Heterologous immunity in organ transplantation

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CHAPTER

6

ALLO-HLA CROSS-REACTIVITIES OF CYTOMEGALOVIRUS-, INFLUENZA- AND VARICELLA ZOSTER VIRUS-SPECIFIC MEMORY T CELLS ARE SHARED BY DIFFERENT INDIVIDUALS

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ABSTRACT

Virus-specific T cells can recognize allogeneic HLA (allo-HLA) through TCR cross-reactivity. The allospecificity often differs by individual (*private* cross-reactivity), but can also be shared by multiple individuals (*public* cross-reactivity); however, only a few examples of the latter have been described. Because these could facilitate alloreactivity prediction in transplantation, we aimed to identify novel public cross-reactivities of human virus-specific $CD8^+$ T cells directed against allo-HLA by assessing their reactivity in mixed lymphocyte reactions. Further characterization was done by studying TCR usage with primer-based DNA sequencing, cytokine production with ELISA, and cytotoxicity with ^{51}chromium-release assays. We identified three novel public allo-HLA cross-reactivities of human virus-specific $CD8^+$ T cells. CMV B35/IPS $CD8^+$ T cells cross-reacted with HLA-B51 and/or HLA-B58/B57 (23% of tetramer-positive individuals), FLU A2/GIL $CD8^+$ T cells with HLA-B38 (90% of tetramer-positive individuals) and VZV A2/ALW $CD8^+$ T cells with HLA-B55 (two unrelated individuals). Cross-reactivity was tested against different cell types including endothelial and epithelial cells. All cross-reactive T cells expressed a memory phenotype, emphasizing the importance for transplantation. We conclude that public allo-HLA cross-reactivity of virus-specific memory T cells is not uncommon and may create novel opportunities for alloreactivity prediction and risk estimation in transplantation.

INTRODUCTION

Alloreactive T cells are a major cause of graft loss after solid organ transplantation and acute graft-versus-host disease (aGVHD) after hematopoietic stem cell transplantation (HSCT) (114, 184). In particular alloreactive T cells expressing a memory phenotype are a potential threat, since their activation threshold is substantially lower compared with their naïve counterpart, while their effector function is enhanced (161, 185). Interestingly, alloreactive memory T cells are present in all individuals, even without prior exposure to alloantigen. This can be explained by heterologous immunity of virus-specific T cells: the inherent capacity of T-cell receptors (TCRs) to cross-react with multiple antigens. Heterologous immunity thereby arms virus-specific T cells with a TCR that can recognize a range of related and unrelated viral peptides, creating a survival benefit to the host. This scenario describes the recognition of different peptides presented by a self-HLA. TCR cross-reactivity, however, can also be directed against allogeneic HLA (allo-HLA). As a consequence, memory T cells that are primed by viral infections may contribute to allograft rejection. Alarmingly, such TCR cross-reactivity of virus-specific memory T cells against allogeneic HLA is indeed common (43).

The TCR repertoire differs by individual due to both intrinsic (thymic selection) and extrinsic (allergens / viral infections) factors. Although most TCRs appear to be unique to individuals (*private* TCRs), dominant TCR sequences have also been found in multiple individuals (*public* TCRs). Public TCRs are selected for optimal recognition of immunodominant viral epitopes (186), and cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) in particular are known for inducing public TCR responses (6, 7, 187). Inherent to their structural differences, the allospecificity of cross-reactive private TCRs (even with the same viral specificity) is directed toward different alloepitopes (private cross-reactivity) (188), and alloreactivity by private TCR cross-reactivity is therefore impossible to predict. In contrast, cross-reactivity of public TCRs is directed against the same alloepitopes in multiple individuals and thus could facilitate the prediction of alloreactivity by memory T cells. A classic example of such public cross-reactivity is found in HLA-B8^{+} individuals: when infected with the EBV virus, they select a public TCR that, in addition to the viral epitope (HLA-B8/FLR), recognizes allogeneic HLA-B*44:02 (11). Such cross-reactive virus-specific T cells were also identified in HLA-B8^{+}B44^{-} lung-transplant recipients transplanted with an HLA-B*44:02 graft (39, 40).

To date, public cross-reactivity of virus-specific T cells is considered uncommon because it requires strict TCR preservation in unrelated individuals; however, reports of other TCR cross-reactivities with similar allospecificity in unrelated individuals give reason to challenge this

conception (189-192). Furthermore, Nguyen et al. recently identified cross-reactivity of CMV A2/NLV-specific T cells with nearly identical TCR usage against HLA-B27 in two unrelated individuals (86).

To the best of our knowledge, these indications of public cross-reactivity of virus-specific T cells are the only ones described so far. Identification of additional public cross-reactivities of virus-specific T cells and knowledge concerning their prevalence and functional characteristics could enable monitoring and facilitate risk estimation in a transplantation setting. Therefore, we aimed to identify novel public cross-reactivities of virus-specific $CD8^+$ memory T cells directed against allogeneic HLA antigens.

MATERIALS AND METHODS

Collection of responder and target cells

Responder and target peripheral blood mononuclear cells (PBMCs) were derived from healthy donors with informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated from whole blood by standard density gradient centrifugation (Ficoll-Isopaque separation) and cryopreserved until usage.

EBV-transformed lymphoblastoid cell lines (EBV-LCLs) were generated according to standard protocol. Supernatant of the EBV-producing marmoset cell line B95.8 was added to bulk PBMCs, incubated for 1.5 h at 37°C, and cultured in RPMI 1640 Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine.

Phytohemagglutinin (PHA) blasts were generated from PBMCs by culturing in RPMI 1640 medium (Gibco) supplemented with penicillin/streptomycin, glutamine, 15% human serum (HS), and PHA (4mg/mL; Murex Biotech Ltd).

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium (Gibco) supplemented with 10% FCS, sodium pyruvate (Gibco), penicillin/streptomycin (Gibco) and 0.01% β-mercaptoethanol (0.05M; Sigma Aldrich, St. Louis, MO). HUVECs were used at passages 1-4.

Proximal tubular epithelial cells (PTECs) were cultured in DMEM ham F12 medium (Lonza) supplemented with 26µg/mL Hydrocortisone (Sigma Aldrich), 1% 1x insulin-transferrin-sodium selenite media supplement (ITS; Sigma Aldrich), 10µg/mL human Epidermal Growth Factor (hEGF; Sigma Aldrich), 0.08 pg/mL 3,3',5-Triiodo-L-thyronine sodium salt (Sigma Aldrich), and penicillin/streptomycin (Gibco). PTECs were used at P1-P4.

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.

Proliferation assays

Proliferation of virus-specific $CD8^+$ T cells was determined at bulk level by mixed lymphocyte reactions (MLRs). Responder PBMCs were labeled with carboxyfluorescein succinimidyl ester (5mM; Molecular Probes) and incubated for 8 days with 1 x 10^6 irradiated stimulator PBMCs (3000 Rad, responder:stimulator (R:S) ratio 1:1) in RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine, 15% HS and IL-2 (10 U/mL). Analysis was done by flow cytometry (FACS Calibur; BD Biosciences) using viral tetramers conjugated with phycoerythrin (PE) and CD8 antibody conjugated with allophycocyanin (APC) (Protein facility of the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands).

In addition, proliferation of $CD8^+$ T-cell clones was determined by thymidine (^3H) incorporation. $CD8^+$ T-cell clones (0.5 x 10^6 cells; responders) were stimulated with irradiated PBMCs and EBV-LCLs (0.5 x 10^6 cells; stimulators) and incorporated with ^3H-thymidine (20 µCi) at day 4. After 24 h, cells were harvested using a Tomtec cell harvester (Tomtec, Hamden, CT) and read by liquid scintillation spectrophotometry on a 1450 LSC MicroBeta TriLux microplate scintillation and luminescence counter (Perkin Elmer, Waltham, MA). The stimulation index (SI) was calculated as: mean counts per minute (CPM) of experimental wells / mean CPM unstimulated (medium-only) wells.

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

Virus-specific $CD8^+$ memory T-cell clones were generated by fluorescence-activated cell sorting (FACS; FACSAria; BD Biosciences), as described previously (118). PBMCs of healthy donors were stained with PE-labeled viral tetramers CMV pp65(123-131) HLA-B*35:01/IPSINVHHY (CMV B35/IPS), influenza (FLU) IMP(58-66) HLA-A*02:01/GILGFVFTL (FLU A2/GIL), and varicella zoster virus (VZV) IE62(593-601) HLA-A*02:01/ALWALPHAA (VZV A2/ALW) (Protein facility of the Leiden University Medical Center) and fluorescein isothiocyanate (FITC)-labeled mAb against CD4, CD19, CD45-RA, CD14, CD40, CD16 and CD56 (BD Pharmingen, San Diego, CA). The FL1 channel was used as a dump channel to avoid direct CD8 mAb staining, as simultaneous CD8/MHC staining triggers TCR internalization. TCR usage was determined by primer-based DNA sequencing using primers against the Vβ and Vα alleles (119).

For optimal conditioning, $CD8^+$ T-cell clones were cultured with irradiated PBMCs (4000 rad) from anonymous buffy coats (Sanquin, Leiden, the Netherlands) 8 days prior to functional testing.

Cytokine production assays

Interferon γ (IFNγ) production of $CD8^+$ T-cell clones was measured by ELISA according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, Utrecht, the Netherlands). Overall, 5×10^3 $CD8^+$ T cells were stimulated with 5×10^4 EBV-LCLs for 24 h at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% FCS (Lonza), 5% HS, and IL-2 (10 U/mL) in triplicate wells.

Cytotoxicity assays

Cytotoxicity was determined by ^{51}chromium (^{51}Cr)-release assay, as previously described (118, 121). In short, $CD8^+$ T-cell clones were stimulated with ^{51}Cr-labeled PHA blasts, EBV-LCLs, PTECs and HUVECs for 4 h at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% FCS (Lonza), 5% HS, and IL-2 (10 U/mL). ^{51}Cr release was measured on a γ-counter (PerkinElmer 2470 Wizard^2, PerkinElmer) and specific lysis was determined by the following calculation: (experimental ^{51}Cr release - spontaneous ^{51}Cr release) / (maximum ^{51}Cr release - spontaneous ^{51}Cr release) x 100. Spontaneous ^{51}Cr release of the target cells was determined in medium alone, and maximum ^{51}Cr release was determined by adding Triton. Values for specific ^{51}Cr lysis represent the mean ± standard deviation of triplicate wells.

RESULTS

Virus-specific CD8$^+$ T cells from different individuals proliferate in response to the same allo-HLA antigens

A cohort comprising 30 healthy individuals was screened for allo-HLA reactivity of virus-specific CD8$^+$ T cells in MLRs against a panel of stimulators expressing the most frequent HLA class I antigens (>5%) in the Western population. In multiple responders, CD8$^+$ T cells with the same viral specificity showed a similar proliferative pattern against the panel (Table 1), and additional MLRs with different stimulators confirmed corresponding allo-HLA specificity. First, CD8$^+$ T cells directed against self-HLA-B35 presenting CMV-derived peptide IPSINVHHY (pp65 123-131) proliferated in response to HLA-B51$^+$ and HLA-B58$^+$ allogeneic cells in 23% (3 of 13) of CMV B35/IPS tetramer-positive individuals. Second, CD8$^+$ T cells directed against self-HLA-A2 presenting FLU-derived peptide GILGFVFTL (MP 58-66), proliferated in response to HLA-B38$^+$ allogeneic targets in 90% (18 of 20) of FLU A2/GIL tetramer-positive individuals. Third, CD8$^+$ T cells directed against self-HLA-A2 presenting VZV-derived peptide ALWALPHAA (IE62 593-601) proliferated in response to HLA-B55$^+$ allogeneic targets in two unrelated individuals. The percentage of VZV A2/ALW tetramer-positive individuals showing this cross-reactivity could not be determined because the low precursor frequency of VZV A2/ALW-specific T cells in peripheral blood hampers their accurate detection by tetramer staining. Representative FACS plots are shown in Figure 1A, and heat maps of all MLRs are shown in Data S1.

Furthermore, CMV B35/IPS, FLU A2/GIL, and VZV A2/ALW CD8$^+$ T cells from individuals that expressed the cross-reactive HLA antigen themselves did not proliferate in response to the latter, indicating self-tolerance. Interestingly, CMV B35/IPS CD8$^+$ T cells from an HLA-B35$^+$B51$^+$ heterozygous individual did not proliferate against syngeneic HLA-B51 but did against allogeneic HLA-B58 (Figure 1B).

Table 1. Cohort of healthy individuals

	Relevant self-HLA	Tetramer-positive	Cross-reactive
CMV B35/IPS	16	13	3
FLU A2/GIL	22	20	18
VZV A2/ALW	22	n.a.	1[1]

CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; n.a. = not applicable

[1]This cross-reactivity has been identified in two additional individuals outside the cohort

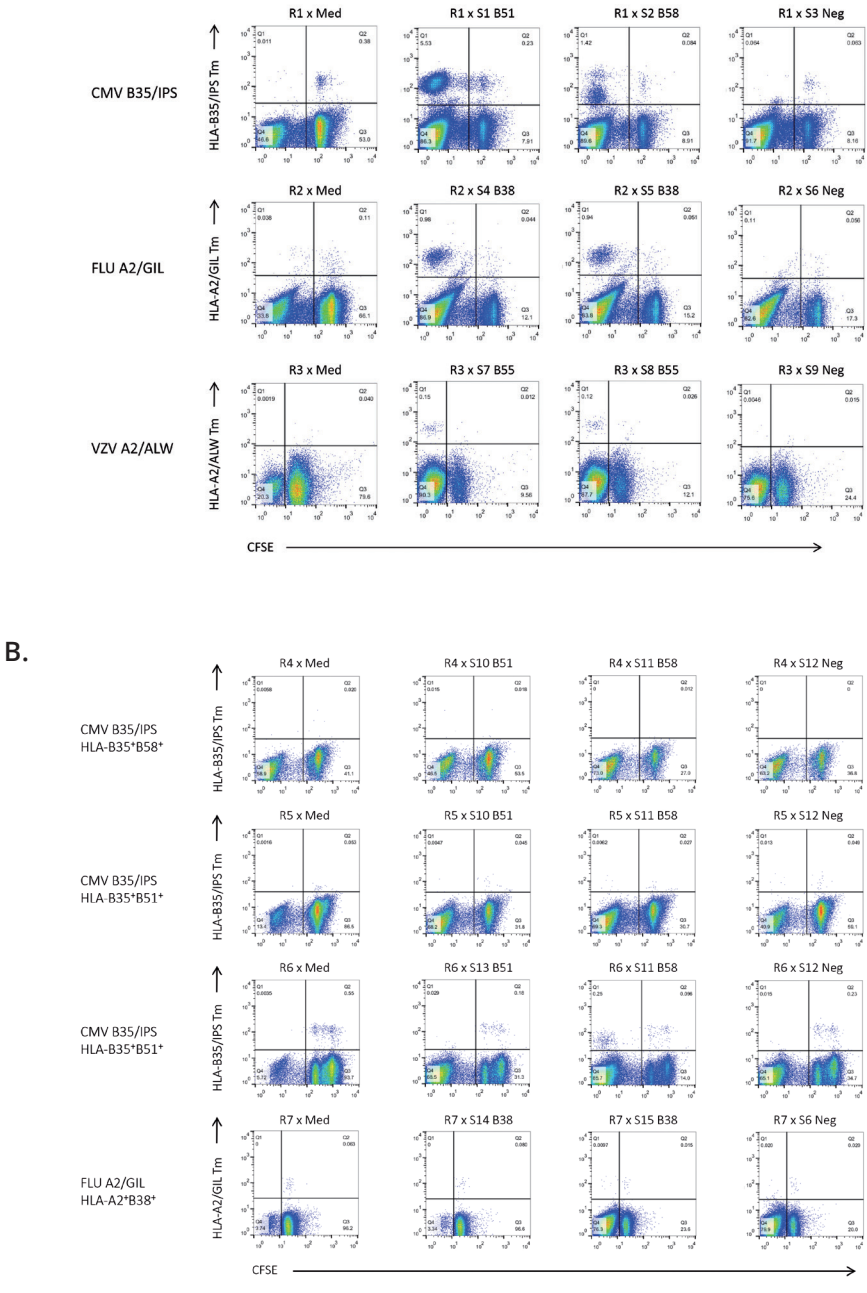

Figure 1. Proliferation of virus-specific CD8⁺ T cells directed at dominant viral epitopes in response to allogeneic HLA. (A) Proliferation of CMV B35/IPS, FLU A2/GIL, and VZV A2/ALW CD8⁺ T cells against respectively allogeneic HLA-B51 and -B58, HLA-B38 and HLA-B55 in mixed lymphocyte reaction. Shown plots are representative examples of positive responders that showed CMV B35/IPS T-cell proliferation (3

of 13 responders), FLU A2/GIL T-cell proliferation (18 of 20 responders) and VZV A2/ALW T-cell proliferation (2 of 2 responders). (B) Proliferation of CMV B35/IPS and FLU A2/GIL CD8$^+$ T cells of individuals expressing the cross-reactive HLA antigen in response to allogeneic cells expressing the antigen. In total, 4 HLA-B35$^+$B51$^+$, 1 HLA-B35$^+$B58$^+$ and 4 HLA-A2$^+$B38$^+$ responders were tested against 5 HLA-B51$^+$, 5 HLA-B58$^+$ and 6 HLA-B38$^+$ stimulators respectively. Representative examples are shown. All plots were gated on PBMCs and CD8$^+$ antibody staining. Full HLA typing of the responders and stimulators is included in Data S2. CFSE, carboxyfluorescein succinimidyl ester; CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Shared allo-HLA cross-reactivity is assigned to public TCR usage of virus-specific CD8$^+$ memory T cells

CMV B35/IPS-, FLU A2/GIL-, and VZV A2/ALW-specific CD8$^+$ memory T-cell clones were generated from the individuals that showed proliferation in response to identical allo-HLA antigens, and highly conserved TCR Vα and Vβ usage was observed. Remarkably, the TCR usage of CMV B35/IPS- and FLU A2/GIL-specific T-cell clones derived from individuals also expressing the cross-reactive HLA antigen highly resembled the TCR usage of allo-HLA cross-reactive T-cell clones. Only minor amino acid differences within the CDR3α (FLU A2/GIL) and CDR3β (CMV B35/IPS) regions were able to abrogate the occurrence of cross-reactivity (Table 2, Data S2).

Stimulation with allogeneic PBMCs confirmed that T-cell clones expressing public TCRs were able to proliferate in response to the cross-reactive alloantigen. Allorecognition of HLA-B51, in contrast to HLA-B58, induced robust proliferation of cross-reactive CMV B35/IPS T cells, similar to stronger compared with cognate antigen (B35 + IPS). Allorecognition of HLA-B38 also induced comparable proliferation of cross-reactive FLU A2/GIL T cells as cognate antigen (A2 + GIL), whereas HLA-B55 allorecognition induced less pronounced proliferation of VZV A2/ALW T cells compared to cognate antigen (A2 + ALW). T-cell clones derived from individuals that also expressed the cross-reactive HLA antigen (CMV B35/IPS 8A2, FLU A2/GIL 4A2) did not proliferate in response to syngeneic cross-reactive HLA. Of interest, HLA-B38 allorecognition of FLU A2/GIL T cells was hampered by differences in Va usage (FLU A2/GIL 3F8), and even in CDR3a usage alone (FLU A2/GIL 4A2) (Figure 2).

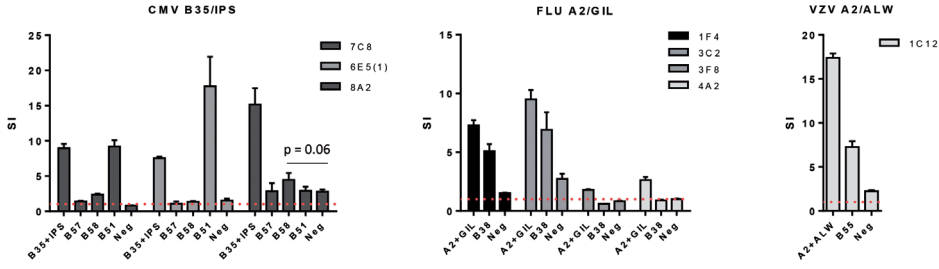

Figure 2. Allo-HLA cross-reactive cytomegalovirus-, influenza-, and varicella zoster virus-specific CD8⁺ memory T-cell clones are able to proliferate in response to allogeneic PBMCs. Clones derived from individuals expressing the cross-reactive HLA antigen (CMV B35/IPS 8A2, FLU A2/GIL 4A2) did not. SI = stimulation index. All experiments were performed in triplicate, and error bars represent the standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; SI, stimulation index; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Virus-specific CD8⁺ memory T-cell clones with public cross-reactive TCRs produce IFNγ on stimulation with allogeneic targets

IFNγ production of the T-cell clones was determined by ELISA. CMV B35/IPS-, FLU A2/GIL-, and VZV A2/ALW-specific CD8⁺ memory T-cell clones bearing public TCRs produced IFNγ in response to EBV-LCLs expressing self-HLA and viral peptide and EBV-LCLs expressing cross-reactive allo-HLA antigens. Interestingly, minor HLA-B57 cross-reactivity was observed for HLA-B58 cross-reactive CMV B35/IPS T cells (Figure 3A and 3B), whereas no significant proliferation against HLA-B57 was observed in MLR at the bulk or clonal level (data not shown; Figure 2). Cross-reactive FLU A2/GIL T-cell clones produced IFNγ mainly on HLA-B*38:01 allorecognition, whereas HLA-B*38:02 allorecognition resulted in only limited IFNγ production. FLU A2/GIL T-cell clones with the same Vb but different Va usage did not cross-react against HLA-B38. Cross-reactive VZV A2/ALW T-cell clone 1C12 produced IFNγ on HLA-B55⁺ allorecognition, comparable to VZV A2/ALW T-cell clone 12, as published previously (193). Furthermore, despite highly similar TCR usage, virus-specific T cells isolated from individuals carrying cross-reactive HLA antigens did not produce IFNγ when stimulated with syngeneic HLA. Interestingly, CMV B35/IPS T-cell clone 8A2 (derived from the heterozygous HLA-B35⁺B51⁺ individual that showed minor proliferation in response to HLA-B58) produced high levels of IFNγ on recognition of HLA-B58⁺ EBV-LCLs, and limited levels on recognition of HLA-B57⁺ EBV-LCLs (Figure 3B). Heat maps of the IFNγ ELISAs are included in Data S1, and the allospecificity of all T-cell clones is indicated in Table 2.

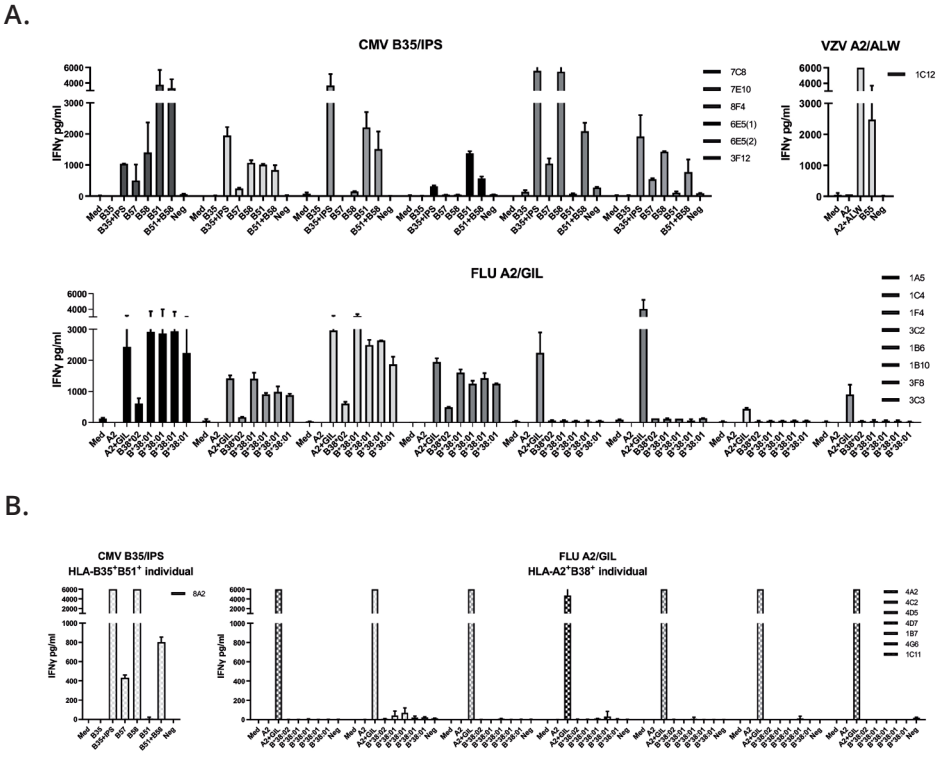

Figure 3. Allo-HLA cross-reactive virus-specific CD8⁺ memory T cells from different individuals produce IFNγ in response to allogeneic EBV-LCLs. (A) Cross-reactive CMV B35/IPS T-cell clones produced IFNγ upon HLA-B51 and HLA-B58 allorecognition. In addition, HLA-B58 cross-reactive CMV B35/IPS T cells consistently produced limited amounts of IFNγ upon HLA-B57 allorecognition. Cross-reactive FLU A2/GIL T-cell clones produced IFNγ upon HLA-B*38:01, and to a limited extent, HLA-B*38:02 allorecognition. Finally, HLA-B55 allorecognition induced IFNγ production of cross-reactive VZV A2/ALW T-cell clone 1C12. (B) Virus-specific CD8⁺ memory T-cell clones derived from individuals expressing the cross-reactive HLA antigen do not produce IFNγ against EBV-LCLs expressing syngeneic HLA. All experiments were performed in triplicate at E:T ratio 1:10, and error bars represent the standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; IFNγ, interferon γ; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Public allo-HLA cross-reactive CMV B35/IPS-, FLU A2/GIL-specific CD8$^+$ memory T cells are cytotoxic, unlike public allo-HLA cross-reactive VZV A2/ALW-specific CD8$^+$ memory T cells

^{51}Cr-release assays showed efficient lysis of HLA-B51$^+$ EBV-LCLs and PHA blasts by HLA-B51 cross-reactive CMV B35/IPS T cells, whereas HLA-B58/B57 cross-reactive CMV B35/IPS T cells (including T-cell clone 8A2 derived from the heterozygous HLA-B35$^+$B51$^+$ individual) were unable to lyse HLA-B58$^+$ and HLA-B57$^+$ EBV-LCLs and PHA blasts**.** Interestingly, the same applied for T cells that recognized all three allo-HLA antigens. Furthermore, FLU A2/GIL T-cell clones efficiently lysed HLA-B*38:01$^+$ EBV-LCLs and PHA blasts, whereas HLA-B*38:02$^+$ EBV-LCL lysis was considerably lower. None of the FLU A2/GIL T-cell clones derived from the HLA-A2$^+$B38$^+$ individual were cytotoxic toward HLA-B38 targets. VZV A2/ALW T-cell clones showed only minor cytotoxicity towards HLA-B55$^+$ EBV-LCLs and PHA blasts, below the generally applied cut-off level of 10% (194) (Figure 4A).

Finally, additional ^{51}Cr-release assays were performed with PTECs and HUVECs as targets to determine possible tissue specificity of the alloimmune response. HLA-B51 cross-reactive CMV B35/IPS T cells were able to lyse HLA-B51$^+$ PTECs and HUVECs with efficiency comparable to PHA-blasts and EBV-LCLs. Similar to HLA-B58$^+$ EBV-LCLs and PHA blasts, HLA-B58/B57 cross-reactive CMV B35/IPS T cells were incapable of lysing HLA-B58$^+$ and HLA-B57$^+$ PTECs. HLA-B58$^+$ HUVECS were not available for testing, but no cytotoxicity was observed against HLA-B57$^+$ HUVECs. Furthermore, HLA-B38 cross-reactive FLU A2/GIL T cells efficiently lysed HLA-B38$^+$ PHA-blasts, EBV-LCLs, HUVECs and PTECs, although the latter to a lesser extent. VZV A2/ALW cross-reactive T cells showed consistent limited cytotoxicity towards HLA-B55$^+$ PHA blasts, EBV-LCLs, PTECs, and HUVECs. Finally, FLU A2/GIL T-cell clones generated from the HLA-A2$^+$B38$^+$ individual again showed no recognition of HLA-B38$^+$ targets (Figure 4B). Cytotoxicity results of the T-cell clones that were able to lyse target cells expressing the cross-reactive HLA antigen are summarized in Table 3.

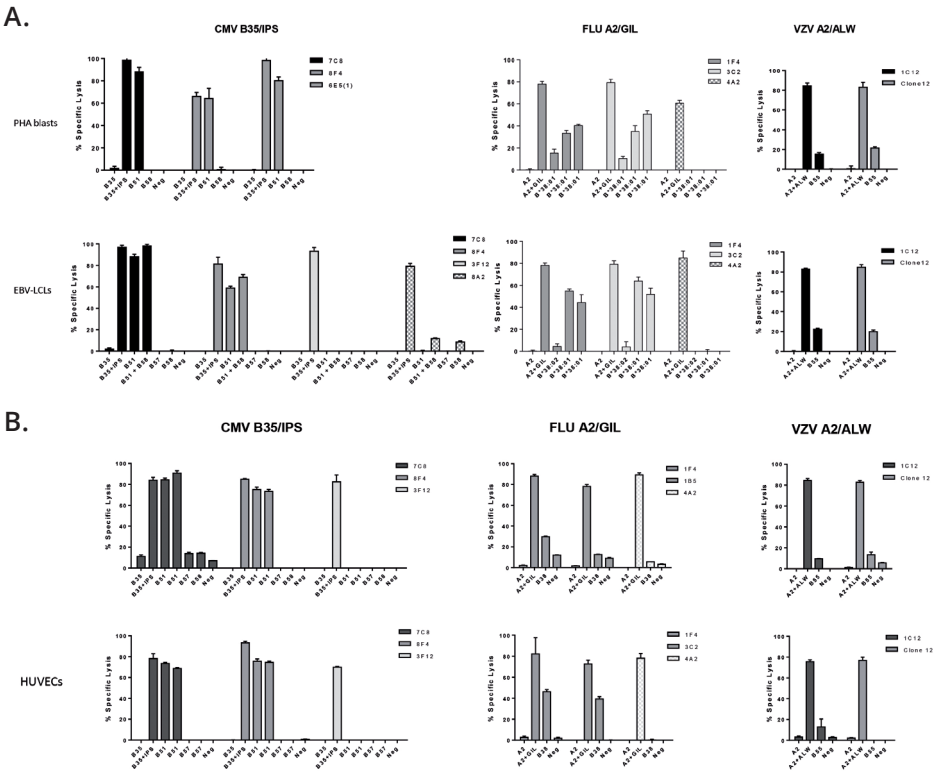

Figure 4. Allo-HLA cross-reactive virus-specific CD8⁺ memory T cells from different individuals are cytotoxic to allogeneic targets. Cytotoxicity was observed to (A) PHA blasts and EBV-LCLs, and (B) PTECs and HUVECs expressing the cross-reactive allo-HLA molecules. Non-cross-reactive T cells (including T cells from individuals expressing the cross-reactive HLA) were not cytotoxic to allogeneic targets. Plots are representative examples, all experiments were performed in triplicate and at different effector:target (E:T) ratios (30:1; 10:1; 1:1; 0.1:1); E:T ratio 30:1 is shown. Error bars represent standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; HUVEC, human umbilical vein endothelial cell; PHA, phytohemagglutinin; PTEC, proximal tubular epithelial cell; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

DISCUSSION

Because public cross-reactivity hinges on strict TCR preservation in multiple individuals, it is allegedly uncommon. Nevertheless, using a small cohort and a restricted set of viral tetramers, we were able to identify three novel public allo-HLA cross-reactivities of virus-specific memory T cells. All TCRs of HLA-B51- and HLA-B58/57-cross-reactive CMV B35/IPS T cells shared strong public features (187) and only minor amino acid differences within their CDR3 loops differentiated between HLA-B51 and HLA-B58/B57 allorecognition. TCR clonotypes recognizing HLA-B51, HLA-B58/B57, and the combination were detected alongside each other in different individuals. FLU A2/GIL cross-reactivity against HLA-B38 was observed in almost all (18 of 20) tetramer-positive individuals, and TCR usage was identical to an abundant public FLU A2/GIL TCR clonotype described in literature (12, 195). Although public TCR usage has not been described for VZV A2/ALW-specific T cells, $CD8^+$ T-cell responses against the VZV A2/ALW epitope are shared by the majority of HLA-$A2^+$ individuals (196) and HLA-B55 cross-reactivity of VZV A2/ALW-specific T cells with corresponding TCR Vβ usage has in fact been identified previously in yet another individual (43). The nearly identical TCR usage in three unrelated individuals again firmly points toward the involvement of a public TCR.

FLU A2/GIL and CMV B35/IPS T cells sorted from HLA-$A2^+B38^+$ and HLA-$B35^+B51^+$ individuals, respectively, did not recognize HLA-B38 and HLA-B51 alloantigens, which is likely the result of thymic TCR selection to avoid autoimmunity. This confirms the observation in HLA-$B8^+B^*44{:}02^+$ heterozygous individuals, which select oligoclonal EBV B8/FLR TCRs that are completely disparate from the public HLA-B^*44:02-cross-reactive TCR (197, 198). Furthermore, even individuals heterozygous for HLA-B^*08:01 and HLA-B^*44:03 (which highly resembles HLA-B^*44:02 but is not functionally cross-reactive) select EBV B8/FLR TCRs that are highly distinct from the public HLA-B^*44:02-cross-reactive TCR (199). In contrast, we did not find such cautious TCR selection. First, unlike HLA-B^*44:03 skewing of the EBV B8/FLR TCR repertoire, self-expression of HLA-B51 did not abrogate HLA-B58 cross-reactivity of CMV B35/IPS cross-reactive T cells in a heterozygous HLA-$B35^+B51^+$ individual. Moreover, FLU A2/GIL and CMV B35/IPS TCRs from HLA-$A2^+B38^+$ and HLA-$B35^+B51^+$ individuals, respectively, were nearly identical to the public cross-reactive TCRs, and only small amino acid insertions within both CDR3 regions (CMV B35/IPS) and even within the CDR3α region alone (FLU A2/GIL) were able to abrogate cross-reactivity. Plausibly, this exceptionally conserved TCR usage illustrates the superiority of these TCR clonotypes in generating antiviral immune responses.

The mechanisms by which the newly identified public TCRs cross-react to allo-HLA remain unknown, however, because HLA-B35 is structurally highly similar to HLA-B51 and HLA-B58/B57, and HLA-B35 and HLA-B51 present a similar peptide repertoire (200, 201), the CMV-B35/IPS cross-reactivity against HLA-B51 and HLA-B58/B57 may be explained by structural mimicry. Structural mimicry between an allo- and viral epitope is the most described mechanism of TCR cross-reactivity and underlies EBV B8/FLR cross-reactivity against HLA-B*44:02 as well as HLA-B*35:01 (44, 135). Both FLU A2/GIL and VZV A2/ALW public cross-reactivities were directed against allo-HLA antigens with only little similarity to the cognate HLA antigen. Although this does not exclude structural mimicry (after all HLA-B*08:01 and HLA-B*44:02 show substantial polymorphism and divergent peptide expression), the finding that FLU A2/GIL cross-reactivity appears to depend on CDR3a usage, whereas TCR docking to the cognate viral epitope depends primarily on binding of the Vβ chain (9, 202), could indicate a different mechanism for allorecognition and points toward an altered TCR docking mode, but this should be investigated further.

The strong functional difference in HLA-B51 versus HLA-B58/B57 allorecognition by CMV B35/IPS T-cell clones is remarkable. A possible explanation may be different TCR affinities for these alloantigens. The small amino acid differences in the α_1 domain between HLA-B51 and HLA-B58/B57 for example, may affect TCR binding (203), especially because these involve the "generic" MHC class I restriction elements on positions 65, 66, and 69 (190). These restriction elements are conserved for HLA-B35 and HLA-B51. In addition, variations in peptide presentation between HLA-B51 and HLA-B58/B57 could affect TCR avidity and lead to suboptimal or alternative TCR signaling and T-cell activation in response to HLA-B58/B57 (204, 205). However, for conclusive statements on the mechanisms behind TCR cross-reactivity, the (allo)peptides presented in the cross-reactive HLA should be identified and crystal structures need to be assembled, which was beyond the scope of this research.

We recently identified FLU A2/GIL cross-reactivity to donor HLA-B38 in an HLA-A2$^+$ renal transplant patient (38), emphasizing that (public) cross-reactivities can indeed present in a clinical setting. As one of the key endeavors in transplantation research is to predict alloreactivity, public cross-reactivity of virus-specific TCRs could provide a useful tool. However, potential predictive value will differ by public cross-reactivity.

The prevalence of the public cross-reactive TCR clonotypes, and thus the percentage of patients that harbor these TCRs, greatly affects prediction. The here-described FLU A2/GIL public cross-reactivity against HLA-B38 is highly abundant, with 90% of FLU A2/GIL tetramer-

positive individuals expressing the public HLA-B38 cross-reactive TCR. In fact, this public TCR is considered the most abundant human TCR clonotype (9). This high prevalence is facilitated by the facts that HLA-A2 is the most common HLA allele worldwide, that infection with the influenza virus occurs in virtually all individuals, and that the $CD8^+$ T-cell response to influenza is dominated by T cells that recognize the HLA-A2/GIL epitope. The CMV B35/IPS HLA-B51 and HLA-B58/B57 cross-reactive TCR clonotypes were found in 23% of CMV B35/IPS tetramer-positive individuals. Although HLA-B35 is less predominant than HLA-A2, CMV affects approximately 40-100% of the world population and is one of the most prevailing infections after transplantation. The prevalence of the HLA-B55 cross-reactive VZV A2/ALW TCR cross-reactivity remains to be established, because the low precursor frequency of VZV A2/ALW-$tetramer^+$ T cells in the peripheral blood hampers its accurate detection. Yet, VZV also affects nearly all individuals, the majority of $HLA\text{-}A2^+$ individuals show $CD8^+$ T-cell responses against the VZV A2/ALW epitope (196), and the identification of highly identical TCRs in different unrelated individuals suggests it is indeed common.

Furthermore, functionality differs by cross-reactivity. CMV B35/IPS cross-reactivity against HLA-B51 and HLA-B58/B57 were both characterized by proliferation and IFNγ production, but only HLA-B51 cross-reactivity induced cytotoxicity. FLU A2/GIL T cells were able to proliferate, produce IFNγ, and exert cytotoxicity toward $HLA\text{-}B38^+$ targets, whereas VZV A2/ALW-specific T cells expressed limited to no cytotoxicity but were able to proliferate and produce IFNγ upon allorecognition. Nevertheless, it is still unclear which functional characteristics of cross-reactive T cells in vitro affect clinical outcome: where cytotoxic responses may harm the allograft directly, cytokine production could harm the allograft indirectly by inducing an inflammatory environment (113). In addition, one should keep in mind that functionality may differ in response to different cell types (tissue specificity), as shown for the public EBV-B8/FLR cross-reactivity to allo-$HLA\text{-}B^*44{:}02$ (87). When the cross-reactive allopeptide has a restricted tissue distribution, (public) TCR cross-reactivity may be restricted to specific organs, whereas if it is expressed in multiple cell types, it could have broader implications for organ transplantation. In our experiments, there were no indications for tissue-specificity of CMV B35/IPS cross-reactivity against HLA-B51 or VZV A2/ALW cross-reactivity against HLA-B55. HLA-B38 cross-reactive FLU A2/GIL T cells, however, appeared less potent in lysing PTECs compared with HUVECs, EBV-LCLs, and PHA blasts, suggesting there may be a certain degree of tissue specificity.

Furthermore, precursor frequencies should be determined because they could affect clinical outcome. Unlike low precursor frequencies, high precursor frequencies of alloreactive memory T cells are associated with impaired tolerance induction and contribution to graft rejection

and graft-versus-host disease (61, 84, 109, 206, 207); therefore, cross-reactive T-cell precursor frequencies strongly determine alloreactivity predictive value and should be determined per individual. Of note, precursor frequencies of virus-specific T cells have been shown to be subject to some fundamental principles that apply to most individuals and that could aid in initial risk estimation. FLU-specific memory T cells, for example, compose a small proportion of the T-cell repertoire, plausibly due to clearance of the virus after infection. CMV-specific memory T cells, in contrast, make up a substantial part of the $CD8^+$ T-cell repertoire (208), likely because CMV is (like EBV) a herpes virus that latently persists after infection. In addition, they have been shown to dominate T-cell repopulation following lymphodepletion (209) and are even suggested to promote solid organ rejection (42). Despite VZV also being a persistent virus, the precursor frequency of VZV A2/ALW-specific memory T cells is low: perhaps due to VZV "hiding" from the immune system in nerve cells. Nevertheless, the precursor frequency of HLA-B55 cross-reactive VZV A2/ALW T cells could increase as a result of routine vaccination against VZV in transplantation recipients. Indeed, one of the HLA-B55 cross-reactive VZV A2/ALW T-cell clones studied in this paper (Clone 12) was derived from a transplant recipient that was vaccinated against VZV (193). In addition, adoptive transfer of enriched virus-specific T cells may introduce cross-reactive virus-specific T cells at high precursor frequencies.

In conclusion, we were able to identify three novel public cross-reactivities of virus-specific memory T cells in a small cohort of individuals, while subjected to a restricted number of known dominant viral epitopes and tetramers. It is therefore expected that this is only the tip of the iceberg - and public cross-reactivity of virus-specific T cells is much more common than anticipated. Increasing the knowledge concerning public cross-reactivities could benefit the prediction of anti-donor reactivity, enable monitoring of potentially harmful alloresponses, and ultimately support clinical decision making.

Table 2. TCR usage of virus-specific CD8⁺ memory T cells in different individuals

	Donor	Clone	TRAV	TRAJ	CDR3α	TRBV	TRBJ	TRBD	CDR3β	Allo-HLA cross-reactivity
CMV B35/IPS	*#1*	7C8	TRAV17*01	TRAJ33*01	C A T E T S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S L S T S P N E K L F F	B51+B58
		6E5(1)	TRAV17*01	TRAJ33*01	C A V E D S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S D G T G P N E K L F F	B51
		6A4	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P A G G P N E K L F F	B58
	#2	3F12	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5D12	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5F8	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5G11	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
	#3	6C8	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		6B11	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		6E5(2)	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S Q T G P N E K L F F	B58
		8F4	TRAV17*01	TRAJ33*01	C A T E D S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S A G T G P N E K L F F	B51
		8F11	TRAV17*01	TRAJ53*01	C A T E S S N Y K L T F	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		7E10	TRAV17*01	TRAJ33*01	C A T E H S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S L S T A P N E K L F F	B51+B58
	#4	8A2	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S T T S G G P N E K L F F	B58
FLU A2/GIL	*#1*	1A5	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		1B6	TRAV29DV5*01	TRAJ38*01	n.d.	TRBV19*01/02/03	TRBJ2-7*01	TRBD2*02	C A S S I R S T G E L F F	-
		1B10	TRAV29DV5*01	TRAJ38*01	n.d.	TRBV19*01/02/03	TRBJ2-7*01	TRBD2*02	C A S S I R S T G E L F F	-
		1C4	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38

Table 2. Continued

	Donor	Clone	TRAV	TRAJ	CDR3α	TRBV	TRBJ	TRBD	CDR3β	Allo-HLA cross-reactivity
		1F4	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
	#3	1B5	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		1B7	TRAV8-6*01	TRAJ42*01	C A V S G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	C A S S L R S S Y E Q Y F	-
		1C11	TRAV17*01	TRAJ42*01	C A T D E G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-2*01	TRBD1*01	C A S S M R S T G E L F F	-
	#5	3C2	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		3C3	TRAV38-1*01/04	TRAJ52*01	C A F M I G A G G T S Y G K L T F	TRBV19*01/02/03	TRBJ1-2*01	TRBD1*01	C A S S I G A Y G Y T F	-
		3F8	TRAV41*01	TRAJ42*01	C A V D A G G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
	#6	4A2	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4C2	TRAV27*03	TRAJ42*01	C A G A G G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-2*01	TRBD1*01	C A S S G R A T G E L F F	-
		4D5	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4D7	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4G6	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
VZV A2/ALW	#7	1C12	TRAV12-2*01	TRAJ40*01	C A A F T S G T Y K Y I F	TRBV27	TRBJ1-1*01	TRBD1*01	C A S S S D R G Y E A F F	B55
	#8	Clone 12	TRAV12-2*01	TRAJ40*01	C A V F T S G T Y K Y I F	TRBV27	TRBJ2-7*01	n.d.	C A S S S D R G Y E Q Y F	B55

Sequences indicated in bold: identical CDR3 regions (indicated per viral specificity)

Table 3. Cytotoxic potential of virus-specific memory CD8+ T-cell clones with public TCR usage able to lyse allogeneic cells expressing cross-reactive HLA antigens[a,b]

	Donor	Clone	Allo-HLA	PHA-blast	EBV-LCL	PTEC	HUVEC
CMV B35/IPS	1	7C8	B51	92 (±2.1)[1]	83 (±17.7)[1]	104	98 (±9.2)[1]
		6E5(1)	B51	49	44 (±7.1)[1]	n.d.	76
	3	8F4	B51	97	73 (±15.4)[1]	87	91 (±15.6)[1]
FLU A2/GIL	1	1A5	B38	43	53	n.d.	58
		1F4	B38	47	63	34	56 (±0.7)[1]
		1C4	B38	48	65 (±2.1)[1]	67	n.d.
	3	1B5	B38	5	33	16	n.d.
	4	3C2	B38	54	63 (±14.1)[1]	n.d.	54
VZV A2/ALW	5	Clone 12	B55	24(±2.9)[1]	21 (±9.1)[1]	16	0
	6	1C12	B55	14(±6.1)[1]	11 (±0.7)[1]	11	13 (±8.7)[1]

CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; HUVEC, human umbilical vein endothelial cell; PHA, phytohemagglutinin;
PTEC, proximal tubular epithelial cell; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA. n.d. = not determined.

[a]Numbers represent the allo-HLA response as a percentage of the anti-virus response

[b]All experiments were performed in triplicate and at different effector: target (E:T) ratios (30:1, 10:1, 1:1, 0.1:1) of which E:T ratio 30:1 is shown

[1]Means of two independent experiments with standard deviation

SUPPLEMENTARY MATERIAL

Data S1

Data S1 can be found at: https://tinyurl.com/yyx8ygj4

Data S2.

Figure 1	HLA typing
R1 (= Donor #1 Table 2)	A*02:01 A*11:01 **B*35:01** B*40:01 C*03:04 C*04:01 DRB1*15:01 DRB1*11:01 DQB1*06:02 DQB1*03:01
R2 (= Donor #3 Table 2)	**A*02:01** A*24:02/24:09N/24:11N+ B*08:01/08:05/08:08N+ B*35:01/35:07/35:11+ C*03:04/03:05/03:08+ C*04:01/04:09N/04:05+ DRB1*03:01 DRB1*13:02 DRB3*01:01 DRB3*03:01 DQB1*02:01 DQB1*06:04 DQA1*01:02 DQA1*05:01/05:03 DPB1*04:01 DPB1*10:01
R3 (= Donor #7 Table 2)	**A2** A3 B51(5) B7 Cw2 Cw7 DR15 DR13 DQ6 DQ1
R4	A2 A24(9) **B35 B58(17)** Cw3 DR2 DR13 DR6 DR51 DR52 DQ6 DQ1
R5	A*03:01 A*31:01 **B*35:01 B*51:0**1 C*04:01/04:09N/04:05 C*15 DRB1*01:01 DRB1*09 DRB4*01:03 DQB1*03:03 DQB1*05:01 DPB1*04:02
R6 (= Donor #4 Table 2)	A*03:01/03:03N/03:04+ A*24:02/24:05/24:09N+ **B*35:01/35:07/35:11+ B*51:01/51:03/51:09+** C*07:02/07:03/07:10+ C*14:02/14:07N DRB1*04:07 DRB1*15:01 DRB4*01:03 DRB5*01:01, DQB1*03:01 DQB1*06:02
R7 (= Donor #6 Table 2)	**A*02:01** A*26:01 **B*38:01** B*55:01 C*01:02 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03
S1	A2 A3 **B51(5)** B7 Cw2 DR15(2)(51) DR11(5)(52) DQ6(1) DQ7(3)
S2	A11 A31(19) **B58(17)** B18 Cw7 DR11(5)(52) DR8 DQ7(3) DQ4(3)
S3	A3 A31(19) B60(40) Cw10 Cw3 DR4(53) DR11(5)(52) DQ7 DQ3
S4	A1 A24(9) B8 **B38(16)** DR4 DR14(6) DQ5(1) DQ8(3)
S5	A*24:02 A*26:01 **B*38:01** B*35:03 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03 DQB1*06:03
S6	A*03:01 A*26:01 B*07:02 B*40:01 C*03:04 C*07:02 DRB1*15 DRB1*04:04 DQB1*06:02 DQB1*03:02
S7	A*03 A*24 B*15:01/15:33/15:34+ **B*55:01/55:02/55:05+** C*03 DRB1*04 DRB1*13:01/13:02/13:06+ DRB3*03 DRB4 DQB1*03:02/03:07/03:08 DQB1*06:04/06:08/06:17
S8	A24(9) A30(19) B13 **B55(22)** Cw9 Cw3 Cw6 DR11 DR5 DR13 DR6 DR52 DQ6 DQ1 DQ7 DQ3
S9	A24(9) A11 B35 B60 (40) Cw10 Cw3 Cw4 DR15 DR2 DR4 DR51 DR53 DQ6 DQ1 DQ7 DQ3
S10	A24(9) A68(28) **B51(5)** B45(12) DR13 DR6 DR10 DR52 DQ5 DQ6 DQ1
S11	A*0301 A*2402 B*0702 **B*58:01** Cw*0701 DRB1*01 DRB1*1301
S12	A*01:01 A*02:01 B*08:01 C*07:01/07:06/07:07 DRB1*03:01/03:04/03:05+ DRB3*01:01 DQB1*02:01/02:02/02:04 DQA1*05:01/05:03 DPB1*01:01 DPB1*04:02
S13	A*02:01 A*03:01 B*07:02 **B*51:01** C*02:02 C*07:02 DRB1*11:01 DRB1*15:01 DRB3*02:02 DRB5*01:01
S14	A*02:01 A*26:01 **B*38:01** B*55:01 C*01:02 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03
S15	A2 **B38(16)** B72(70) Cw2 DR3 DR13(6) DQ1

Data S2. Continued

Table 2	HLA typing
Donor 1	**A*02:01** A*11:01 **B*35:01** B*40:01 C*03:04 C*04:01 DRB1*15:01 DRB1*11:01 DQB1*06:02 DQB1*03:01
Donor 2	A1 A11 B8 **B35** Bw6 Cw4 Cw7 DRB1*0103 DRB1*0301 DQ5 DQ2
Donor 3	**A*0201** A*2402 B*0801 **B*3501** Cw*0304 Cw*0401 DRB1*0301 DRB1*1302
Donor 4	A3 A24(9) **B51(5) B35** C*0702 C*14:02 DRB1*04:07 DRB1*15:01 DRB4*01:03 DRB5*01:01 DQB1*03:01 DQB1*06:02
Donor 5	**A*02** A*03 B*07 B*35 C*04 C*07 DRB1*01 DRB1*08 DQB1*04:02 DQB1*05:01
Donor 6	**A*02:01** A*03:01 B*37:01 **B*38:01** C*06:02 C*12:03 DRB1*03:01 DRB1*13:01 DQB1*02:01 DQB1*06:03
Donor 7	**A2** A3 B51(5) B7 Cw2 Cw7 DR15 DR13 DQ6 DQ1
Donor 8	**A*02:01** B*13:02 B*40:01 C*03:04 C*06:02 DRB1*07:01 DRB4 DQB1*02:01