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Stimulation of Human EBV- and CMV-Specific Cytolytic Effector Function Using Allogeneic HLA Molecules

Lloyd J. D’Orsogna,*†‡,§ Heleen van den Heuvel,* Ellen M. W. van der Meer-Prins,* Dave L. Roelen,* Ilias I. N. Doxiadis,* and Frans H. J. Claas*

Viral infection is a major cause of morbidity and mortality, and there are few therapeutic options available to augment a virus-specific T cell response. Although allo-HLA cross-reactivity from virus-specific memory T cells is common, it is unclear whether priming with specific allogeneic cells could conversely elicit a viral peptide/self-HLA restricted cytotoxic T cell response in humans. First, we used the previously described allo-HLA-B*44:02 cross-reactivity of EBV peptide/HLA-B8 restricted T cells, to determine whether allogeneic HLA stimulation can elicit a cytolytic immune response against EBV. EBV-B8* HLA-B44* EBV-seropositive PBMCs were stimulated with either HLA-B*44:02* or HLA-B*44:03* mismatched irradiated PBMCs in a 7–10 d MLR. The allo-HLA stimulated responder cells were then evaluated for cytotoxicity using EBV peptide loaded autologous target cells and unloaded HLA-B8* EBV LCL target cells. PBMCs from EBV-seropositive donors gained EBV-specific cytolytic effector function following specific allo-HLA stimulation. Finally, we also elicited cytolytic CMV-specific responses using specific allogeneic cell stimulation, to confirm that this technique can be used to elicit viral peptide/self-HLA restricted responses even from nonpublic TCR responses. Allogeneic cell stimulation used as a cell therapy may be a potential tool to augment antiviral T cell response in patients with EBV or CMV infection. The Journal of Immunology, 2012, 189: 4825–4831.

Control of viral replication depends primarily on virus-specific memory T-lymphocyte activity (1, 2). In the normal course of viral infections, antiviral immunity and noninfecitivity correlates with the development of virus-specific effector memory T cells. Absence of HIV-specific CD8 T cells is associated with progression to AIDS in HIV-infected individuals (3), and the use of lymphocyte-targeted biologic therapies has recently been associated with viral reactivation that might not respond to antiviral antibiotics (4). For example, whereas allogeneic marrow depleted of T cells prevents acute and chronic forms of graft-versus-host disease (GVHD) posttransplant, the risk of infections, particularly with EBV and CMV, is increased (5). Furthermore, viral infection can cause severe morbidity and mortality, even in healthy individuals without specific immune defects.

New therapies are therefore required to increase the number and/or effector function of virus-specific T cells. Antiviral prophylaxis can be toxic and does not result in an increase in virus-specific T cells, nor does it achieve long-term eradication. Adoptive transfer of third-party cell lines may be associated with GVHD or failure due to allogeneic rejection (6), and is technically difficult (7). Peptide stimulation does not induce a polyclonal T cell response and can fail to induce cytotoxic CD8 T cell responses (8). Furthermore, although Ag-specific T cell responses are actively maintained, they are reversible and short lived in the absence of stimulating Ag (9–11).

We have recently confirmed that alloreactivity from virus-specific T cells is common, and that the allo-HLA reactivity and virus specificity is mediated via the same TCR (12). Forty-five percent of virus-specific CD4 and CD8 T cell clones were shown to be cross-reactive against allo-HLA molecules. For example, EBV infection in a HLA-B8* individual always selects for a dominant "public" Vb6S2 TCR (13), which cross-reacts against allo-HLA-B*44:02 (14). We confirmed the previously described alloreactivity of this EBV EBNA3A-specific T cell (HLA-B8/FLRGRAYGL restricted) against allogeneic HLA-B*44:02 (12, 15). Allo-HLA cross-reactivity was also shown for CMV, varicella-zoster virus (VZV) and influenza virus–specific T cells, which express nonpublic TCRs (12).

A high level of cross-reactivity against allo-HLA molecules is therefore an essential feature of the virus-specific memory TCR (12–22). This allo-HLA cross-reactivity by virus-specific T cells can be reproducibly detected in vitro. However, it is currently unknown whether stimulation with allogeneic-HLA molecules could conversely and specifically augment an HLA-restricted virus-specific T cell response.

The purpose of this study was to assess whether allogeneic HLA challenge could be a useful tool to augment an HLA-restricted antiviral CD8 T cell response, as determined by cytolytic functional assays. We used virus-specific tetramers to confirm that in vitro allogeneic challenge of EBV- and CMV-seropositive individuals resulted in proliferation of human virus–specific CD8 T cells. Furthermore, we confirmed that this proliferation was associated with increased cytolytic effector function from the allo-HLA primed cells against viral Ags. Our proof-of-principle results demonstrate that allo-HLA stimulation may be a potential tool to augment cyto-
to lytic antiviral CD8 T cell effector responses in patients with viral infection. This approach should be investigated further.

Materials and Methods
Preparation of responder, stimulator, and target cells
Responder and stimulator cells were both obtained using blood samples from healthy donors after informed consent. PBMCs were isolated from heparinized blood by standard density gradient centrifugation and were subsequently cryopreserved until use. EBV-transformed lymphoblastoid cell lines (EBV LCLs) were generated using standard procedures and were cultured in RPMI 1640 (Cambrex) with 10% FCS. The HLA type of all cells used in our experiments was determined molecularly by sequence-specific oligonucleotide and sequence-specific primer genotyping at the Leiden University Medical Centre, Department of Immunohematology and Blood Transfusion, The Netherlands.

Proliferation assays for EBV EBNA3A–specific T cell responses
For the proliferation assays, 1 × 10⁶ CFSE-labeled PBMCs from an HLA-B8⁺ HLA-B44⁺ EBV seropositive healthy donor were cocultured with 1 × 10⁶ HLA-B*44:02⁺ or HLA-B*44:03⁺ mismatched irradiated PBMCs (3000 rad) also from healthy donors, in a 24-well flat-bottom plate. Cells were incubated for 7–10 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/ml). Next, 4-1BB FcγRIIIA receptor recruitment assay was performed after staining the cells with CD8-APC (Becton Dickinson) and PE-labeled HLA-B8/FLR tetrameric complexes to detect cell division. In all experiments, HLA-A2/GLC and HLA-B8/RAK tetrameric complex staining served as negative controls. The proliferation assays for EBV EBNA3A–specific T cell responses were repeated using 20 different HLA-B*44⁺ responders HLA-B8⁺ B*44:02⁺ stimulator pairings, and eight different HLA-B8⁺ B*44:03⁺ stimulator pairings. The HLA typing of the selected responder-stimulator example is given below Fig. 1A.

Proliferation assays for CMV-specific T cell responses
To determine whether allo-HLA stimulation could elicit an antiviral response against any virus or specificity, we had to first determine a new method whereby specific allogeneic cells stimulating the proliferation of virus-specific T cells from any given individual could be identified easily. CFSE-labeled PBMCs (1 × 10⁶) from CMV-seropositive healthy donors were first cocultured with a pool of 1 × 10⁶ total mismatched irradiated PBMCs (3000 rad) from four different healthy donors (0.25 × 10⁶ cells of each individual stimulator), in a 24-well flat-bottom plate. Each responder was screened against four different pools of four PBMCs. The 16 total different allogeneic stimulus cells were selected to cover the most commonly occurring HLA molecules. Cells were incubated for 7–10 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/ml). Fluorescence-activated cell sorter analysis was performed after staining the cells with PE-labeled CMV-specific tetrameric complexes to detect cell division. If proliferation of CMV specific cells was detected after stimulation with a screening pool of four different allogeneic PBMCs, then the same responder PBMCs were tested individually against the four stimulator PBMCs to determine which allogeneic cells elicited proliferation of the CMV-specific T cells. The proportion of CMV-specific tetramer-positive T cells within the total CD8 T cell population were also determined before and after allogeneic cell stimulation using routine FACS analyses. The CMV-seropositive responder cells were then stimulated with the individual relevant PBMCs (or control) in a new assay (without CFSE labeling), following which the allo-HLA–primed responder cells were harvested, stained for cytokine activity (see below). The HLA typing of the selected responder-stimulator example is given below Figs. 2B and 3.

Proliferation assay for combined stimulation of two virus specificities from one responder
T cell alloresponses are polyclonal and polyclonal antiviral T cell responses targeting different viral epitopes are required for effective antiviral immunity. To confirm that allo-HLA stimulation could induce a polyclonal antiviral T cell response targeting different viral epitopes, we stimulated a single responder PBMC with a combination of two allogeneic PBMCs, expressing HLA molecules that were known to stimulate different CMV-specific T cell responses, from within that individual responder. CFSE-labeled PBMCs (0.85 × 10⁶) from a CMV-seropositive healthy donor responder were first cocultured with a pool of 1 × 10⁶ total mismatched irradiated PBMCs (3000 rad) from two different healthy donors S1 and S2 (0.5 × 10⁶ cells of each individual stimulator), in a 24-well flat-bottom plate. S1 and S2 expressed allogeneic HLA molecules (HLA-A*02:05 and HLA-B*51:01, respectively) known to stimulate different CMV-specific T cell response (pp65/A2 and pp65/B35 respectively) from the responder. A single stimulator cell expressing both HLA-A*02:05 and HLA-B*51:01 was not available. Cells were incubated for 7–10 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/ml). Fluorescence-activated cell sorter analysis was performed after staining the cells with PE-labeled CMV pp65/A2 and CMV pp65/B35 specific tetrameric complexes to detect cell division.

Cytotoxicity assays
To confirm that allogeneic cell stimulation resulted in increased virus-specific cytolytic effector function from the stimulated PBMCs, and not just proliferation, we performed cytolytic assays using autologous cells loaded with the relevant viral peptide or unloaded EBV LCLs as target cells. Responder PBMCs from EBV- or CMV-seropositive healthy donors were first specifically stimulated in a 7–10 d MLR with allogeneic irradiated cells to stimulate a virus-specific memory T cell of interest (see above). The stimulated PBMCs were then evaluated for cytotoxicity by incubating serial dilutions with 2000 viral peptide loaded autologous target cells or EBV LCL target cells, in a 4 h [51Cr] release assay. Cognate viral peptide was directly added to the autologous target cells and incubated for 60 min, simultaneously with chromium incubation, and then washed three times. Supernatants were harvested for gamma counting: percent-specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100%.

Statistical analysis
Values for specific lysis are presented as the mean of triplicate wells with SD. Comparative analyses were nonparametric (unpaired) t tests, and p < 0.05 was considered significant. Statistics were derived using Graph Pad Prism 4 for Windows (version 4.02, 2004).

Results
EBV-specific CD8 T cells proliferate following specific allo-HLA stimulation
To determine whether an allogeneic HLA challenge could specifically stimulate a virus-specific CD8 T cell response within whole blood, a modification of the MLC assay was used. EBV EBNA3A–specific T cells proliferated only in response to stimulation with HLA-B*44:02⁺ and not HLA-B*44:03⁺, mismatched irradiated PBMCs implying specific stimulation of cross-reactive virus-specific T cells by allogeneic HLA molecules (Fig. 1). EBV EBNA3A–specific T cells did not proliferate in response to stimulation with allogeneic HLA-B8⁺ HLA-B44⁺ PBMCs, excluding the possibility that the cells could be responding to EBV peptides contained within the culture medium or presented via stimulator cells (data not shown). Proliferation was associated with a specific increase in the proportion of EBV EBNA3A–specific T cells within the CD8 T cell compartment (Supplemental Fig. 1), and no proliferation of HLA-A2/GLC or HLA-B8/RAK restricted T cells was detected (data not shown), thereby excluding bystander proliferation and confirming the allo-HLA dependency of the stimulation. These results confirm that virus-specific CTL can directly recognize and proliferate in response to allo-HLA HLA to which they are cross-reactive and have never been exposed.

CMV-specific CD8 T cells proliferate following specific allo-HLA stimulation
To determine whether allo-HLA stimulation can elicit proliferation of CMV-specific T cells, we screened for responder CMV-specific T cell proliferation using pools of PBMC stimulator cells from four different donors who were chosen to cover the most commonly occurring HLA molecules. Proliferation of CMV-specific CD8 memory T cells was detectable using pools of four different PBMC stimulators together (Supplemental Table I). The individual PBMC giving the specific stimulation was then easily determined in
Allo-HLA stimulation is associated with an increase in the total number, not just proportion, of virus-specific memory T cells

To confirm that specific allo-HLA stimulation was associated with an increase in the total number of virus-specific T cells, not just an increased proportion of virus-specific T cells, we extrapolated the total number of CMV-specific T cells based on the total number of harvested lymphocytes and the proportion of tetramer positive T cells before and after stimulation. In two separate experiments after stimulation with S13, the number of CMV specific T cells increased from 320 to 35,200 and from 2,280 to 33,110, respectively (Table I). These data are consistent with specific in vitro allo-HLA stimulation being associated with a 10- to 100-fold increase in the total number of virus-specific T cells, as compared with the number of virus-specific T cells after stimulation with an allogeneic control cell (Table I).

Allo-HLA stimulation may stimulate polyclonal antiviral T cell responses

Polyclonal T cell responses targeting different viral epitopes are required for effective antiviral immunity. We therefore stimulated a single responder PBMC with a combination of two allogeneic PBMCs, expressing HLA molecules that were already known to stimulate different CMV-specific T cell responses, from within that one individual responder. CMV A2/pp65- and CMV B35/pp65-specific T cells from the same responder both proliferated in response to stimulation with two different HLA molecules, present on the surface of two different allogeneic PBMCs, within the same assay (Fig. 4). Negative controls gave appropriate results (Fig. 4), suggesting that allo-HLA stimulation may be capable of stimulating multiple different virus-specific T cells from within the one responder.

EBV- and CMV-specific CD8 memory T cells gain viral peptide/self-HLA-restricted cytolytic effector function following specific allo-HLA stimulation

For viral protection, it is essential that the proliferation of virus-specific T cells following allogeneic stimulation is associated with a gain of cytolytic effector function against the original viral peptide/self-HLA restricted target Ag. We therefore performed a cytolytic assay using responder HLA-B8+ EBV sero-positive healthy donor PBMCs following in vitro stimulation with either homozygote HLA-B*44:02 or HLA-B*44:03 mismatched irradiated PBMCs, and with viral peptide loaded autologous cells and unloaded EBV transformed B cells (EBV LCLs) as target cells. Following 7–10 d of stimulation with HLA-B*44:02 mismatched irradiated PBMCs, primed responder cells from an HLA-B8+ EBV-sero-positive healthy donor showed increased cytolytic effector function against both HLA-B8+ EBV LCLs and FLR peptide loaded autologous target cells, but not HLA-B8+ EBV LCLs nor RAK peptide loaded autologous target cells (Fig. 5), as compared with the same PBMCs cocultured with either HLA-B*44:03 mismatched PBMCs or culture with IL-2 containing medium alone. This increased cytolytic effector function was associated with proliferation and an increase in the proportion of EBV EBNA3A-specific CD8 T cells (Supplemental Fig. 1).

Allogeneic HLA challenge can indeed increase the (in vitro) cytolytic effector function of human CMV and EBV specific CD8 T cells against their original cognate viral Ag. We argue that these
proof-of-principle results may have important implications for treatment of viral infections, if confirmed in vivo.

Discussion

This study demonstrates that human virus-specific memory T cells gain cognate viral Ag specific cytolytic effector function following stimulation with allogeneic HLA molecules against which they are cross-reactive. Stimulation of peripheral blood from a nonsensitized HLA-B8+ EBV seropositive healthy donor with HLA-B*44:02 mismatched irradiated PBMCs increases (in vitro) cytolytic effector function against EBV. Furthermore, we show that this technique can be used to elicit cytolytic effector function against any potential viral Ag, as shown for CMV. These results provide proof-of-principle evidence that stimulation with specific allogeneic HLA molecules could be useful for treatment of viral infections.

FIGURE 2. Screening for allo-HLA cross-reactivity of virus-specific memory T cells. A representative example is shown. (A) CMV pp65-specific CD8 memory T cells (A2/NLV restricted) from Responder 2 (R2) proliferate following stimulation with a pool of four PBMCs (pool 4 containing stimulators 13–16), but not other pools of four different stimulator PBMCs (pool 2 shown). (B) R2 was then tested individually against all four stimulators present in pool 4 (S13–16). R2 proliferated only when stimulated with S13 and not when stimulated with the other three stimulators present in pool 4 (S15 shown), thereby confirming that the CMV pp65-specific T cells from R2 were specifically stimulated by only S13 allogeneic cells. HLA typing of responder 2 HLA-A*02, 11; B*35, 40; DRB1*11, 15. Stimulator 13 HLA-A*02:01, 02:05; B*18, 50; DRB1*11, 13. Stimulator 15 HLA-A*23, 29; B*15, 53; DRB1*11, 13.

FIGURE 3. The proportion of CMV-specific CD8 T cells is specifically increased following allogeneic cell stimulation. A representative example is shown. CMV pp65-specific CD8 T cells accounted for 2.1% of total CD8 T cells from responder 2 (R2), following 8 d co-culture with stimulator 13 (S13). The proportion of CMV pp65-specific CD8 T cells was unaltered by co-culture with stimulator 15 (S15) or IL-2 containing medium alone. The primed responder cells shown here were then harvested and used as effector cells in the cytolytic assay shown in Fig. 6. HLA typing of responder 2 HLA-A*02, 11; B*35, 40; DRB1*11, 15. Stimulator 13 HLA-A*02:01, 02:05; B*18, 50; DRB1*11, 13. Stimulator 15 HLA-A*23, 29; B*15, 53; DRB1*11, 13.
The importance of our findings is reinforced by functional studies showing that the proliferation of EBV- and CMV-specific CD8 memory T cells corresponded with a specific increase of cytolytic effector function against viral peptide-loaded autologous cells, which was not detectable without specific allo-HLA stimulation. Cytolysis of the EBV LCLs by the HLA-B*44:02–primered effector cells suggests that virus-infected cells can spontaneously process and present viral peptides via HLA class I molecules in the course of normal infection, and that the amount of peptide present is sufficient to trigger killing from allo-HLA primed effector cells.

EBV infection in an HLA-B8* HLA-B44* individual selects for a public BV6S2 TCR, which cross-reacts against allogeneic HLA-B*44:02 (13). Although not all virus-specific immune responses give rise to a public TCR, the allo-HLA cross-reactivity of virus-specific T cells from a given individual can be detected easily in vitro using techniques we have described here and elsewhere (12, 15). Indeed, successful stimulation of cytolytic effector function against CMV Ag reveals that this technique could potentially be useful to elicit T cell cytolytic effector function against any virus or specificity. Furthermore, stimulation of two different CMV-specific T cell responses from one individual suggests that allo-HLA stimulation may also be capable of stimulating a polyclonal antiviral T cell response targeting different viral epitopes, which does not occur with single peptide stimulation. Techniques described here should be reproducible in most routine laboratories.

We have confirmed that these effects are mediated by leukocytes present in the blood components and are related to the expression of HLA Ags. We used irradiated isolated PBMCs for stimulation of the virus-specific memory T cells, thereby excluding any contributions by plasma, platelets, or erythrocytes. Therefore, we suggest that allogeneic cell therapy should be investigated using only isolated leukocytes as stimulators.

Immunologic memory is one of the hallmarks of the adaptive immune response. Functional virus-specific memory T cells are essential for proper host defense because, in the periphery, infected cells can be targeted for immediate killing, both during the initial infection and on subsequent reinfecion or viral reactivation.

The results presented in this study suggest that specific allogeneic cell therapy could prime or maintain virus-specific memory. The proportion and total number of virus-specific T cells in the CD8 compartment increased significantly following specific allo-HLA stimulation. CFSE dilution and counting experiments confirmed that the increase in the proportion of virus-specific memory T cells was secondary to proliferation, and not just better survival of memory T cells in culture, with the number of virus-specific memory T cells increasing between 10- and 100-fold. Data from preliminary clinical studies suggest that CMV-specific CD8 T cell levels greater than $1 \times 10^5/L$ of peripheral blood may correlate with protection (23); therefore, the total number of virus-specific T cells induced by proliferation following allogeneic cell stimulation may be important in isolation.

However, others have also shown that the memory T cell state of readiness is actively maintained and reversible, requiring ongoing specific TCR signaling (9, 11). Transfer of memory T cells to naive mice, in the presence or absence of priming Ag, reveals that maintenance of T cell memory is short lived in the absence of TCR-mediated signaling (9). Furthermore, recently activated memory T cells can bypass the requirement for CD28/CD80/CD86 costimulation, as compared with resting memory T cells that are still dependent on CD28 triggering for their activation (24). Although at baseline in our EBV-specific cytolytic assay 1.5% of CD8 T cells in the peripheral blood of the individual were EBV EBNA3A specific T cells, no cytolysis of FLR peptide loaded autologous cells could be detected prior to allo-HLA-B*44:02

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Allo-HLA stimulation may stimulate polyclonal antiviral T cell responses. (A) CMV A2/pp65 and (C) CMV B35/pp65-specific T cells from the same responder both proliferated in response to stimulation with two different HLA molecules, present on the surface of two different allogeneic stimulator PBMCs, in the same assay. Negative controls gave appropriate results (B, D). Thereby suggesting that allo-HLA stimulation may be capable of stimulating multiple different virus-specific T cells, targeting different viral epitopes, from within the one responder. HLA typing responder PBMCs HLA-A*02:01, 11; B*35, 40; DR*11, 15. Stimulator 1 HLA-A*02:01, 02:05; B*18, 50; DRB1*11, 13. Stimulator 2 HLA-A*02, 36; B*51, 72; DRB1*03, 10.
stimulation, suggesting specific allogeneic cell priming was important to induce the observed cytolysis. Therefore, the allogeneic stimulation used in our assays may also have increased cytolytic effector function of the virus-specific T cells via triggering TCR signaling or abrogating costimulation requirements, regardless of the changes to the total number of cells.

To evade these cytolytic CD8 T cell responses, viruses have evolved many different strategies for immune evasion (25–27), most of which interfere with the various steps necessary for MHC class I restricted Ag presentation. For example, CMV evades MHC class I Ag presentation by reducing the stability of class I heavy chains (28) and also by dislocating MHC class I heavy chains from the endoplasmic reticulum (29). The coordinated function of murine CMV genes can completely inhibit CTL lysis (30). Among others, the EBV EBNA1 protein contains an element that interferes with its proteasomal proteolysis, and the HSV ICP47 protein inhibits the TAP complex (31, 32). HIV is highly efficient at evading immune responses through mechanisms such as modulation of MHC class II presentation (33) and downregulation of MHC class I molecules (3, 33–35). Many other viral immune evasion strategies are also described (36–40).

Allogeneic cell therapy may be capable of bypassing all these viral strategies of immune evasion as the virus-specific memory T cells are directly stimulated via molecular mimicry (41). The allo-HLA molecule against which the virus-specific T cell is cross-reactive is constitutively expressed and occupied by the stimulating self-peptide. Theoretically allogeneic cell therapy could even stimulate additional virus-specific responses other than the specificity of interest. Steffens et al. (42) demonstrated that preemptive CMV specific CD8 T cell immunotherapy, guided by viral DNA load, prevented lethal disease, and reduced the risk of virus recurrence. Similarly, allogeneic cell therapy may ensure a high proportion of preexisting activated virus-specific memory T cells to prevent disease and accelerate the resolution of productive infection.

Finally, we acknowledge that further work is required before allogeneic cell therapy can be used in the clinical setting to treat viral infections. In these experiments, we have used healthy blood donors as responder PBMCs, not cells from immunosuppressed patients. Whereas infusion of irradiated leukocytes should not be associated with chimerism or engraftment, this possibility should be considered
in an extremely immunodeficient recipient. Repeated allogeneic cell therapy may also cause sensitization of a recipient to future transplantations. It is also unclear whether such treatment would stimulate a de novo virus-specific response from naive T cells (43–45). Nonetheless, results demonstrated in this study suggest allo-HLA stimulation may have potential as an alternative to adoptive transfer or pharmacological therapy to treat viral infections.

The high frequency of allog-HLA cross-reactivity by virus-specific T cells is increasingly being recognized. We provide (in vitro) evidence that allogeneic cell therapy may be useful to conversely stimulate a beneficial antiviral cytolytic effector response for treatment of viral infection. This proof-of-principle technique could provide important future options for the treatment of viral infections. This approach should be investigated further.

Acknowledgments
We thank Prof. Christian Clausen for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary table I: Screening for allo-HLA crossreactivity using pools of allogeneic cells. Pools of 4 different allogeneic cells were first used to screen for allo-HLA crossreactivity of CMV specific CD8 T-cells within whole blood, using CFSE staining of proliferating responder cells. The specific allogeneic cell giving the stimulation was then easily identified in a second assay. Specific allogeneic stimulation was associated with not only proliferation but also increased cytolytic activity against the original cognate viral antigen. Specific allogeneic cells stimulating a virus-specific T-cell response were identifiable for most responders and specificities. + Specific Proliferation Detected. – No Proliferation Detected. PBMCs from responder 2 were used in the assays described in figures 2, 3 and 6.

<table>
<thead>
<tr>
<th>Responder</th>
<th>Medium</th>
<th>Pool 1 (S1-4)</th>
<th>Pool 2 (S5-8)</th>
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After stimulation with homozygote HLA-B*4402 mismatched PBMCs

Culture with medium only

After stimulation with homozygote HLA-B*4403 mismatched PBMCs
Supplementary figure 1: The proportion of EBV specific CD8 T-cells is specifically increased following allogeneic cell stimulation. EBV EBNA3A specific CD8 T-cells accounted for 20.8% of total CD8 T-cells, following 8 day co-culture with homozygote HLA-B*44:02 mismatched irradiated PBMCs. The proportion of EBV EBNA3A specific CD8 T-cells was unaltered by co-culture with homozygote HLA-B*44:03+ PBMCs. FACS plots gated on total CD8 T-cell population. The primed responder cells shown here were then harvested and used as effector cells in the cytolytic assays shown in Figure 5. Responder HLA-A*01, 02; B*08, -; DRB1*03, -. HLA-B*44:02+ stimulator HLA-A*02, 68; B*44:02, -; DRB1*07, 14. HLA-B*44:03+ stimulator HLA-A*02, 32; B*44:03, -; DRB1*01, 08.