Heterologous immunity in organ transplantation

Heuvel, H. van den

Citation

Heuvel, H. van den. (2019, April 25). *Heterologous immunity in organ transplantation*. Retrieved from https://hdl.handle.net/1887/71941

Version:	Not Applicable (or Unknown)
License:	Leiden University Non-exclusive license
Downloaded from:	https://hdl.handle.net/1887/71941

Note: To cite this publication please use the final published version (if applicable).

Cover Page

The handle http://hdl.handle.net/1887/71941 holds various files of this Leiden University dissertation.

Author: Heuvel, H. van den
Title: Heterologous immunity in organ transplantation
Issue Date: 2019-04-25

CHAPTER

5

STIMULATION OF HUMAN EBV- AND CMV-SPECIFIC CYTOLYTIC EFFECTOR FUNCTION USING ALLOGENEIC HLA MOLECULES

Lloyd J. D'Orsogna[1,2,3]

Heleen van den Heuvel[1]

Ellen M. W. van der Meer-Prins[1]

Dave L. Roelen[1]

Ilias I. N. Doxiadis[1]

Frans H. J. Claas[1]

[1] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden 2300 RC, The Netherlands; [2] School of Pathology and Laboratory Medicine, University of Western Australia, Perth 6009, Western Australia, Australia; [3] Department of Clinical Immunology and PathWest Laboratory Medicine, Royal Perth Hospital, Perth 6000, Western Australia, Australia; and Institute for Immunology and Infectious Diseases, Murdoch University, Perth 6150, Western Australia, Australia

J Immunol. 2012 Nov 15;189(10):4825-31

ABSTRACT

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. HLA-B8^{+} HLA-B*44^{-} 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 an antiviral T-cell response in patients with EBV or CMV infection.

INTRODUCTION

Control of viral replication depends primarily on virus-specific memory T-lymphocyte activity (148, 149). In the normal course of viral infections, antiviral immunity and non-infectivity 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 (150), and the use of lymphocyte-targeted biologic therapies has recently been associated with viral reactivation that might not respond to antiviral antibiotics (151). For example, whereas allogeneic marrow depleted of T cells prevents acute and chronic forms of graft-versus-host disease (GVHD) post-transplant, the risk of infections, particularly with EBV and CMV, is increased (152). 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 (153), and is technically difficult (154). Peptide stimulation does not induce a polyclonal T-cell response and can fail to induce cytotoxic $CD8^+$ T-cell responses (155).

Furthermore, although Ag-specific T-cell responses are actively maintained, they are reversible and short lived in the absence of stimulating Ag (156-158). 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 (43). 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 (7), which cross-reacts against allo-HLA-B*44:02 (11). We confirmed the previously described alloreactivity of this EBV EBNA3A–specific T cell (HLA-B8/FLRGRAYGL restricted) against allogeneic HLA-B*44:02 (43, 121). Allo-HLA cross-reactivity was also shown for CMV, varicella-zoster virus (VZV) and influenza virus–specific T cells, which express nonpublic TCRs (43).

A high level of cross-reactivity against allo-HLA molecules is therefore an essential feature of the virus-specific memory TCR (7, 11, 24, 35, 43, 112, 121, 128, 159-161). 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

5

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 cytolytic 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 Center, Department of Immunohematology and Blood Transfusion, The Netherlands.

Proliferation assays for EBV EBNA3A–specific T-cell responses

For the proliferation assays, 1 x 10^6 CFSE-labeled PBMCs from an HLA-$B8^+$ HLA-B^*44^- EBV-seropositive healthy donor were co-cultured with 1 x 10^6 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, fluorescence-activated cell sorter analysis 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-$B8^+B44^-$ responder - HLA-$B8^-B^*44{:}02^+$ stimulator pairings, and eight different HLA-$B8^+B44^-$ responder - HLA-$B8^-B^*44{:}03^+$ stimulator pairings. The HLA typing of the selected responder-stimulator example is given below Figure 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 x 10^6) from CMV-seropositive healthy donors were first co-cultured with a pool of 1 x 10^6 total mismatched irradiated PBMCs (3000 rad) from four different healthy donors (0.25 x 10^6 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 stimulator cells were selected to cover the most common 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 and used as effector cells in the cytotoxicity assays (see below). The HLA typing of the selected responder-stimulator examples is given below Figures 2B and 3.

5

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 x 10^6) from a CMV-seropositive healthy donor responder were first co-cultured with a pool of 1 x 10^6 total mismatched irradiated PBMCs (3000 rad) from two different healthy donors S1 and S2 (0.5 x 10^6 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 responses (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 4h [51Cr] release assay. Cognate viral peptide or control 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 2 spontaneous release) / (maximum release – spontaneous release) x 100%. Values for specific lysis are presented as the mean of triplicate wells with SD.

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 (Figure 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 Figure 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 allogeneic HLA to which they are cross-reactive and have never been exposed.

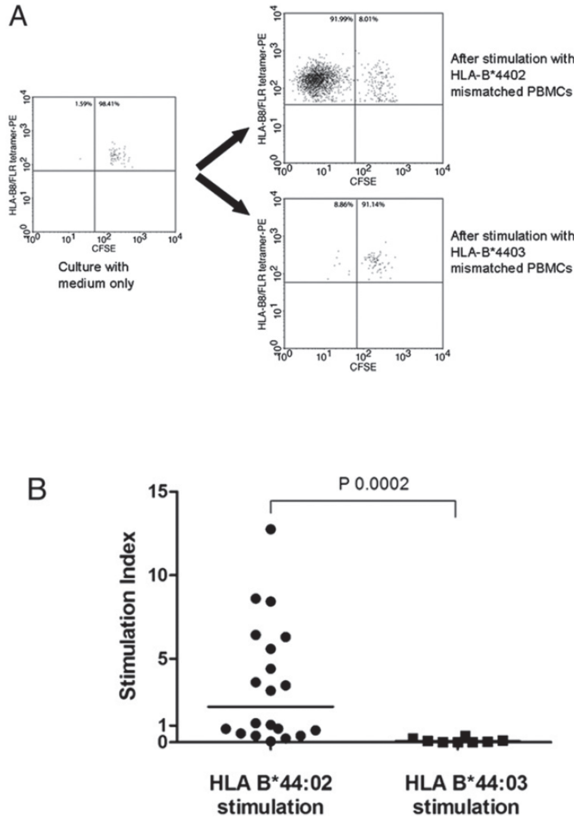


Figure 1. EBV-specific $CD8^+$ memory T cells specifically proliferate after allogeneic cell stimulation. (A) EBV EBNA3A-specific T cells are specifically stimulated to proliferate after 7-10 d in vitro co-culture with HLA-B*44:02$^+$, but not HLA-B*44:03$^+$, mismatched irradiated PBMCs. Bystander activation was excluded. FACS plots gated on total HLA-B8/FLR-tetramer-complex-positive lymphocytes. Assay repeated multiple times with different responder-stimulator pairings, with similar results. A representative result is shown. Responder: HLA-A*02, A31; B*08, B39; DRB1*03, DR16. HLA-B*44:02$^+$ stimulator: HLA-A*11, -; B*44:02, B51; DRB1*12, DR15. HLA-B*44:03$^+$ stimulator: HLA-A*02, A68; B*44:03, B51; DRB1*08, DR13. (B) Proliferation of EBV EBNA3A-specific T cells is reproducible across different responder-stimulator pairings. Assay repeated with 20 different HLA-B8$^+$B44$^-$ responder - HLA-B8$^-$B*44:02$^+$ stimulator pairings, and 8 different HLA-B8$^+$B44$^-$ responder - HLA-B8$^-$B*44:03$^+$ stimulator pairings. Results are expressed as a relative proportion of EBV EBNA3A-specific T cells within the $CD8^+$ T-cell compartment after HLA-B*44:02 or HLA-B*44:03 stimulation, as compared with the non-stimulated PBMCs. HLA-B*44:02 stimulation significantly increased the proportion of EBV EBNA3A-specific T cells, as compared with HLA-B*44:03 stimulation (p = 0.0002).

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 a second assay. For example, CMV pp65-specific T cells (HLA-A2/NLV restricted) from a healthy donor (responder 2) proliferated in response to a PBMC pool of four different PBMCs (pool 4; Figure 2A, Supplemental Table I). The same responder was then tested individually against the stimulators present in the screening pool to identify the specific stimulator (Figure 2B).

Proliferation was associated with a specific increase in the proportion of CMV pp65-specific T cells within the CD8$^+$ T-cell compartment (Figure 3). Screening experiments were repeated multiple times with different responders and for different CMV CD8 T-cell specificities. Using this technique, proliferation of HLA-A2/NLV- and HLA-B35/IPS-restricted CD8$^+$ T cells from different responders was elicited (Supplemental Table I). Furthermore, this stimulation is demonstrable without the need to generate virus-specific T-cell clones from the responder, even when the virus-specific T cell of interest does not express a public TCR, thereby suggesting that allogeneic cells stimulating viral-peptide/HLA-restricted T cells from any given responder are readily identifiable in the routine laboratory.

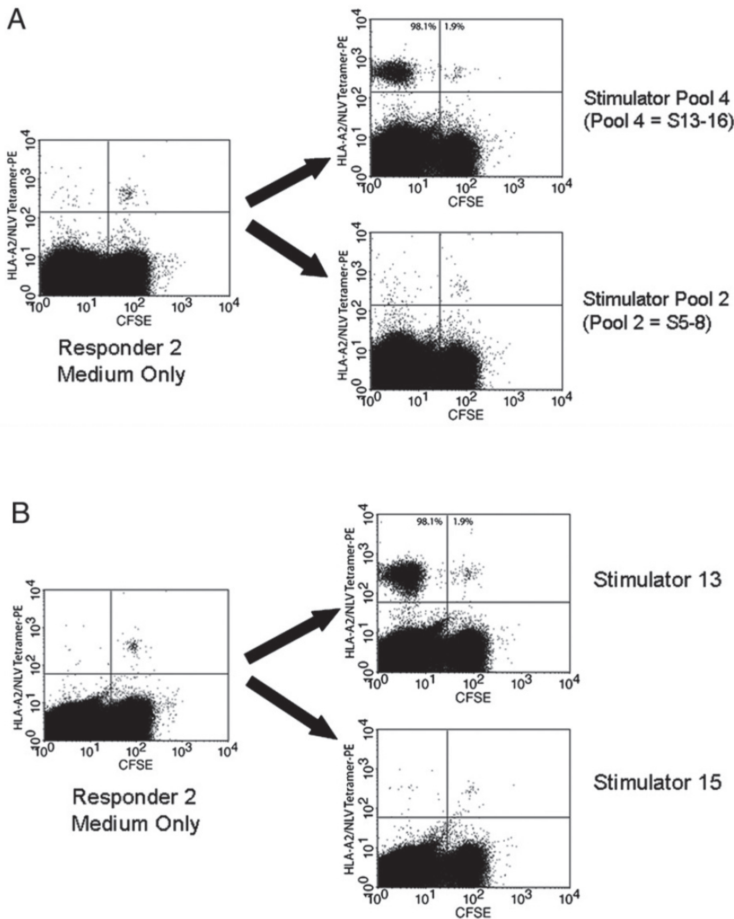


Figure 2. Screening for allo-HLA cross-reactivity of virus-specific memory T cells. A representative example is shown. (A) CMV pp65-specific CD8+ memory T cells (A2/NLV restricted) from Responder 2 (R2) proliferate following stimulation with a pool of four PBMCs (pool 4 containing stimulators 13-16), 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, A11; B*35, B40; DRB1*11, DR15.
Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.

5

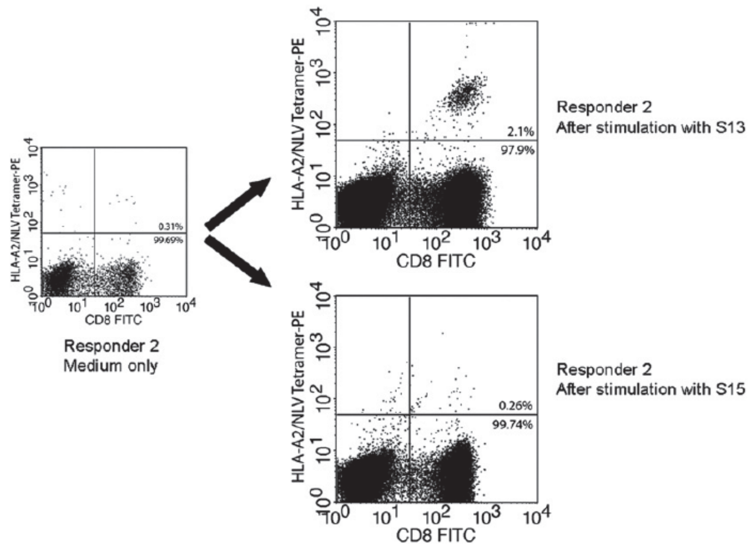


Figure 3. The proportion of CMV-specific CD8+ T cells is specifically increased following allogeneic cell stimulation. A representative example is shown. CMV pp65-specific CD8+ T cells accounted for 2.1% of total CD8+ T cells from responder 2 (R2), following 8 d co-culture with stimulator 13 (S13). The 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 Figure 6.

HLA typing of responder 2: HLA-A*02, A11; B*35, B40; DRB1*11, DR15.
Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.

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 2280 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 1).

Table 1. Specific allo-HLA stimulation is associated with an increase in the total number, not just proportion, of virus-specific memory T cells

Responder	Stimulator	Total No. of Lymphocytes before Stimulation	Total No. of Lymphocytes after Stimulation	% of Tetramer-Positive Cells within Total Lymphocyte Population after Stimulation	Estimated Total No. of CMV-Specific T Cells after Stimulation	Fold Increase in Total No. of CMV-Specific T Cells after Allo-HLA Stimulation[a]
R2	Medium only	2×10^6	2.9×10^6	0.015	435	1.36
	S13	1×10^6	3.2×10^6	1.1	35,200	110
	S15	1×10^6	3.2×10^6	0.01	320	1
R2	Medium only	2×10^6	0.5×10^6	0.04	200	0.09
	S13	3×10^6	7.7×10^6	0.43	33,110	14.5
	S15	3×10^6	5.7×10^6	0.04	2280	1

[a]*Relative to control stimulation with allogeneic S15*

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. Negative controls gave appropriate results, suggesting that allo-HLA stimulation may be capable of stimulating multiple different virus-specific T cells from within the one responder (Figure 4).

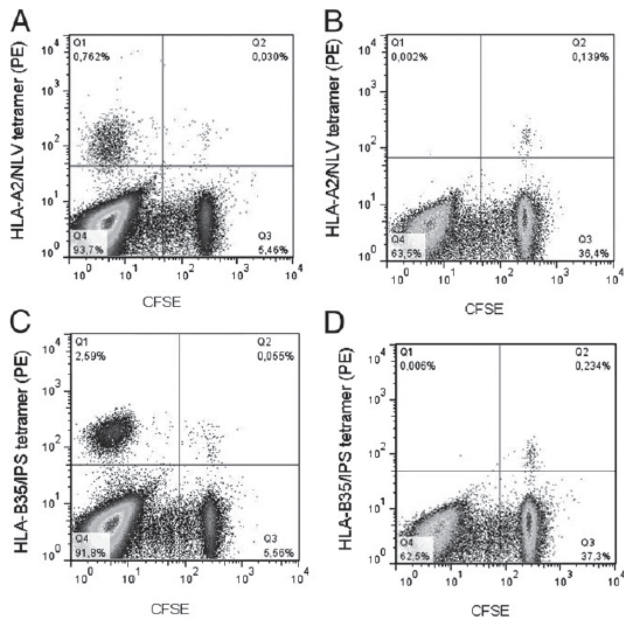


Figure 4. Allo-HLA stimulation may stimulate polyclonal antiviral T-cell responses. (A) CMV A2/pp65- and (C) CMV B35/pp65-specific T cells from the same responder both proliferated in response to stimulation with two different HLA molecules, present on the surface of two different allogeneic stimulator 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, A11; B*35, B40; DR*11, DR15.
Stimulator 1: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 2: HLA-A*02, A36; B*51, B72; DRB1*03, DR10.

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 $HLA\text{-}B8^+$ EBV-seropositive 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 $HLA\text{-}B8^+$ EBV-seropositive 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 (Figure 5), as compared with the same PBMCs co-cultured with either HLA-B*44:03-mismatched PBMCs or cultured 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 Figure 1). Likewise, specific stimulation of CMV-specific CD8^{+} T cells with allo-HLA resulted in increased cytolytic effector function against CMV-peptide-loaded autologous cells (Figure 6). Confirming that 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 non-sensitized 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.

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-primed 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 (7). 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 (43, 121).

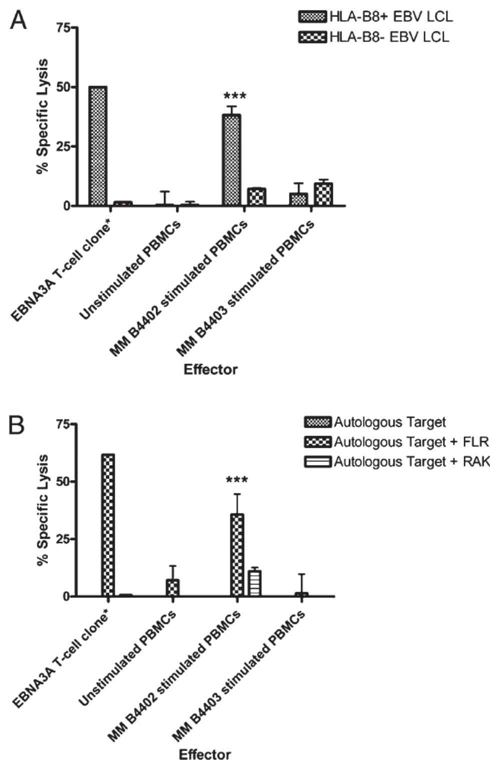


Figure 5. EBV-specific cytolytic effector function of allo-HLA primed cells using EBV-LCL target cells and viral peptide-loaded autologous target cells. (A) PBMCs from a HLA-B8+ EBV-seropositive donor gain EBV-specific cytolytic effector function following allogeneic HLA-B*44:02+ cell stimulation. Unstimulated HLA-B8+ PBMCs and HLA-B*44:03-stimulated HLA-B8+ PBMCs do not demonstrate cytolytic effector function against HLA-B8+ EBV-LCLs. E:T ratio 50:1; targets 2000 and EBV EBNA3A-specific T cells accounted for 20.8% of effector cell population after HLA-B*44:02 stimulation. Positive control EBNA3A T-cell clone is described previously (121), and responder PBMCs used in this assay are also obtained from the same donor.

HLA typing of responder PBMCs and EBNA3A T-cell clone: HLA-A*01, A*02; B*08:01, -; DRB1*03, -.

HLA-B8+ EBV-LCL: HLA-A*01, -; B*08, -; DRB1*03, -.

HLA-B8- EBV-LCL: HLA-A*03, -; B*07, -; DRB1*15, -.

***$p < 0.0001$ versus HLA-B8- EBV-LCL.

(B) PBMCs from an HLA-B8+ EBV-seropositive donor gain HLA-B8/FLR-restricted cytolytic effector function following allogeneic HLA-B*44:02 stimulation. Unstimulated HLA-B8+ PBMCs and HLA-B*44:03-stimulated HLA-B8+ PBMCs do not demonstrate cytolytic effector function against FLR-peptide-loaded autologous cells. E:T ratio 50:1; targets 2000 and EBV EBNA3A-specific T cells accounted for 20.8% of effector cell population after HLA-B*44:02 stimulation. Positive control EBNA3A T-cell clone is described previously (15), and responder PBMCs used in this assay are also obtained from the same donor.

HLA typing of responder PBMCs, autologous target PBMCs, and EBNA3A T cell clone: HLA-A*01, A*02; B*08:01, -; DRB1*03, -.

***$p = 0.0094$ versus RAK-peptide-loaded autologous cells.

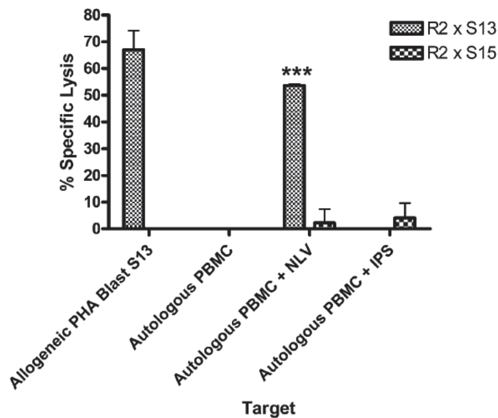


Figure 6. CMV-specific cytolytic effector function of allo-HLA primed cells. PBMCs from a CMV-seropositive HLA-A2+ donor (responder 2; R2) gain HLA-A2/NLV-restricted cytolytic effector function following heterozygote allogeneic cell stimulation with stimulator 13 cells (R2 x S13). S15-stimulated PBMCs do not demonstrate cytolytic effector function against NLV-peptide-loaded autologous cells (R2 x S15). A strong secondary response against stimulator 13 (S13) is demonstrated from R2 responder cells primed with S13 (positive control), but not S15. E:T ratio 100:1; targets 2000 and CMV A2/NLV-specific T cells accounted for 2.1% of effector cell population after stimulation with S13.

HLA typing of responder 2: HLA-A*02, A11; B*35, B40; DRB1*11, DR15.
Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.
***$p < 0.0001$ versus IPS-loaded autologous cells.

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 reinfection 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 1×10^7 / L of peripheral blood may correlate with protection (162); 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 (156, 158). 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 (156). 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 (163). 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 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 (164-166), 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 (167) and also by dislocating MHC class I heavy chains from the endoplasmic reticulum (168). The coordinated function of murine CMV genes can completely inhibit CTL lysis (169). Among others, the EBV EBNA1 protein contains an element

that interferes with its proteasomal proteolysis, and the HSV ICP47 protein inhibits the TAP complex (170, 171). HIV is highly efficient at evading immune responses through mechanisms such as modulation of MHC class II presentation (172) and downregulation of MHC class I molecules (150, 172-174). Many other viral immune evasion strategies are also described (175-179).

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 (44). 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. (180) 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 pre-existing 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 (181-183). 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 allo-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

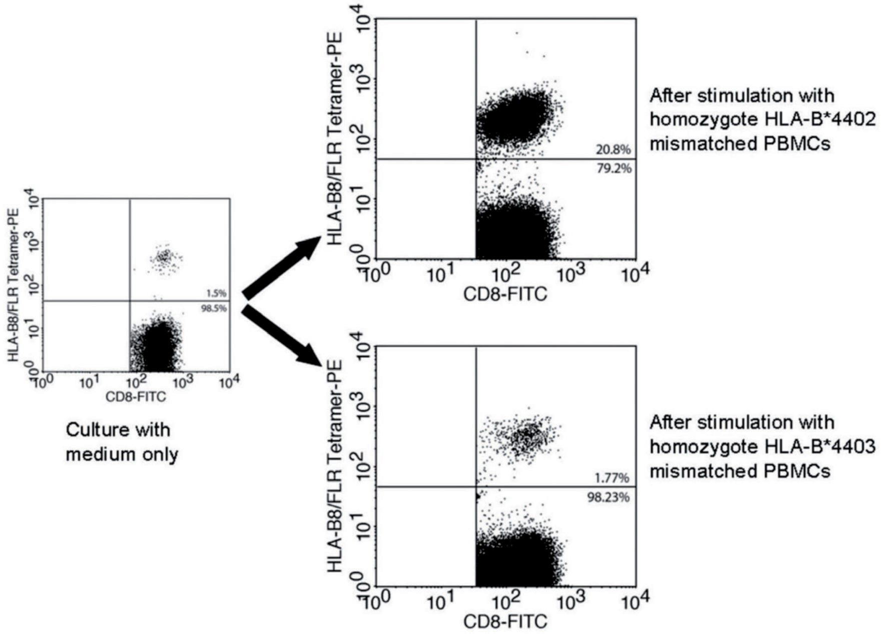
We thank Prof. Frank Christiansen for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

Supplemental Table 1. Screening for allo-HLA cross-reactivity using pools of allogeneic cells.

	Medium	Pool 1 (S1-4)	Pool 2 (S5-8)	Pool 3 (S9-12)	Pool 4 (S13-16)
Responder 1 A2/NLV	-	-	+	-	+
Responder 2 A2/NLV	-	-	-	-	+
Responder 3 A2/NLV	-	-	-	-	-
Responder 4 B35/IPS	-	-	-	-	+

Pools of 4 different allogeneic cells were first used to screen for allo-HLA cross-reactivity 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.



Supplemental 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, A*02; B*08, -; DRB1*03, -.

HLA-B*44:02⁺ stimulator: HLA-A*02, A68; B*44:02, -; DRB1*07, DR14.

HLA-B*44:03⁺ stimulator: HLA-A*02, A32; B*44:03, -; DRB1*01, DRB1*08.