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**ARTICLE**

# Prophylactic and therapeutic adenoviral vector-based multivirus-specific T-cell immunotherapy for transplant patients

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Viral infections including cytomegalovirus, Epstein-Barr virus, adenovirus, and BK virus are a common and predictable problem in transplant recipients. While cellular immune therapies have been successfully used to tackle infectious complications in transplant recipients, manufacturing immunotherapies to address the multitude of possible pathogens can be technically challenging and labor-intensive. Here we describe a novel adenoviral antigen presentation platform (Ad-MvP) as a tool for rapid generation of multivirus-specific T-cells in a single step. Ad-MvP encodes 32 CD8+ T-cell epitopes from cytomegalovirus, Epstein-Barr virus, adenovirus, and BK virus as a contiguous polyepitope. We demonstrate that Ad-MvP vector can be successfully used for rapid *in vitro* expansion of multivirus-specific T-cells from transplant recipients and *in vivo* priming of antiviral T-cell immunity. Most importantly, using an *in vivo* murine model of Epstein-Barr virus-induced lymphoma, we also show that adoptive immunotherapy with Ad-MvP expanded autologous and allogeneic multivirus-specific T-cells is highly effective in controlling Epstein-Barr virus tumor outgrowth and improving overall survival. We propose that Ad-MvP has wide ranging therapeutic applications in greatly facilitating *in vivo* priming of antiviral T-cells, the generation of third-party T-cell banks as "off-the-shelf" therapeutics as well as autologous T-cell therapies for transplant patients.

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## INTRODUCTION

Stem cell or solid organ transplantation (SOT) is essential treatments for patients with hematological malignancies or organ failure. Treatment success can be limited by infectious complications caused by common pathogens such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK virus (BKV) or adenovirus (ADV) that arise as a result of profound immunosuppression after transplantation.[1–3] Antiviral drugs given either prophylactically or as early therapy for patients with detectable viral loads are an effective strategy for reducing viral infections.[4–6] However, long-term treatment with these drugs is associated with significant toxicity, expense and the appearance of drug-resistant virus isolates, which ultimately results in treatment failure.[7–9] Cellular immunotherapy has emerged as an effective alternative treatment that can prevent or reduce virus-associated transplant complications while being associated with much lower toxicity.[10–17] One of the major limitations of autologous or donor-derived T-cell therapy is that the process of generating these effector cells often takes many weeks or months. This limits the use of this approach therapeutically, because the patients often succumb to progressive disease or lose their graft before the T-cells are ready for infusion. Ideally, a T-cell therapy that can be offered as an "off-the-shelf" treatment would be more suitable for these patients. A second limitation is that T-cell preparations often only target a single pathogen which restricts their utility for patients presenting with multiple infections and makes the generation of T-cell banks more laborious and costly. While recent studies have successfully developed strategies to expand multivirus-specific T-cells,[18–20] one major limitation in the manufacture of these effector cells is that the precise epitope specificity of T-cells expanded using a complex mixture of synthetic peptides remains poorly defined. Moreover, the use of mixtures of overlapping peptides from multiple antigens increases the potential risk of expansion of allogeneic T-cells which may be reactive against engrafted organ. This is particularly relevant for SOT patients where the risk of graft rejection by allogeneic T-cells is much higher when compared with stem cell transplant recipients. To overcome these limitations, we have developed a novel replication-deficient adenoviral antigen presentation system which encodes multiple human leukocyte antigen (HLA) class I-restricted minimal T-cell epitopes from EBV, CMV, BKV, and ADV as a polyepitope protein (referred to as Ad-MvP). We demonstrate that the Ad-MvP platform can be used for the rapid expansion of multivirus-specific cytotoxic T-cells from SOT recipients following

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**Table 1** Clinical characteristics of SOT recipients

| *Patient ID* | *Age/sex* | *Organ* | *Drugs* | *Serological status* | *Antiviral treatment* | *CMV/EBV/BKV reactivations post tx* | *CMV/EBV/BKV disease* |
|---|---|---|---|---|---|---|---|
| SOT02 | | lung | FK, MMF, P | R+/D+ (CMV) | Val | 2 (CMV) | Yes (eye) |
| SOT06 | | lung | CsA, MMF, P | R+/D+ (CMV) | Gan, Val | 2 (CMV) | Yes (lung) |
| SOT26 | | lung | FK, MMF, P | R+/D+ (CMV) | Val | 2 (CMV) | No |
| SOT35 | | lung | CsA, MMF, P, FK | R−/D+ (CMV) | Val | 1 (CMV) | No |
| SOT56 | | kidney | TK, MMF, P | R+/D− (CMV) | Val | 0 | No |
| SOT58 | | kidney | FK, MMF, P,B | R−/D+ (CMV) | Gan | 1 (CMV) | No |
| SOT62 | | kidney | CsA, MMF, P, FK | R+/D− (CMV) | None | 3 (CMV) | No |
| SOT68 | | kidney | CsA, MMF, P | R−/D+ (CMV) | Val | 2 (CMV) | No |
| SOT75 | | kidney | CsA, MMF, P, FK | R−/D+ (CMV) | Gan, Val | 3 (CMV) | No |
| SOT22 | | Lung | CsA, P, MMF, AZA | R−/D+ (EBV) | Gan | 2 (EBV) | Yes (PTLD) |
| SOT33 | | Heart | CsA, P AZA | R−/D+ (EBV) | Val | 2 (EBV) | Yes (PTLD) |
| SOT59 | | Lung | CsA, AZA, P | R−/D+ (EBV) | NA | 1 (EBV) | Yes (PTLD) |
| SOT15 | | Kidney | FK, P, E | R−/D+ (BKV) | None | 1 (BKV) | Yes (BKVAN) |
| SOT22 | | Kidney | FK, P | R−/D+ (BKV) | Lef | 1 (BKV) | Yes (BKVAN) |

ADV, adenovirus; AZA, azathioprine; B , basiliximab; BKV, BK virus; BKVAN, BK-associated nephropathy; CMV, cytomegalovirus; CsA, cyclosporin A; D, donor; EBV, Epstein-Barr virus; E, Everolimus; FK, tacrolimus; Gan, ganciclovir; Lef, Leflunomide; MMF, mycophenolate mofetil; P, prednisone; PTLD, post-transplant lymphoproliferative disorder; R, recipient; SOT, solid organ transplantation; tx, transplantation; Val, valganciclovir , , .

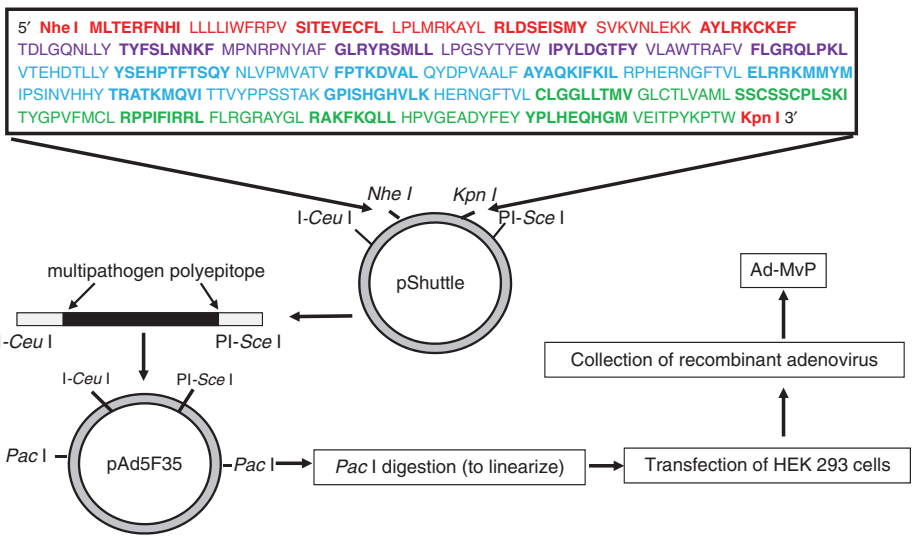


**Figure 1** Schematic outline for the construction of Ad-MvP Synthetic DNA sequence encoding a polyepitope protein containing contiguous 32 HLA class I-restricted CTL epitopes from BKV (red text), ADV (violet text), CMV (blue text), and EBV (green text) was cloned into a pShuttle vector and then subcloned into the Ad5F35 expression vector. The recombinant Ad5F35 vector was packaged into infectious adenovirus by transfecting HEK 293 cells, and recombinant adenovirus (referred to as Ad-MvP) was harvested from transfected cells by repeated freeze-thawing cycles. ADV, adenovirus; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

single stimulation and that these T-cells are highly effective in controlling virus-associated B cell lymphoma. In addition, Ad-MvP can also be used successfully for priming and/or boosting multivirus-specific T-cells *in vivo*. Use of the Ad-MvP vector will greatly facilitate the rapid production of multivirus-specific T-cell banks to make autologous or "off-the-shelf" T-cell therapy readily available for transplant patients.

## RESULTS

### Single stimulation with Ad-MvP is sufficient to expand polyfunctional multivirus specific T-cells from transplant recipients

In the first set of experiments, we explored the potential application of the Ad-MvP antigen presentation system for transplant recipients. We recruited a cohort of SOT recipients who had either ongoing

or a previous history of recurrent viral reactivation/disease (CMV, EBV or BKV; Table 1). Peripheral blood mononuclear cells (PBMC) from these SOT recipients were stimulated with Ad-MvP (Figure 1) at a multiplicity of infection at 10:1 and then cultured for 14 days. Representative data from two different transplant recipients presented in Figure 2a shows that a single stimulation with Ad-MvP was sufficient to induce the rapid expansion of T-cells specific for ADV, BKV, CMV, and EBV epitopes. T-cells expanded from SOT33 showed strong reactivity toward CMV and EBV, while T-cells expanded from SOT15 showed strong reactivity against CMV but also EBV, BKV, and ADV. A comprehensive summary of T-cell expansions following Ad-MvP stimulation from 14 SOT recipients is presented in Figure 2b. These analyses showed that CMV, BKV, EBV, and ADV-specific T-cell expansions were observed in 86, 71, 86, and 29% of SOT patients respectively (Figure 2b). More importantly, the majority of these *in vitro* expanded T-cells showed a polyfunctional profile (Figure 2c). Taken together, these studies showed that Ad-MvP is highly efficient in expanding multivirus-specific T-cells from transplant recipients and this expansion is not impacted by underlying immunosuppression or ongoing viral reactivation/disease.

*In vivo* priming of multivirus-specific T-cells with Ad-MvP

In addition to the potential application of Ad-MvP as a tool for *in vitro* expansion of pre-existing memory/effector T-cells, using a mouse model we also explored the utility of this vector for *in vivo* priming of multivirus-specific T-cells in seronegative transplant recipients/donors. Transgenic mice expressing the HLA A*0201 allele (referred to as HHD II mice) were immunized with Ad-MvP ($0.5\times10^8$ pfu/mouse) and then one group was boosted with the same dose on day 21. On day 50 postimmunization, these mice were assessed for antigen-specific T-cell responses. While *ex vivo* analysis revealed strong T-cell response to EBV epitopes and a low or undetectable response toward epitopes from CMV, BKV, and ADV, a 6–240 fold increase in antigen-specific T-cells was observed following *in vitro* stimulation with BKV, ADV, CMV, or EBV-specific HLA-A*0201-restricted peptide pools (Figure 3a). A comprehensive summary of multiple HLA-A2-restricted T-cell responses in HHD II mice following Ad-MvP prime alone and prime-boost immunization is shown in Figure 3b. This analysis also showed that while in both the prime alone and prime-boost setting EBV-specific T-cell responses were the dominant component of *ex vivo* analysis, a significant change in the composition of antigen-specific T-cells was observed following *in vitro* stimulation. Taken together, these experiments clearly demonstrated that Ad-MvP vector is highly efficient in inducing multivirus-specific T-cells *in vivo*.

Expansion of multivirus-specific T-cells from healthy donors with Ad-MvP for third-party T-cell bank

While autologous T-cell therapy has been successfully used to treat many SOT recipients, many patients are not amenable to this therapy due to severe lymphopenia or transplant-related clinical

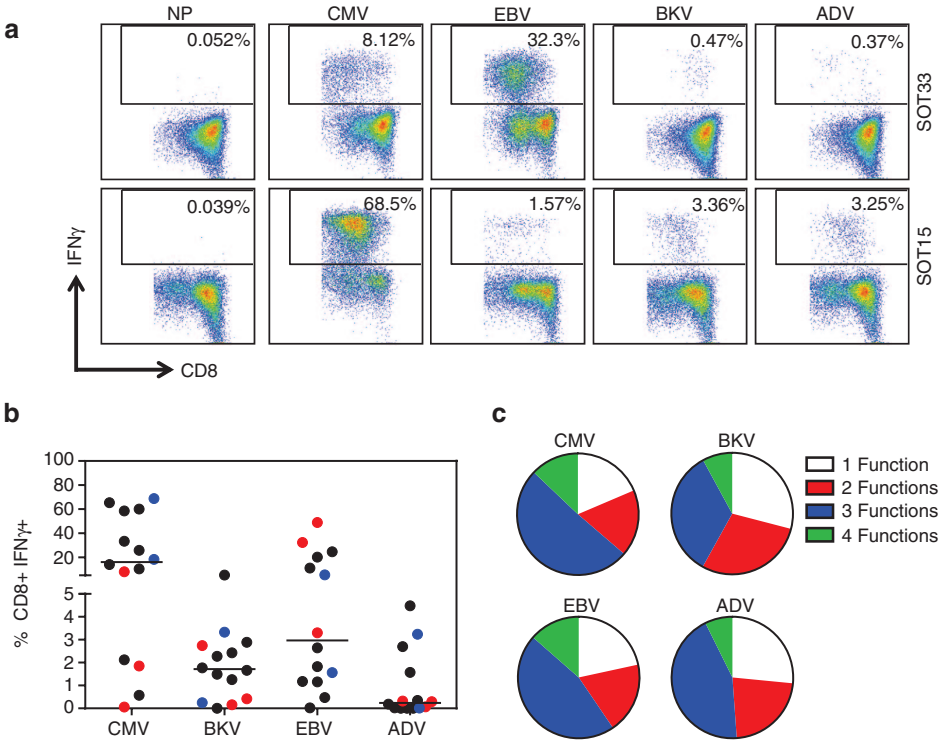


**Figure 2** Expansion of multivirus-specific T-cells from solid-organ transplant recipients with Ad-MvP. PBMC from 14 SOT patients were stimulated with Ad-MvP and cultured for 14 days in the presence of IL-2. The frequency of epitope specific CTL was determined by measuring IFNγ production in response to stimulation with virus-specific peptide pools containing epitopes encoded in Ad-MvP. (**a**) Representative dot plots following recall with CMV, EBV, BKV or ADV peptide epitopes is shown. (**b**) Data represents a summary of the number of virus-specific IFNγ-producing $CD8^+$ T-cells from all SOT patients. Black symbols represent patients recruited with CMV-associated complications, red symbols represent patients with EBV-associated PTLD, and blue symbols represent patients with BKV viremia (**c**) Ad-MvP expanded CTL were assessed for the intracellular production of IFNγ, TNF, IL-2, and externalization of CD107a following *in vitro* stimulation with the virus-specific peptide pools. Boolean Analysis was performed using FlowJo Software (Ashland, OR). Pie Charts represent the proportion of T-cells specific for each virus capable of generating monofunctional and polyfunctional $CD8^+$ T-cells that produce various combinations of IFNγ, TNF, IL-2, and externalisation of CD107a simultaneously. ADV, adenovirus; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; IFNγ, interferon gamma; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; SOT, solid organ transplantation; TNF, tumor necrosis factor.

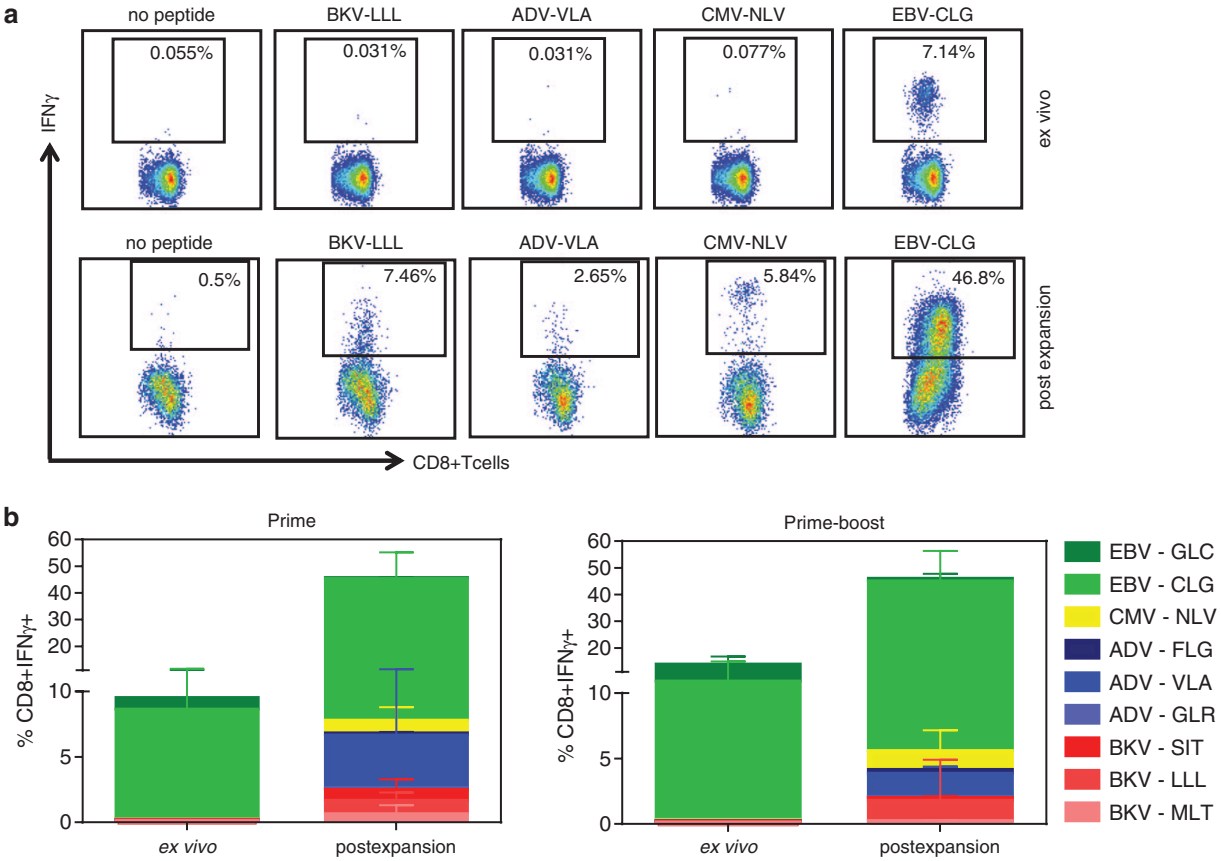


**Figure 3** Priming of multivirus-specific T-cells following immunization with Ad-MvP. (**a**) Representative data showing *ex vivo* and *in vitro* expanded virus-specific T-cells from HHD II transgenic mouse immunized with Ad-MvP. (**b**) Stacked bar graph showing percentage of multivirus-specific CD8$^+$ T-cells expressing IFNγ in HLA*A02 transgenic mice immunized with Ad-MvP. Splenocytes from immunized mice were isolated on day 50 postvaccination and stimulated *in vitro* with HLA-A*02-restricted CD8$^+$ T-cell peptide epitopes from BKV, ADV, CMV or EBV. T-cell specificity was assessed using an intracellular cytokine assay. ADV, adenovirus; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

complications. More recently, third-party HLA matched virus-specific T-cell therapy has emerged as an excellent alternative to autologous cellular therapy. To assess Ad-MvP as a potential tool for manufacturing T-cell banks, PBMCs from a panel of healthy volunteers were stimulated with autologous PBMCs infected with Ad-MvP at a multiplicity of infection of 10:1 and then cultured for 14 days. *Ex vivo* analyses showed that the mean T-cell response specific for EBV, CMV, BKV, and ADV were 0.33% (range 0–1.12%), 0.49% (0–1.519%), 0.03% (range 0–0.013%), and 0.006% (range 0–0.054%) respectively. A comprehensive summary of T-cell expansions following Ad-MvP stimulation from 20 healthy donors is presented in Figure 4a. These analyses showed that in all healthy donor samples T-cells specific for at least three different viruses were detected. The mean expansions of CD8$^+$IFNγ$^+$ T-cells specific for CMV, EBV, BKV, and ADV were 33.83, 15.91, 1.70, and 1.12% respectively. The polyfunctional profiling of these *in vitro* expanded effector cells showed that 60–80% of EBV, CMV, BKV, and ADV-specific T-cells expressed IFNγ, TNF, and/or IL-2 with strong cytotoxic potential as assessed by CD107a mobilization (Figure 4b).

To further refine the culture conditions required for optimal yield of multivirus- specific T-cells, we assessed T-cell expansion potential in the presence of different cytokine combinations in comparison to the standard supplementation with IL-2 alone. PBMCs from healthy donors were stimulated with Ad-MvP and expanded in the presence of combinations of IL-2, IL-21, IL-7, and/or IL-15/IL-7. While the overall T-cell expansions and polyfunctional profile was slightly improved when cells were cultured in the presence of IL-2 in combination with IL-21 and IL-15, there was no statistically significant difference when compared with T-cell expansion in IL-2 alone (Figure 4c,d). Similarly, no statistical difference was observed in absolute percentage of CD3$^+$/CD4$^+$/CD8$^+$ T-cells following healthy donor PBMC stimulation with Ad-MvP and expansion in the presence of combinations of IL-2, IL-21, IL-7, and/or IL-15/IL-7, however, there was a modest increase in the multivirus-specific CD8$^+$ T-cell percentage when expanded in the presence of IL-2, IL-7, and IL-15 or IL-2 and IL-21 cytokine combinations (Figure 5).

### Autologous and allogeneic adoptive immunotherapy with Ad-MvP-expanded T-cells

Having established the *in vitro* and *in vivo* immunogenicity of the Ad-MvP vector, the next set of experiments were designed to assess the potential therapeutic application of the Ad-MvP vector in a mouse model of EBV-associated lymphoma. A group of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were engrafted with EBV-transformed lymphoblastoid cells (Donor code: D01; HLA A1, A11, B8, and B35). Autologous T-cells from D01 were expanded using Ad-MvP and which included CD8$^+$ T-cells specific for three EBV epitopes (HLA B8 and B35-restricted) as well as CMV and ADV (Figure 6a). On day 6 after EBV lymphoma induction, mice were adoptively treated with a single injection of autologous Ad-MvP expanded T-cells. Data presented in Figure

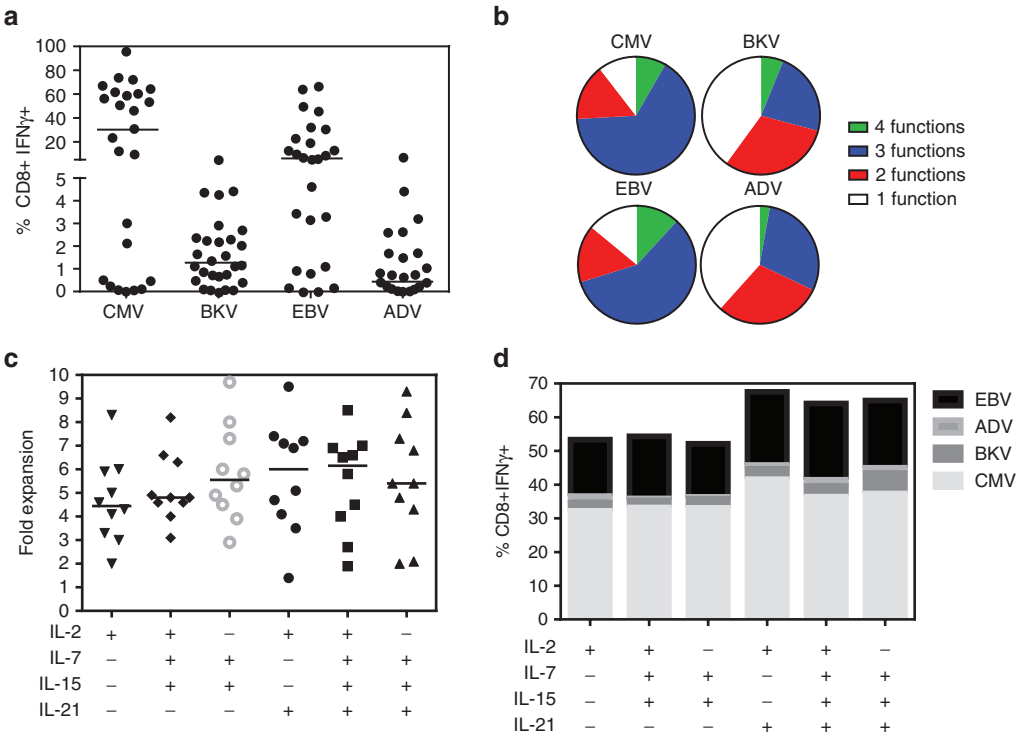


**Figure 4** Expansion of multivirus-specific T-cells using Ad-MvP in healthy volunteers. PBMC from healthy volunteers were stimulated with Ad-MvP and expanded in the presence of IL-2 for 14 days. The frequency of epitope specific CTL was determined by measuring IFNγ production in response to stimulation with HLA-matched epitopes contained in Ad-MvP. (**a**) Summary of the frequency of multivirus specific T-cells in a cohort of healthy donors. (**b**) Ad-MvP expanded CTL were stimulated with peptide pools corresponding to the epitopes contained in the polyepitope for each virus. Production of IFNγ, TNF, IL-2, and externalization of CD107a were measured as markers of polyfunctionality. (**c**) *In vitro* expansion of multivirus-specific CD8⁺ T-cells from healthy donors using Ad-MvP in the presence of different cytokine combinations. (**d**) The frequency of antigen-specific T-cells following *in vitro* culture in the presence of different cytokines was assessed using intracellular cytokine assays. IFNγ, interferon gamma; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor.

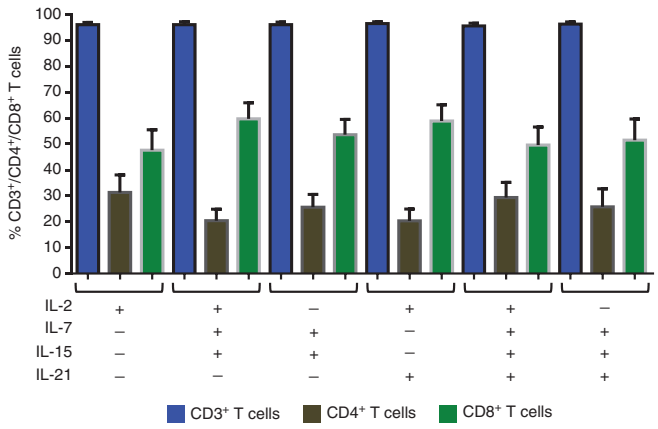


**Figure 5** Evaluation of percentage of expanded multivirus-specific CD3⁺/CD4⁺/CD8⁺ in healthy volunteers. PBMC from healthy volunteers were stimulated with Ad-MvP and expanded in the presence of IL-2, IL-21, IL-7, and/or IL-15/IL-7 for 14 days. The percentage of CD3⁺/CD4⁺/CD8⁺ T-cell expansion was evaluated using cell surface markers. Results are shown as mean cell percentage ± SEM. IL, interleukin; PBMC, peripheral blood mononuclear cells.

6b,c shows that following adoptive immunotherapy, a significant delay in lymphoma outgrowth was observed in mice treated with Ad-MvP-expanded autologous T-cells when compared with mock-treated mice ($P = 0.033$). Considering the broader applicability of allogeneic antigen-specific T-cell therapy, we also assessed the therapeutic efficacy of Ad-MvP expanded T-cells from a HLA-matched donor (Donor code: D055; HLA A1, A2, B8, and B40). The expanded T-cells from D055 included T-cells specific for CMV, ADV, and four EBV epitopes restricted through HLA B8 and HLA A2. T-cells specific for HLA B8-restricted epitopes (FLR and RAK) matched to the EBV lymphoma in NOD/SCID mice (Figure 6d). Tumor bearing mice treated with allogeneic multivirus-specific T-cells also showed significantly delayed tumor growth (Figure 6e,f; $P = 0.0065$).

## DISCUSSION

T-cell therapy has emerged as an effective treatment tool for viral morbidity and mortality after transplant that provides an alternative to costly prophylactic/pre-emptive antiviral therapy which is often limited by toxicity and resistance.[10,12,18,21–24] However, transplant patients are often vulnerable to multiple infections and one challenge for the broader application of T-cell therapy is the number of pathogens that can be targeted.[25–28] We describe here a novel antigen presenting platform designed to stimulate T-cell responses to four common pathogens (CMV, EBV, BKV, and ADV) which have been identified as the major causes of viral complications after transplant. The Ad-MvP platform encodes multiple CD8⁺ T-cell epitopes from the four different viruses as a polyepitope protein. We demonstrate that stimulation with Ad-MvP consistently achieved twofold to 10-fold expansions of multivirus-specific T-cells both from healthy volunteers as well as transplant patients who have been heavily pretreated with immunosuppressants and have ongoing EBV or CMV-associated clinical complications. Furthermore, we show that both autologous and allogeneic Ad-MvP T-cells can control the growth of EBV-associated lymphoma in a xenogeneic

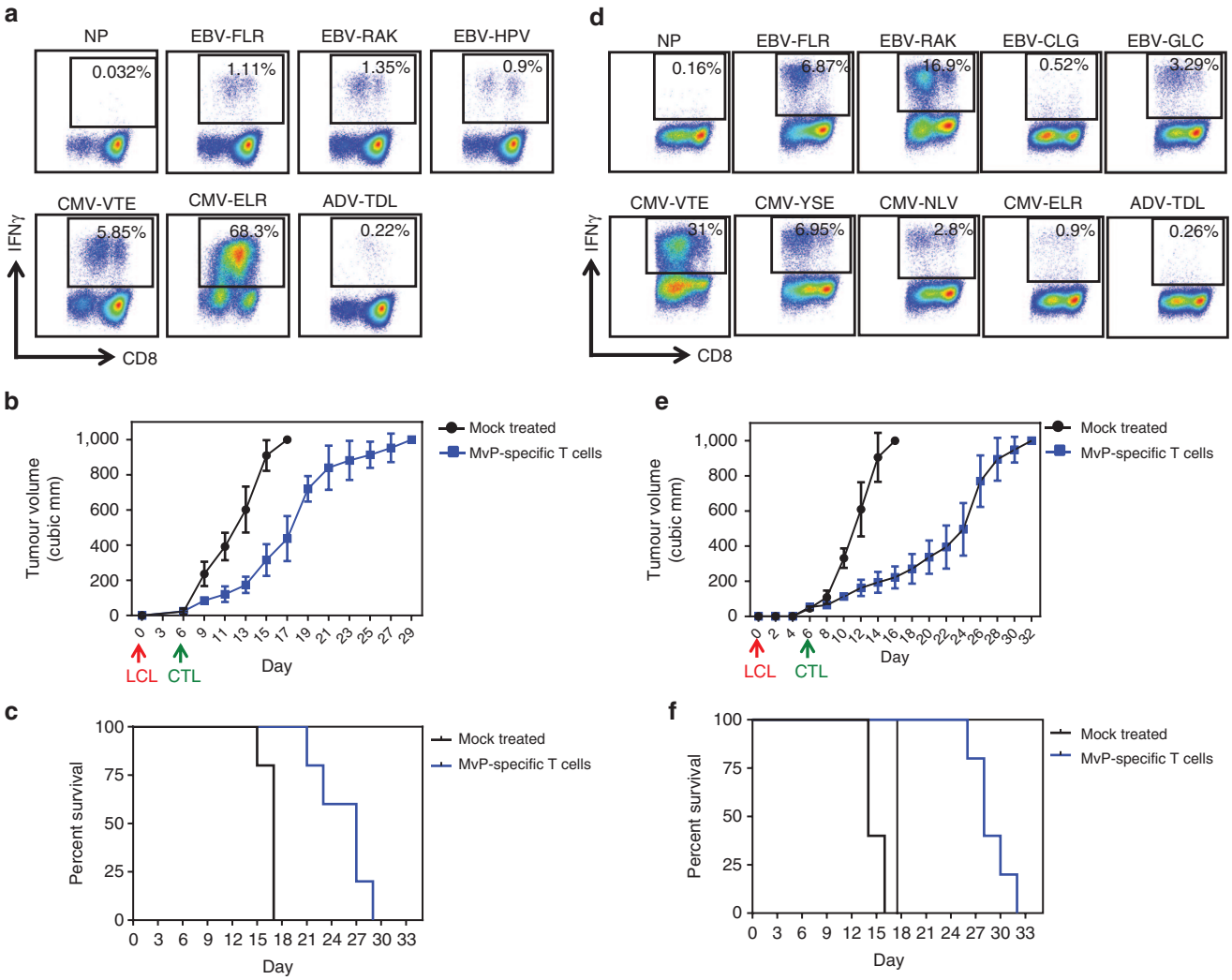


**Figure 6** Adoptive immunotherapy for EBV-associated B-cell lymphoma using Ad-MvP expanded autologous or allogeneic multivirus-specific T-cells. **(a, d)** Epitope-specificity analysis of Ad-MvP expanded T-cells from donors D01 (HLA A1, A11, B8, B35) and D055 (HLA A1, A2, B8, B40) using intracellular cytokine assays (**b**) NOD/SCID mice ($n = 10$) were engrafted with EBV transformed lymphoblastoid cells (LCLs) from donor H002 to induce B cell lymphoma. On day 6 after engraftment, mice were either mock treated ($n = 5$) or adoptively infused with autologous $2 \times 10^7$ Ad-MvP expanded CTL ($n = 5$; shown in panel **a**). Tumor volume was measured using vernier calipers. (**c**) Kaplan-Meier survival graph of EBV tumor bearing mice after mock treatment or autologous T-cell therapy. (**e**) NOD/SCID mice ($n = 10$) were engrafted with EBV transformed LCL from donor H002 to induce B-cell lymphoma. On day 6 after engraftment, mice were either mock treated ($n = 5$) or adoptively infused with HLA matched allogeneic Ad-MvP expanded T-cells from donor H005 ($n = 5$; shown in panel **b**). Tumor volume was measured using vernier calipers. Each data points in panels **b** and **e** shows mean ± SEM of tumor size as measured in multiple mice using vernier calipers. **(f)** Kaplan–Meier survival graph of EBV tumor bearing mice after mock treatment or allogeneic T-cell therapy. EBV, Epstein-Barr virus; LCL, lymphoblastoid cells.

mouse model, demonstrating the potential application of the MvP vector for use in both an autologous or HLA-matched allogeneic setting.

Recent studies from a number of groups have begun to explore the potential application of multivirus-specific T-cells for the treatment of a range of viral diseases in transplant patients.[16,17,29] The initial protocols established to generate multivirus-specific T-cells were very laborious and dependent upon the production of EBV-transformed lymphoblastoid cells as a source of antigen presenting cells (APCs) for repeated antigenic stimulation. More recent protocols have refined these approaches and now typically employ a single stimulation with pools of overlapping peptide libraries from immunodominant viral antigens. These multivirus-specific T-cells expanded with peptide libraries were recently used in stem cell transplant patients and provided a 94% virological and clinical response rate.[30] While this approach has accelerated the production of multivirus-specific T-cells, precise characterization of epitope specificity of these T-cell populations may remain a challenge due to the complexity of the antigenic stimuli used. In contrast, the Ad-MvP vector simplifies the characterization of the clinical product by using a polyepitope of fully defined HLA-restricted peptide epitopes. In addition, the Ad-MvP approach also provides a platform for the selection of immunodominant T-cell epitopes irrespective of the antigen source. This is of particular importance for large viruses, including EBV and CMV, which encode between 160–250 open reading frames. Overlapping peptide based approaches targeting single immunodominant antigens, such as CMV-pp65, have the potential to exclude immunodominant T-cell responses encoded by other antigens.

The capacity to fully characterize T-cell specificity and HLA-restriction is likely to be of particular importance in the application of immunotherapy using allogeneic T-cell banks. This is particularly relevant for advanced stage development and formal

**Table 2** List of HLA class I restricted T-cell epitopes included in Ad-MvP

| *Virus* | *Sequence*[a] | *Antigen* | *HLA restriction* |
|---|---|---|---|
| **BKV** | **MLTERFNHIL** | large T antigen | A*02 |
| | **LLLIWFRPV** | large T antigen | A*02:01 |
| | **SITEVECFL** | VP1 | A*02:01 |
| | **LPLMRKAYL** | large T antigen | B*07:02, B*08 |
| | **RLDSEISMY** | large T antigen | A*01 |
| | **SVKVNLEKK** | large T antigen | A*03 |
| | **AYLRKCKEF** | large T antigen | A*24 |
| **ADV** | TDLGQNLLY | hexon protein | A*01 |
| | TYFSLNNKF | hexon protein | A*24:02 |
| | MPNRPNYIAF | hexon protein | B*07, B*35 |
| | GLRYRSMLL | hexon protein | A*02:02 |
| | LPGSYTYEW | hexon protein | B*53:01 |
| | IPYLDGTFY | hexon protein | B*35, B*53:01 |
| | **VLAWTRAFV** | DNA polymerase | A*02 |
| | **FLGRQLPKL** | DNA binding protein | A*02 |
| **CMV** | **VTEHDTLLY** | pp50 | A*01 |
| | **YSEHPTFTSQY**[b] | pp65 | A*01, *B*44* |
| | **NLVPMVATV** | pp65 | A*02:01 |
| | **FPTKDVAL** | pp65 | B*35:02, B*35:08 |
| | **QYDPVAALF** | pp65 | A*24:02 |
| | **AYAQKIFKIL** | IE-1 | A*23:01, A*24:02 |
| | **RPHERNGFTVL** | pp65 | B*07:02 |
| | **ELRRKMMYM** | IE-1 | B*08:01 |
| | **IPSINVHHY** | pp65 | B*35:01 |
| | **TRATKMQVI** | pp65 | C*06:02 |
| | **TTVYPPSSTAK** | pp150 | A*03:01, A*68:01 |
| | **GPISHGHVLK** | pp65 | A*11 |
| | **HERNGFTVL** | pp65 | B*40:01 |
| **EBV** | **CLGGLLTMV** | LMP2a | A*02:01 |
| | **GLCTLVAML** | BMLF1 | A*02:01 |
| | **SSCSSCPLSKI** | LMP2a | A*11:01 |
| | **TYGPVFMCL** | LMP2a | A*24:02 |
| | **RPPIFIRRL** | EBNA3A | B*07:02 |
| | **FLRGRAYGL** | EBNA3A | B*08:01 |
| | **RAKFKQLL** | BZLF1 | B*08:01 |
| | **HPVGEADYFEY**[b] | EBNA1 | B*35:01, B*35:08, B*53:01 |
| | **YPLHEQHGM** | EBNA3A | B*35:01, B*35:02, B*35:03 |
| | **VEITPYKPTW** | EBNA3B | B*44:02 |

ADV, adenovirus; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus.
[a]Bold epitopes are included in the polyepitope, nonbold epitopes are delivered by the adenoviral vector. [b]Underlined sequences indicate epitopes that are embedded in the larger sequence and their respective HLA restrictions.

implementation of these therapies in clinical settings. Allogeneic antigen-specific T-cell therapy, which is reliant upon the use of healthy seropositive blood donors to generate "off-the shelf" T-cell products, has been successfully employed in a number of clinical centers using both single and multivirus-specific T-cells.[18,19] Using a xenogeneic EBV lymphoma model we demonstrated that allogeneic T-cells displayed a similar level of protection against tumor burden as that afforded by autologous T-cells. This was in spite of the fact that the allogeneic MvP-specific T-cell product matched only 50% of HLA class I alleles expressed by lymphoma cells.

Another potential application of the Ad-MvP is its use to prime virus-specific T-cell responses in seronegative transplant recipients prior to transplant. It has been suggested that vaccination of seronegative patients before transplant could provide protection against common infectious complications post-transplant.[31–34] While a significant amount of preclinical and clinical research has been undertaken to explore the use of vaccination in transplant patients, particularly in the context of CMV, the capacity to induce T-cell memory against multiple potential infectious complications using a single vaccine vector has the potential to reduce the cost associated with developing a vaccine platform for each disease. Using a HLA transgenic mouse model, we demonstrated that Ad-MvP induced robust responses to multiple viruses that could be recalled and expanded following restimulation with viral peptide epitopes. These observations provide preclinical evidence that in addition to its potential use a platform to rapidly generate multivirus-specific T-cells, the Ad-MvP vector also has potential use as a vaccine vehicle to induce memory T-cell immunity against multiple viruses in a single dose.

Adoptive immunotherapy with either autologous or allogeneic HLA matched multivirus-specific T-cells has the potential to provide a simplified platform for the treatment of multiple infectious complications associated with organ transplantation. For this approach to be broadly applicable several criteria must be fulfilled including simple and rapid manufacturing protocols, broad HLA-coverage, targeting of multiple viral epitopes and effectiveness against more than one common pathogen. We have demonstrated that the Ad-MvP vector addresses all of the above criteria, and is therefore likely to be a valuable tool to make T-cell therapy readily available to treat viral complications after transplantation.

## MATERIALS AND METHODS

### Construction of multivirus adenoviral vector (Ad-MvP)

The amino acid sequence of the 32 contiguous HLA class-I restricted $CD8^+$ T-cell epitopes as a polyepitope from CMV, EBV, ADV, and BKV (Table 2) was translated into the nucleotide sequence using human universal codon usage. These epitopes have been previously described.[35–38] The nucleotide acid sequence encoding the polyepitope with *Nhe I* and *Kpn I* restriction sites at 5′ and 3′ respectively was cloned into the pShuttle expression vector. Following amplification, the expression cassette from pShuttle was subcloned into an Ad5F35 expression vector. The recombinant Ad5F35 vector was transfected into human embryonic kidney HEK293 cells, and recombinant adenovirus (referred to as Ad-MvP) stocks were produced in HEK293 cells (Figure 1).

### *In vitro* expansion of multivirus-specific T-cells

PBMCs were isolated from peripheral blood by Ficoll gradient, washed and resuspended in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% foetal bovine serum (FBS) (growth medium) or revived from frozen stocks and rested for at least 1 hour at 37°C before being used in T-cell assays. The cells were divided into responder and stimulator cells at a responder to stimulator ratio of 2:1. The stimulator cells were infected with Ad-MvP at a multiplicity of infection of 10:1 for 1 hour at 37°C. Unbound virus particles were washed off and the stimulator cells were cocultured with the responder cells in the presence of different cytokines as indicated (interleukin-2, IL-2 =

120 IU/ml, IL-21 = 30 ng/ml, IL-7 = 10 ng/ml, and/or IL-15 = 10 ng/ml). Every 3–4 days, the cultures were supplemented with growth medium containing the respective cytokines. Virus-specific T-cell expansion was tested on day 14 using an intracellular cytokine assay.

### Characterization of multivirus specific CTL by intracellular cytokine assay and flow cytometry

PBMCs or cultured T-cells were stimulated with 1 µg/ml peptides corresponding to defined HLA class I-restricted $CD8^+$ T-cell epitopes derived from CMV, EBV, BKV or ADV proteins and incubated in the presence of a CD107a-antibody, Brefeldin A and Monensin for 5 hours. After surface staining for CD8 and CD4, cells were fixed and permeabilized with cytofix/cytoperm and stained for IFNγ, IL-2, and TNF. Stained cells were resuspended in phosphate-buffered saline containing 2% paraformaldehyde and acquired using a FACSCanto II or LSR Fortessa with FACSDiva software (BD Biosciences,San Jose, CA). Postacquisition analysis was conducted using FlowJo software (version 10.1r7).

### Ad-MvP immunization in HLA transgenic mice

All animal immunization protocols were conducted in compliance with the QIMR Berghofer Medical Research Institute Animal Ethics Committee. HLA-A*02 transgenic mice (HHD II) were maintained in a pathogen–free animal facility at QIMR Berghofer. Three groups (placebo, prime, and prime-boost) of 6–8 week old female mice were injected intramuscularly with 50 µl phosphate-buffered saline or 50 µl Ad-MvP ($1 \times 10^9$ pfu/ml). A booster dose was given on day 21 to the prime-boost group. Mice were sacrificed on day 50, splenocytes from all the groups were stimulated *in vitro* with BKV, ADV, CMV or EBV-specific HLA-A*02 restricted peptide pools. Splenocytes were cultured in a 24-well plate for 10 days at 37˚C, 10% $CO_2$. On days 3 and 6, cultures were supplemented with growth medium containing recombinant IL-2. T-cell specificity was assessed using an intracellular cytokine staining assay.

### Adoptive transfer of multivirus specific T-cells in an EBV lymphoma model

Two groups of adult (6–10 week-old) NOD/SCID mice irradiated with a single dose of 230 cGy were engrafted subcutaneously with $10^7$ EBV-transformed lymphoblastoid cells per mouse. Tumor growth was monitored every 2–3 days using vernier calipers. Six days after engraftment of lymphoblastoid cells, mice were either mock treated or infused with $2 \times 10^7$ Ad-MvP-expanded T-cells. These *in vitro*-expanded T-cells included EBV-, CMV-, ADV-, and BKV-specific T-cells. Tumor burden was monitored after adoptive T-cell therapy and mice were sacrificed when tumor volume reached 1,000 $m^3$.

### Statistical analysis

The group difference between mice treated with Ad-MvP-expanded autologous or allogeneic antigen-specific T-cells and mock-treated mice was evaluated by a linear mixed-effect model with time, group, and the interaction of time and group as predictors.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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