | DR14 | DQ1 | |
| 6 | A*01:01:01:01 | A*26:01:01 | B*08:01:01 | B*49:01:01 | C*07:01:01 | | DRB1*01 | DRB1*03:04 | DQB1*03:04 | DQB1*05:04 |
| 7 | A*11:01:01 | A*31:01:02 | B*15:01:01 | B*57:01:01 | C*03:03:01 | C*06:02/06:55 | DR7 | DRB1*04:01 | DQB1*03:01 | DQB1*03:03 |
| 8 | A*02:03:01 | A*24:02 | B*38:02:01 | B*40:01:02 | C*03:04:01 | C*07:02:01 | DR2 | DR5 | | |
| 9 | A*29:02:01 | A*31:01:02 | B*18:01/18:17N | B*58:01:01 | C*05:01 | C*07:18/07:01 | DR3 | DR8 | DQ2 | |
| 10 | A*24:03:01 | | B*51:01:01 | | C*15:02:01 | | DRB1*11:04 | | DQB1*03:01 | |
| 11 | A*26:01:01 | | B*38:01:01 | | C*12:03:01 | | DRB1*04:02 | | DQB1*03:02 | |
| 12 | A*24:02:01 | A*30:01:01 | B*51:01:01 | B*58:01:01 | C*01:02:01 | C*03:02:02 | DR1 | DR7 | DQ1 | DQ2 |
| 13 | A*02:01:01 | A*03:01:01 | B*15:01:01:01 | | C*03:03:01 | | DR4 | DR13 | DQ1 | DQ3 |
| 14 | A*68:01:02 | A*68:02:01 | B*44:02/44:19N | B*55:01:01 | C*03:03:01 | C*07:04:01 | DR14 | | DQ1 | |
| 15 | A*24:02 | A*31:01:02 | B*39:01 | B*55:01:01 | C*03:03:01 | C*12:03:01 | DR13 | | DQ1 | |
| 16 | A*30:01:01 | A*68:02:01 | B*42:01:01 | | C*17:01:01 | | DRB1*03:02 | | DQB1*04:02 | |
| 17 | A*01:01:01:01 | | B*41:01 | | C*17:01:01 | | DRB1*11:01 | | DQB1*03:01 | |
| 18 | A*02:01:01 | A*11:01:01 | B*35:01:01 | B*44:03:01 | C*04:01:01 | C*16:01:01 | DRB1*01:03 | | | |
| 19 | A*02:01:01 | A*25:01:01 | B*18:01/18:17N/18:43 | B*44:02/44:19N/44:55 | C*05:01:01 | C*12:03:01 | DR1 | DR4 | DQ1 | DQ7 |
| 20 | A31 | A*24:02 | B7 | | Cw4 | Cw7 | DR12 | DR15 | | |

HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 2

| EBV-LCL | HLA-A | | HLA-B | | HLA-C | | HLA-DR | | HLA-DQ | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A*24:02 | A*33:01:01 | B*14:02:01 | | C*02:02/02:32 | C*08:02/08:29 | DR1 | | DQB1*05:01 | |
| 2 | A*11:01:01 | A*31:01:02 | B*15:01:01 | B*57:01:01 | C*03:03:01 | C*06:02/06:55 | DR7 | DRB1*04:01 | DQB1*03:01 | DQB1*03:03 |
| 3 | A*02:01 | | B*15:01 | B*39:01 | C*03:03/03:11/03:13 | C*07:01 | DRB1*13:03 | DRB1*14:54/14:01/14:07 | DQB1*03:01 | DQB1*05:03 |
| 4 | A2 | | B44(12) | B57(17) | Cw6 | | DR7 | DR9 | | |
| 5 | A*68:01:02 | A*68:02:01 | B*44:02 | B*55:01:01 | C*03:03:01 | C*07:04:01 | DR14 | | DQ1 | |
| 6 | A1 | A*24:03(9) | B52(5) | B49(21) | Cw7 | | DR15(2) | DR11(5) | DQ6(1) | DQ7(3) |
| 7 | A*01:01:01:01 | | B*40:01:01 | | C*06:02:01:01 | | DRB1*13:01:01:02 | | DQB1*06:03:01 | |
| 8 | A31 | A*24:02 | B7 | | Cw4 | Cw7 | DR12 | DR15 | | |
| 9 | A1 | A3 | B37 | | Cw6 | | DR10 | | DQ5(1) | |
| 10 | A3 | A24(9) | B39 | B50 | | | DRB1*03:01 | DRB1*16:01 | DQB1*02:01 | DQB1*05:02 |
| 11 | A*03:01 | A*33:01 | B*14:02 | B*51:24 | C*08:02 | C*15:02 | DRB1*04:01 | DRB1*16:01 | DQB1*03:01 | DQB1*05:02 |
| 12 | A*02:01/02:17/02:04 | | B*18:01/18:03/18:05 | | C*07:01/07:05/07:06+ | | DRB1*14:54/14:01/14:05 | | DQB1*05:03/05:05 | |
| 13 | A*01 | A*02:01 | B*08:01 | B*53:06 | C*02:02 | C*07:01/07:06 | DRB1*03:01 | DRB1*11:01 | DQB1*02:01 | DQB1*03:01 |
| 14 | A*02:10 | A*30:01 | B*13:02 | B*40:06 | C*06:02 | C*08:01 | DRB1*07:01 | DRB1*09:01 | DQB1*02 | DQB1*03:03 |
| 15 | A*24:02 | A*26:01/26:08/26:02 | B*38:01 | B*51:01/51:03/51:11N | C*05 | C*12:03/12:06 | DRB1*07 | DRB1*13:22 | DQB1*02:02 | DQB1*06:03 |
| 16 | A*02:01 | | B*08:01 | B*39:06:02 | C*07:02 | | DRB1*08:01 | DRB1*09:01 | DQB1*03:03 | DQB1*04:02 |
| 17 | A*66:02 | A*30:02 | B*18:01 | B*58:01 | C*07:01 | | | | | |
| 18 | A*01:01 | A*02:07 | B*08:01/08:08N/08:18 | B*27:04/27:68/27:69 | C*07:01/07:06/07:18 | C*12:02/12:17/12:22 | | | | |
| 19 | A*24:02 | A*25:01 | B*15:01/15:12/15:14 | B*55 | C*03:03/03:11/03:12 | | DRB1*08:06 | DRB1*15:01 | DQB1*05:01 | DQB1*06:02 |
| 20 | A*02:17:01 | | B*15:01:01:01 | | C*03:03:01 | | DRB1*14:02:01 | | DQB1*03:01:01 | |
| 21 | A*01:01 | | B*35:02 | | C*04:01 | | DRB1*11:04:00 | | DQB1*03:01 | |
| 22 | A*02:01:01:01 | | B*45:01:01 | | C*16:01:01 | | DRB1*13:01:01 | | DQB1*06:03:01 | |
| 23 | A*02:08 | A*01:01:01:01 | B*50:01:01 | B*08:01:01 | C*07:01:01:01 | C*06:02:01:02 | DRB1*03:01:01:01 | DRB1*07:01:01:01 | DQB1*02:01/02:02/02:04 | |

*HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 2 Continued*

| EBV-LCL | HLA-A | | HLA-B | | HLA-C | | HLA-DR | | HLA-DQ | |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 | A*23:01 | | B*14:01 | | C*08:02 | | DRB1*04:01:01 | | DQB1*03:02:01 | |
| 25 | A*24:02:01:01 | A*26:01 | B*35 | B*38:01 | C*04:01 | C*12:03 | DRB1*03:01 | DRB1*08:06 | DQB1*02:01 | DQB1*06:02 |
| 26 | A*30:01:01 | A*68:02:01 | B*42:01:01 | | C*17:01:01 | | DRB1*03:02 | | DQB1*04:02 | |
| 27 | A2 | A*80:01 | B58 | B70 | Cw2 | Cw6 | DR17 | DR11 | DQ2 | DQ7 |
| 28 | A*02:04 | | B62(15) | | Cw3 | | DRB1*14: 02/14:06 | | DQ8(3) | |
| 29 | A*66:01 | | B*38:01 | | C*12:03 | | DRB1*14:01 | | DQB1*05:03 | |
| 30 | A*02:01:01 | A*02:07 | B*46:01:01 | | C*01:02:01 | | DRB1*09:01:02 | DRB1*12:02:01 | DQB1*03:01 | DQB1*03:03 |
| 31 | A*31:01/31: 02/31:06 | A*32:01/32: 05/32:08 | B*40:01/40: 02/40:11 | B*40:02/40: 27/40:29 | C*02:02/02: 04/02:08+ | C*03:04/03: 05/03:08 | DRB1*04:04/ 04:07/04:05 | DRB1*04:04/04: 03/04:07+ | DQB1*03:02/03: 05/03:07+ | |
| 32 | A*01:01/01 :04N/01:09 | | B*15:17 | B*57:01/57: 06/57:08 | C*04:01/04 09N/04:05 | C*07:01/07: 05/07:06 | DRB1*04:05 | DRB1*08:08 | DQB1*02:01 | DQB1*04:02 |
| 33 | A*24:02 | | B*40:01 | | C*03:04 | | DRB1*09:01:02 | | DQB1*03:03:02 | |

[1] All T-cell lines and clones were tested against EBV-LCL panel 1 except CMV A2/NLV Clone 1 (EBV-LCL panel 2)