HLA alloreactivity by human viral specific memory T-cells

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Stimulation of human viral specific cytolytic effector function using allogeneic cell therapy

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ABSTRACT

Viral infection is a major cause of morbidity and mortality, and there are few therapeutic options available to augment a viral specific T-cell response. While allo-HLA crossreactivity from viral specific memory T-cells is common, it is unclear if priming with allogeneic cells could conversely elicit a viral peptide/self-HLA restricted T-cell response. Firstly we used the previously described allo-HLA-B*44:02 crossreactivity by EBV peptide/HLA-B*08:01 restricted T-cells, to determine if allogeneic HLA stimulation can elicit a cytolytic immune response against Epstein-Barr virus. HLA-B*08:01^{+} 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 day mixed lymphocyte reaction. The stimulated responder cells were then evaluated for cytotoxicity using EBV peptide loaded autologous target cells and unloaded HLA-B*08:01^{+} EBV LCL target cells. PBMCs from EBV seropositive donors gained EBV specific cytolytic effector function following specific allo-HLA stimulation. Finally, as a proof-of-principle, we also elicited cytolytic CMV specific responses using allogeneic cell stimulation, to confirm that this technique can be used to elicit viral peptide/self-HLA restricted responses against any virus or specificity. Allogeneic cell stimulation used as a cell therapy may be a potential tool to augment an anti-viral T-cell response in patients with viral infection.

INTRODUCTION

Control of viral replication depends primarily on viral specific memory T-lymphocyte activity (1,2). In the normal course of viral infections, anti-viral 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 (3), and use of lymphocyte targeted biological therapies has recently been associated with viral reactivation which may not respond to anti-viral antibiotics (4). For example, while allogeneic marrow depleted of T-cells prevents acute and chronic forms of graft versus host disease (GvHD) posttransplant, the risk of infections, particularly with Epstein-Barr virus (EBV) and cytomegalovirus (CMV), are increased (5). Furthermore, viral infection can cause severe morbidity and mortality, even in individuals without defined immune deficiency.

Currently there are no in-vivo autologous therapies to increase the number or effector function of viral specific T-cells. Antiviral prophylaxis can be toxic and does not result in an increase in viral-specific T-cells nor achieve long-term eradication. Adoptive transfer of 3rd party cell lines is associated with GvHD or failure due to allogeneic rejection (6), and is technically difficult (7). While antigen specific T-cell responses are actively maintained, they are reversible and short lived in the absence of antigen (8-10).

We have recently confirmed that alloreactivity from viral specific T-cells is common, and that the allo-HLA reactivity and virus specificity is mediated via the same T-cell receptor (TCR) (11). 45% 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-B*08:01^{+} individual always selects for a dominant "public" Vb6S2 TCR (12), which cross-reacts against allo-HLA-B*44:02(13). We confirmed the previously described alloreactivity of this EBV EBNA3A specific T-cell (HLA-B8/FLRGRAYGL restricted) against allogeneic HLA-B*44:02 (11,14). Allo-HLA cross-reactivity was also shown for cytomegalovirus (CMV), varicella-zoster virus (VZV) and influenza virus specific T-cells (Amir) which express non-public TCRs.

A very high level of cross-reactivity against allo-HLA molecules is therefore an essential feature of the virus specific memory TCR (11-21). This allo-HLA crossreactivity by viral specific T-cells can be reproducibly predicted in-vitro. However, currently it is unknown if stimulation with allogeneic-HLA molecules could conversely specifically augment a HLA-restricted viral specific T-cell response.

The purpose of this study was therefore to assess if allogeneic HLA challenge could be a useful tool to augment a HLA-restricted anti-viral CD8 T-cell response, as determined by cytolytic functional assays. We used viral 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 antigens. Our proof-of-principle results demonstrate that allo-HLA stimulation may be a potential tool to augment cytolytic anti-viral CD8 T-cell effector responses in patients with viral infection.

RESULTS

EBV specific CD8 T-cells proliferate following allogeneic cell stimulation

To determine whether an allogeneic HLA challenge could specifically stimulate a viral 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 viral specific T-cells by allogeneic HLA molecules (Figure 1). EBV EBNA3A specific T-cells did not proliferate in response to stimulation with allogeneic HLA-B*0801⁺ HLA-B*44⁻ 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 (Figure 2 and table 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. The observed response was abrogated when heterozygote HLA-B*08:01⁺ HLA-B*44:02⁺ responder PBMCs were used, consistent with specific thymic editing of the T-cell repertoire (Data not shown). These results confirm that viral specific CTL can directly recognize and proliferate in response to allogeneic HLA to which they are crossreactive and have never been exposed.

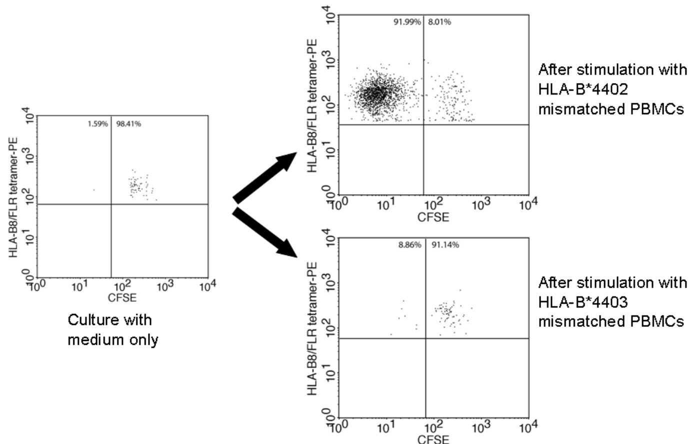

Figure 1. EBV specific CD8 memory T-cells specifically proliferate following allogeneic cell stimulation.

EBV EBNA3A specific T-cells are specifically stimulated to proliferate following 7-10 day in-vitro co-culture with heterozygous 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 4 times with different responder-stimulator pairings, with similar results. A representative result is shown. Responder HLA-A*02,31; B*08:01,39; DRB1*03,16. HLA-B*44:02⁺ stimulator HLA-A*11,-; B*44:02,51; DRB1*12,15. HLA-B*44:03⁺ stimulator HLA-A*02,68; B*44:03,51; DRB1*08,13.

Viral specific T-cell proliferation may be greater following homozygote cell stimulation
To determine if homozygote allo-HLA is a greater stimulus for viral specific T-cells HLA-B*08:01^{+} B*44^{-} responder PBMCs were stimulated with either homozygous or heterozygous, HLA-B*44:02 or HLA-B*44:03, allogeneic cells. EBV EBNA3A specific T-cells accounted for 20.8% of the total CD8 T-cell population following homozygous HLA-B*44:02 allogeneic cell stimulation, but only 5.04% of the total CD8 T-cell population following heterozygous HLA-B*44:02 cell stimulation (Table 1 and Figure 2). The proportion of EBV EBNA3A specific CD8 T-cells was not significantly altered by homozygous or heterozygous allo-HLA-B*44:03 stimulation in the same assay (Table 1). The percentage of EBV EBNA3A specific CD8 T-cells prior to stimulation was 1.5%.

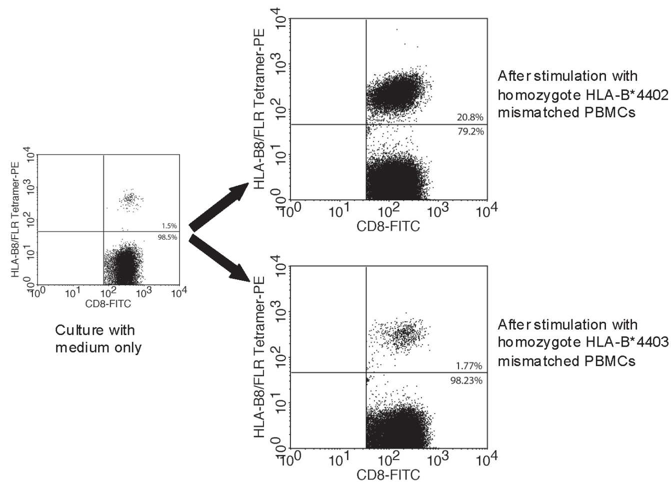

Figure 2. The proportion of EBV specific CD8 T-cells is specifically increased following allogeneic cell stimulation.
EBV EBNA3A specific CD8 T-cells accounted for 20.8% of total CD8 T-cells, following 8 day co-culture with homozygote HLA-B*44:02 mismatched irradiated PBMCs. The proportion of EBV EBNA3A 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*08:01,-; 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.

Table 1. Viral specific T-cell proliferation may be greater following homozygote allogeneic cell stimulation.

	HLA-B*44:02	HLA-B*44:03
Heterozygous stimulation	5.04%	2.19%
Homozygous stimulation	20.80%	1.77%

HLA-B*08:01+ responder PBMCs were stimulated with either homozygous or heterozygous, HLA-B*44:02 or HLA-B*44:03, allogeneic cells. The proportion of EBV EBNA3A specific CD8 T-cells was measured using viral specific tetrameric complexes and results are expressed as percentage of EBV EBNA3A specific CD8 T-cells within the total CD8 T-lymphocyte population. The percentage of EBV EBNA3A specific CD8 T-cells prior to stimulation was 1.5%. The responder PBMCs stimulated with HLA-B*44 homozygous allogeneic cells are shown in figure 2, and were harvested and used as the effector cells in the assays shown in figure 5.

Table 2. Screening for allo-HLA crossreactivity 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 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 viral specific T-cell response were identifiable for most responders and specificities.
+ Specific proliferation detected.
– No proliferation detected.

CMV specific CD8 T-cells proliferate following allogeneic cell stimulation

To determine whether allo-HLA stimulation can elicit proliferation of T-cells specific for any viral peptide/self-HLA restriction of interest, we screened for responder CMV specific T-cell proliferation using pools of PBMC stimulator cells. Proliferation of CMV specific CD8 memory T-cells was detectable using pools of 4 different PBMC stimulators together (Table 2). 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 4 different PBMCs (Pool 4 - Figure 3a and table 2). The same responder was then tested individually against the stimulators present in the screening pool in order to identify the specific stimulator (Figure 3b). Proliferation was associated with a specific increase in the proportion of CMV pp65 specific T-cells within the CD8 T-cell compartment (Figure 4). 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 (Table 2). Furthermore this stimulation is demonstrable without the need to generate viral specific T-cell clones from the responder, even when the viral specific T-cell of interest does not express a public TCR, thereby confirming that allogeneic cells stimulating viral-peptide/HLA restricted T-cells from any given responder are readily identifiable in the routine laboratory.

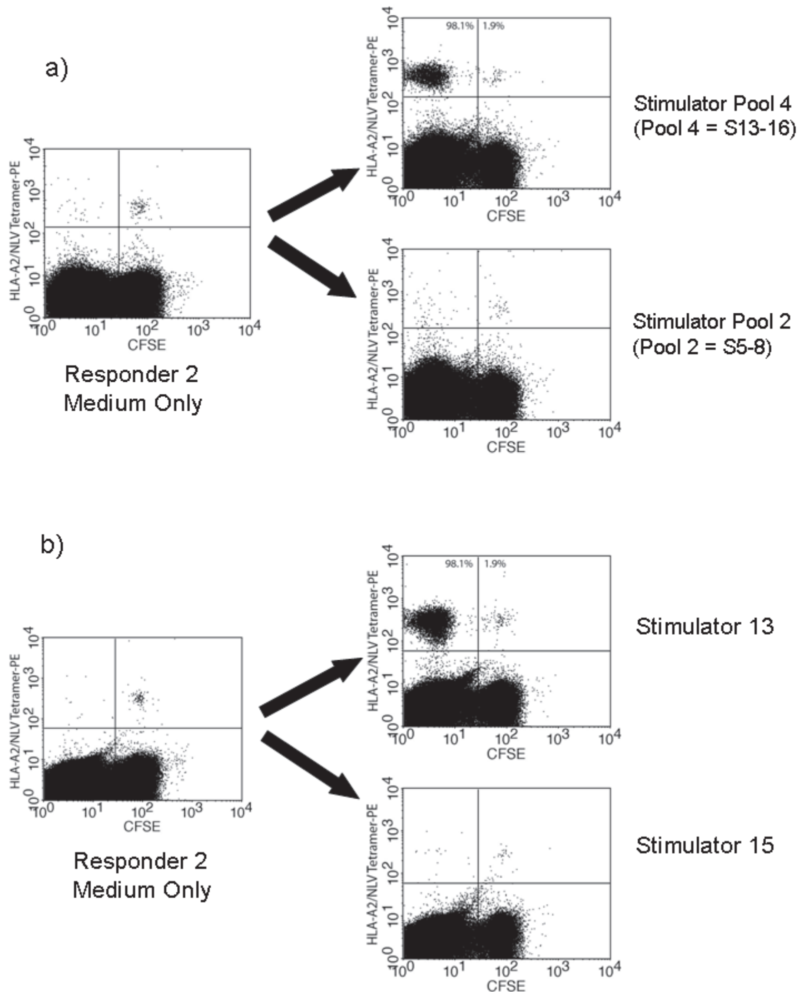

Figure 3. Screening for allo-HLA crossreactivity of viral specific memory T-cells.
(a) CMV pp65 specific CD8 Memory T-cells (A2/NLV restricted) from Responder 2 (R2) proliferate following stimulation with a pool of 4 PBMCs (Pool 4 containing stimulators 13-16), but not other pools of 4 different stimulator PBMCs (Pool 2 shown). (b) Responder 2 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 3 stimulators present in pool 4 (S15 shown). Thereby confirming the CMV pp65 specific T-cells from responder 2 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.

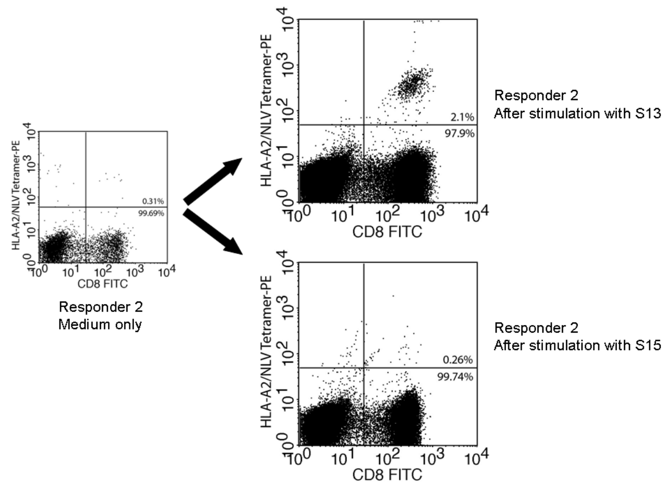

Figure 4. The proportion of CMV specific CD8 T-cells is specifically increased following allogeneic cell stimulation.
CMV pp65 specific CD8 T-cells accounted for 2.1% of total CD8 T-cells from responder 2 (R2), following 8 day co-culture with Stimulator 13 (S13). The proportion of CMV pp65 specific CD8 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*02,11; 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.

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 viral specific T-cells following allogeneic stimulation is associated with a gain of cytolytic effector function against the original viral peptide/self-HLA restricted target antigen. We therefore performed a cytolytic assay using responder HLA-B*08:01$^+$ 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 day stimulation with HLA-B*44:02 homozygote mismatched irradiated PBMCs, primed responder cells from a HLA-B*08:01$^+$ EBV seropositive healthy donor showed increased cytolytic effector function against both HLA-B*08:01$^+$ 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 to the same PBMCs co-cultured 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 (Figure 2). 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). Once again confirming that allogeneic HLA challenge can indeed increase the (in-vitro) cytolytic effector function of human viral specific T-cells against their original cognate viral antigen. We argue these proof-of-principle results may have important implications for treatment of viral infections, if confirmed in-vivo.

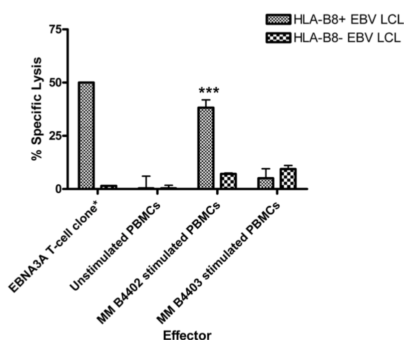

Figure 5a. EBV specific cytolytic effector function of allo-HLA primed cells using EBV LCL target cells. PBMCs from a HLA-B*08:01^{+} EBV seropositive donor gain EBV specific cytolytic effector function following allogeneic HLA-B*44:02^{+} cell stimulation, ***P<0.0001 versus HLA-B*08^{-} EBV LCL. Unstimulated HLA-B*08:01^{+} PBMCs and HLA-B*44:03 stimulated HLA-B*08:01^{+} PBMCs do not demonstrate cytolytic effector function against HLA-B*08*01^{+} EBV LCLs. Effector:target ratio 50:1, targets 2000. *Positive control EBNA3A T-cell clone is previously described (14), 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*01,02; B*08:01,-; DRB1*03,-. HLA-B8^{+} EBV LCL HLA-A*01,-; B*08:01,-; DRB1*03,-. HLA-B8^{-} EBV LCL HLA-A*03,-; B*07,-; DRB1*15,-.

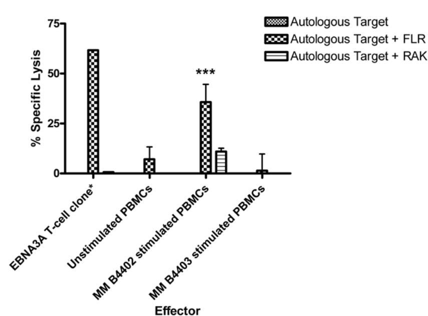

Figure 5b. EBV specific cytolytic effector function of allo-HLA primed cells using viral peptide loaded autologous target cells. PBMCs from a HLA-B*08:01^{+} EBV seropositive donor gain HLA-B8/FLR restricted cytolytic effector function following allogeneic HLA-B*44:02 stimulation. ***P=0.0094 versus RAK peptide loaded autologous cells. Unstimulated HLA-B*08:01^{+} PBMCs and HLA-B*44:03 stimulated HLA-B*08:01^{+} PBMCs do not demonstrate cytolytic effector function against FLR peptide loaded autologous cells. Effector:target ratio 50:1, targets 2000. *Positive control EBNA3A T-cell clone is previously described (14), 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*01,02; B*08:01,-; DRB1*03,-.

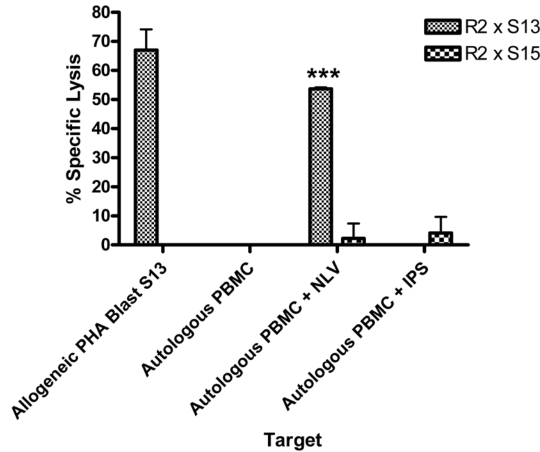

Figure 6. CMV specific cytolytic effector function of allo-HLA primed cells.
PBMCs from a CMV seropositive HLA-A*02:01+ donor (R2) gain HLA-A2/NLV restricted cytolytic effector function following heterozygote allogeneic cell stimulation with stimulator 13 cells (R2 x S13). ***P<0.0001 versus IPS loaded autologous cells. S15 stimulated PBMCs do not demonstrate cytolytic effector function against NLV peptide loaded autologous cells (R2 x S15). A strong secondary response against S13 is demonstrated from R2 responder cells primed with S13 (Positive Control), but not S15. Effector:target ratio 100:1, targets 2000. 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.

DISCUSSION

This study demonstrates that human viral specific memory T-cells gain cognate viral antigen specific cytolytic effector function following stimulation with allogeneic HLA molecules against which they are crossreactive. Stimulation of peripheral blood from a non-sensitized HLA-B*08:01+ EBV seropositive healthy donor with HLA-B*44:02 mismatched irradiated PBMCs increases (in-vitro) cytolytic effector function against EBV. Furthermore, we show this technique can be used to elicit cytolytic effector function against any potential viral antigen, as shown for CMV. These results provide proof-of-principle evidence that specific allogeneic cell therapy could be useful for treatment of viral infections.

The importance of our findings are 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.

For the EBV specific cytotoxicity assays we used homozygote HLA-B*44:02^{+} PBMCs to stimulate the EBV EBNA3A specific T-cell response, as this should provide a larger antigenic stimulus. However our CMV specific cytotoxicity assay clearly demonstrates that heterozygote cell stimulation is sufficient to prime viral specific cytolytic effector functions. Nonetheless, further studies may be required to determine if homozygote allogeneic cell therapy truly provides a significantly better stimulation over heterozygote allogeneic cell therapy.

EBV infection in a HLA-B*08:01^{+} HLA-B*44^{-} individual selects for a public BV6S2 TCR which cross-reacts against allogeneic HLA-B*44:02 (12). While not all viral specific immune responses give rise to a public TCR, the allo-HLA crossreactivity of virus specific T-cells from a given individual can be easily detected in-vitro using techniques we have described here and elsewhere (11,14). Indeed, successful stimulation of cytolytic effector function against CMV antigen reveals that this technique can be reproducibly used to elicit T-cell cytolytic effector function against any virus or specificity. Furthermore, identification of the allogeneic cells that stimulated the anti-viral cytotoxicity did not require generation of virus specific T-cell clones. Techniques described here should therefore 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 antigens. We used irradiated isolated PBMCs for stimulation of the viral specific memory T-cells thereby excluding any contributions by plasma, platelets and/or erythrocytes. Therefore we suggest allogeneic cell therapy should be investigated using only isolated leukocytes as stimulators.

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

Results presented here suggest that specific allogeneic cell therapy could prime and/or maintain viral specific memory. The proportion of EBV EBNA3A specific T-cells in the CD8 compartment increased from 1.5% to 20.8% following stimulation with homozygote HLA-B*44:02^{+} allogeneic cells and the proportion of CMV pp65 specific T-cells from 0.31% to 2.1% following heterozygote allogeneic cell stimulation. Data from preliminary clinical studies suggest that CMV specific CD8 T-cell levels greater than 1x107/L of peripheral blood may correlate with protection (22), therefore the total number of viral 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 (8,10). Transfer of memory T-cells to naïve mice, in the presence or absence of priming antigen, reveals that maintenance of T-cell memory is short lived in the absence of TCR mediated signaling (8). Furthermore, recently activated memory T-cells can bypass the requirement for CD28/CD80/CD86 co-

stimulation, as compared to resting memory T-cells that are still dependent on CD28 triggering for their activation (23). Although at baseline in our EBV specific assays 1.5% of CD8 T-cells in the peripheral blood of the individual were EBV EBNA3A specific T-cells, prior to allo-HLA-B*44:02 stimulation no cytolysis of FLR peptide loaded autologous cells could be detected, suggesting 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 viral specific T-cells via triggering TCR signaling and/or abrogating co-stimulation requirements, irrespective 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 (24-26), most of which interfere with the various steps necessary for MHC class I restricted antigen presentation. For example, CMV evades MHC class I antigen presentation by reducing the stability of class I heavy chains (27) and also by dislocating MHC class I heavy chains from the endoplasmic reticulum (28). The co-ordinated function of murine CMV genes can completely inhibit CTL lysis (29). Amongst others, the EBV EBNA1 protein contains an element that interferes with its proteasomal proteolysis and the HSV ICP47 protein inhibits the TAP complex (30-31). Many other viral immune evasion strategies are also described (32-36).

Allogeneic cell therapy may be capable of bypassing all these viral strategies of immune evasion as the viral specific memory T-cells are directly stimulated via molecular mimicry (37). The allo-HLA molecule against which the virus specific T-cell is crossreactive 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 and colleagues demonstrated that pre-emptive CMV specific CD8 T-cell immunotherapy, guided by viral DNA load, prevented lethal disease and reduced the risk of virus recurrence (38). 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.

HIV specific effector memory CD8 T-cells are present in most HIV infected individuals and play a critical role in controlling viral replication and disease progression, however HIV is also highly efficient at evading immune responses (39). Recent data demonstrate that HIV escape mutations may impair dendritic cell function (3) and that the HIV-1 Vpu protein modulates MHC class II presentation (40), thereby possibly impairing later CD4 and/or CD8 T-cell responses to the same and other epitopes. High viraemia is also associated with in-vivo downregulation of MHC class I in rhesus macaques infected with SIV (41). The maintenance of early differentiated, highly avid HIV specific CD8 T-cells by allogeneic cell therapy could induce a non-progressive course of the disease. Further in-vitro studies are warranted using responder PBMCs from HIV infected individuals.

Poorly controlled viral infections are also associated with malignancy. Post-transplant lymphoproliferative disease (PTLD) is a well recognized complication of both solid organ and allogeneic bone marrow transplantation, and is associated with a deficient cellular response from the host to EBV infected B-cells. Most PTLD occurring in solid organ setting arise from

recipient cells, therefore, allogeneic HLA-B*44:02 cell therapy may elicit an anti-tumour response in a HLA-B*08:01^{+} recipient with PTLD. Results presented here strongly support this hypothesis.

Results presented here demonstrate the stimulation of cytolytic effector function from pre-existing memory T-cells. It is unclear if allogeneic cell therapy could also be used to stimulate a de-novo viral specific response from naïve T-cells. However it is likely that stimulation of de-novo viral specific T-cell responses using allogeneic cells, from naïve T-cells, would require additional co-stimulatory factors than those provided by irradiated allogeneic PBMCs alone (42-44).

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. While 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 cause sensitization of a recipient to future transplantations. Nonetheless results demonstrated here suggest cell therapy may have potential as an alternative to adoptive transfer or pharmacological therapy to treat viral infections.

The high frequency of allo-HLA crossreactivity by viral specific T-cells in the transplantation setting is increasingly being recognised. We provide (in-vitro) evidence that allogeneic cell therapy may be useful to conversely stimulate a beneficial anti-viral 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.

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. PBMC were isolated from heparinized blood by standard density gradient centrifugation, and were subsequently cryopreserved until use. Epstein-Barr virus-transformed B-cell lines (EBV LCLs) were generated using standard procedures, and were cultured in Iscoves Modified Dulbeccos Medium (IMDM, Cambrex) with 10% fetal calf serum (FCS). The HLA type of all cells used in our experiments was determined molecularly by SSO and SSP genotyping at the Leiden University Medical Centre, Dept of Immunohematology and Blood Transfusion, the Netherlands.

Proliferation Assays for EBV EBNA3A specific T-cell responses

For the proliferation assays 1x10*6 Carboxyfluoresceinsuccinimidyl ester (CFSE)-labeled PBMC from a HLA-B*08:01^{+} HLA-B*44^{-} EBV seropositive healthy donor, were co-cultured with 1x10*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 days in IMDM culture medium with 15% human serum and IL-2 (25IU/ml). Then, 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 proportion of EBV EBNA3A specific T-cells within the total CD8 T-cell population of a single responder was also determined before and after homozygous vs. heterozygous allo-HLA-B*44 cell stimulation in a separate assay without CFSE labeling. The HLA typing of the selected responder-stimulator examples is given below the figures.

Proliferation Assays for CMV specific T-cell responses

To determine if allo-HLA stimulation could elicit an anti-viral response against any virus or specificity, we had to first determine a new method whereby specific allogeneic cells stimulating the proliferation of viral specific T-cells from any given individual could be identified. 1x10*6 Carboxyfluoresceinsuccinimidyl ester (CFSE)-labeled PBMC from CMV seropositive healthy donors, were first co-cultured with a pool of 1x10*6 total mismatched irradiated PBMCs (3000 Rad) from 4 different healthy donors (0.25x10*6 cells of each individual stimulator), in a 24 well flat bottom plate. Each responder was screened against 4 different pools of PBMCs. The 16 total different allogeneic stimulator cells were selected to cover the most common occurring HLA molecules. Cells were incubated for 7-10 days in RPMI culture medium with 15% human serum and IL-2 (25IU/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 following stimulation with a screening pool of 4 different allogeneic PBMCs, then the same responder PBMCs were tested individually against the 4 stimulator PBMCs to determine which allogeneic cell(s) 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 al-

logeneic 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 methods below). The HLA typing of the selected responder-stimulator examples is given below the figures.

Cytotoxicity Assays

To confirm that allogeneic cell stimulation resulted in increased viral specific cytolytic effector function, not just proliferation, from the stimulated PBMCs 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 day mixed lymphocyte reaction with allo-HLA mismatched irradiated cells to stimulate a viral specific memory T-cell of interest (see methods 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 hour ^{51}Cr release assay. Cognate viral peptide or control viral peptide was directly added to the autologous target cells and incubated for 60 minutes, simultaneously with chromium incubation, and then washed three times. Supernatants were harvested for gamma counting: *per cent-specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.*

Statistical Analysis

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

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose.

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