Prophylactic infusion of cytomegalovirus specific cytotoxic T-lymphocytes stimulated with Ad5f35pp65 gene modified dendritic cells following allogeneic haemopoietic stem cell transplantation.

Short Title: Infusion of CMV-pp65 specific T-cells post-HSCT.

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ABSTRACT

Cytomegalovirus (CMV) and its therapy continue to contribute to morbidity and mortality in haemopoietic stem cell transplantation (HSCT). Many studies have demonstrated the feasibility of *in-vitro* generation of CMV-specific T-cells for adoptive immunotherapy of CMV. Few clinical trials have been performed showing the safety and efficacy of this approach *in-vivo*.

In this study, donor-derived, CMV-specific T-cells were generated for 12 adult HSCT patients by stimulation with dendritic cells transduced with an adenoviral vector encoding the CMV-pp65 protein. Patients received a prophylactic infusion of T-cells after day 28 post-HSCT. There were no infusion related adverse events. CMV DNAemia was detected in 4 patients post-infusion, but was of low level. No patient required CMV-specific pharmacotherapy. Immune reconstitution to CMV was demonstrated by ELISPOT assay in all recipients with rapid increases in predominantly CMV-pp65 directed immunity in 5. Rates of graft versus host disease, infection and death were not increased compared to expected.

These results add to the growing evidence of the safety and efficacy of immunotherapy of CMV in HSCT, supporting its more widespread use. This study was registered at [www.anzctr.org.au](http://www.anzctr.org.au) under ID Number: ACTRN12605000213640.
INTRODUCTION

Although effective cytomegalovirus (CMV)-specific pharmacotherapy exists, CMV remains a significant problem post-haemopoietic stem cell transplantation (HSCT). Ultimately, only CMV-specific immune reconstitution adequately controls CMV reactivation and disease. Adoptive transfer of CMV-specific T-cells has potential to safely restore CMV-specific immunity, control active CMV and prevent CMV recurrence. Although there are a plethora of in-vitro studies demonstrating the feasibility of producing CMV-specific T-cells for adoptive transfer, the number of clinical trials using such cells remains small\(^1\)\(^-\)\(^7\). Ongoing trials are needed to establish safety, efficacy and optimise clinically successful techniques. Studies examining adoptive immunotherapy of CMV have varied in their source of antigen used to generate the CMV-specific T-cells.

We have previously shown the safety of using the HLA-A2 restricted epitope of the pp65 antigen (NLVPMVATV) to generate a highly specific population of predominantly CD8\(^+\) cytotoxic T-cells. These cells were given to 9 patients ranging from 4 to 65 years of age, the majority receiving a T-cell replete graft after non-myeloablative conditioning. Infusion was not associated with toxicity and there was no increase in graft versus host disease (GVHD) compared to expected rates. CMV reactivation as measured by CMV-PCR occurred in 2 patients, neither of whom required specific anti-CMV therapy. CMV-specific immunity as measured by tetramer analysis was seen to rise transiently in 6 of these patients\(^8\).
There are two significant limitations inherent to this technique: firstly, the HLA restriction of a single epitope makes its broad application to all patients undergoing HSCT difficult; second, while being highly specific for CMV, the infusional product lacks specific CD4+ helper T-cells which has been suggested to be important for maintenance of functional T-cell immunity\textsuperscript{9,10}. These limitations have not been an issue with earlier immunotherapy studies utilising live virus\textsuperscript{1,2} or inactivated viral lysates\textsuperscript{3,4}. However, using these antigens does not conform to good manufacturing practice guidelines, making their inclusion in immunotherapy protocols untenable.

Recently, the Houston group used an adenoviral vector encoding the immunodominant pp65 lower matrix protein antigen (Ad5f35pp65) to simulate CMV-specific T cells. Combined with Epstein-Barr virus (EBV) transformed B-cells as antigen presenting cells, they generated cultures with activity against CMV, adenovirus and EBV. These cells were then given prophylactically to 11 patients, most of whom had undergone \textit{in-vivo} T-cell depletion\textsuperscript{5}.

Given the inherent limitations of using a single epitope to stimulate CMV-specific T-cells, we proceeded to using the Ad5f35pp65 vector to generate CD4+ and CD8+ CMV-specific T-cells for adoptive transfer in HSCT. Here we describe a phase I clinical trial examining the safety and efficacy of this technique in 12 mostly non-T-cell depleted patients infused prophylactically post-allogeneic HSCT, confirming the utility of this technique in a broader range of patients.
PARTICIPANTS AND METHODS

PARTICIPANT DETAILS
Patients of all HLA types receiving an allogeneic HSCT for haematological malignancy from a fully matched or 1 antigen mismatched related or unrelated donor were eligible for inclusion in this study. Patients with a life expectancy of less than 6 months and with CMV seronegative donors were excluded. This study was approved by the institutional ethics committee and informed consent was obtained from the donor and recipient prior to enrollment in accordance with the Declaration of Helsinki. Patients received CMV-specific T cells infusions between the 8th of January and 5th of November 2007. This research was approved by the Human Research Ethics committees of Westmead Hospital and The University of Sydney. In addition, protocols received approval from the Gene and related Therapies Research Advisory Panel of the National Health and Medical Research Council.

T-CELL GENERATION
CMV-pp65 specific T-cells were generated as previously described. Briefly, dendritic cells (DC) were differentiated from donor monocytes isolated from peripheral blood mononuclear cells (PBMC) by adherence. These were cultured for 7 days in Cellgro serum-free medium (Cellgenix, Freiburg, Germany) supplemented with recombinant human IL-4 and GM-CSF 1000u/ml (Chemicon International, Temecula, California, USA). DC were matured on day 6 by the
addition of recombinant human TNF-alpha (Chemicon International, Temecula, California, USA). The Ad5f35pp65 vector was added to the culture simultaneously at an MOI of 20 (supplied by The Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, USA). The MOI was based on pre-clinical experiments studying NLV⁺ tetramer cell expansion in HLA-A2+ CMV seropositive individuals following Ad5f35pp65 transduction of DC. Mature DC were harvested, washed, irradiated 30Gy, suspended in AIMV (Gibco, In-vitrogen, Grand Island, New York, USA) with 10% human AB serum (ARCBS, Sydney, Australia) and used to stimulate autologous PBMC at an effector to stimulator ratio of 10:1. T-cell cultures were re-stimulated on day 7 at which time recombinant human IL-2 (Chemicon International, Temecula, California, USA) was added at 20u/ml. The concentration of IL-2 was increased to 50u/ml on day 14 and cells were harvested and cryopreserved on day 21 for later analysis and infusion.

T-CELL INFUSION

T-cells were infused at 2x10⁷/m² on or after day 28 post-HSCT. This dose was chosen based on prior data from the Houston group regarding prophylaxis of Epstein Barr virus (EBV) infection, the number of non CMV-specific cells in our products and the risk of GVHD with non-specific donor lymphocyte infusions¹²,¹³. The primary end point was infusional safety with secondary end points of CMV reactivation, incidence of GVHD, use of antivirals and CMV-specific immune reconstitution.
Infusion was delayed if there was grade II or greater GVHD, corticosteroid dose equivalent to or greater than 1mg/kg/day prednisolone, antilymphocyte globulin use within the 4 weeks prior to intended infusion and significant renal or liver dysfunction (creatinine or bilirubin greater than twice the upper limit of normal range, serum transaminases greater than 3 times the upper limit of normal range).

Release criteria for cell products included negative cultures for bacteria and fungi, negative testing for Mycoplasma by polymerase chain reaction (PCR), greater than 50% post-thaw recovery and viability by trypan blue exclusion, less than 2% contaminating CD19+ B-cells and CD14+ monocytes as analysed by flow cytometry and negative alloreactivity (less than 10% killing of recipient PHA blasts at an effector to target ratio of 20:1 assessed by 4 hour 51Cr release assay).

POST-INFUSION IMMUNOPHENOTYPING AND TETRAMER STAINING

Immune reconstitution directed against individual CMV-pp65 epitopes post-infusion was monitored by immunophenotype and tetramer binding. All individuals infused had at least one HLA type corresponding to one of 5 specific epitopes for which tetramers were commercially available (Beckman Coulter, San Diego, CA, USA). These included HLA A*0201 (NLVPVMATV), HLA A*2402 (QYDPVAALF), HLA B*0702 (TPRVTGGGAM), HLA B*0801 (ELRRKMMYM) and HLA B*3501 (IPSINVHHY). Antibodies directed against CD3, CD4, CD8,
CD19, CD56 and CD14 were all purchased from Becton Dickinson (San Jose, CA, USA).

Tetramer was added to 100µl of whole blood and incubated at room temperature for 30 minutes. Antibodies for cell surface antigens were added for the final 15 minutes of incubation followed by lysis of red cells using PharmLyse (Becton Dickinson, San Jose, CA, USA). Cells were then washed and resuspended in flow buffer consisting of phosphate buffered saline/ 1% bovine serum albumin. A minimum of 30000 events was acquired on the LSRII flow cytometer with FACSDiva software (Becton Dickinson, San Jose, CA, USA). Results were analysed using FCS Express (De Novo Software, Ontario, Canada).

Absolute tetramer positive cell numbers present in the peripheral blood were calculated by multiplying the percentage of tetramer+ cells by the white cell count obtained from the Advia full blood count analyzer.

POST-INFUSION ENZYME LINKED IMMUNOSPOT ASSAY

Reconstitution to whole pp65, IE1 and adenoviral proteins was assessed by interferon-γ enzyme linked immunospot assay (ELISPOT) analysis as previously described \(^8\). 0.5-1x10^5 cells from each time point were suspended in 200µl AIMV/10% human AB serum and stimulated with pp65, IE1 and adenoviral hexon protein pepmix (JPT Peptide Technologies GmbH, Berlin, Germany) for 18 hours in multiscreen, MAIPS4510 96 well filter plates (Millipore, Bedford, MA, USA) precoated with catcher antibody (m-AB 1-DIK) (Becton Dickinson, San Jose, CA, USA). After washing and incubation with detector antibody (m-AB 7-B6-1-Biotin)
spots were developed using ExtrAvidin and SigmaFast BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, St Louis, MO, USA) according to manufacturers directions. Spots were counted manually and results expressed as spot forming cells (SFC) per $10^5$ cells. Testing was performed in triplicate for each time point. Pre- and post-infusion samples were batched to avoid inter-assay variability.

CMV SURVEILLANCE AND THERAPY
Monitoring for CMV reactivation consisted of weekly qualitative PCR. If positive, a quantitative PCR was then performed. Ganciclovir therapy 5mg/kg twice daily was initiated by an independent treating physician if CMV DNA levels exceeded 1500 copies/ml. Definitions of CMV reactivation, infection and disease are as per Ljungman et al$^{14}$.

MONITORING FOR ADVERSE EVENTS
Patients were monitored for infusional toxicity, infection and GVHD. To assess infusional toxicity, patients were monitored for 4 hours and reviewed 24 hours after T-cell infusion. GVHD was graded according to Glucksberg et al$^{15}$.
RESULTS

SAFETY OF CMV-SPECIFIC T-CELL INFUSION

Between January and December of 2007, 12 patients received a single dose of 2x10^7/m^2 CMV specific T-cells. Patient age ranged from 18 to 65 years, 6 received myeloablative conditioning and 4 received in-vivo T-cell depletion (2 with alemtuzumab and 2 with antithymocyte globulin, see Table 1). In all cases, in-vitro cultures produced adequate numbers for the target cell dose. With a median starting number of PBMC of 14x10^6 (range 8x10^6-20x10^6), cultures expanded almost 20 fold (median 19.5, range 8.5-30) over a 21 day period to a median of 211x10^6 (range 136x10^6-505x10^6) total cells. Cells infused were predominantly CD3^+ (median 94.5%, range 26%-99.8%), with a greater proportion of CD8^+ (median 73.9%, range 23%-91%) compared to CD4^+ (median 19%, range 2.5%-59%) T-cells. Single CMV epitope restriction based on tetramer binding ranged from 0.2% to 64% (median 7.7%). This varied depending on the individual epitope. Cultures from 4 HLA A*0201^+ donors had a higher proportion of tetramer^+ cells (median 32.2%, range 3.3%-64%) than the 4 HLA B*0702^+ donors (median 17.6%, range 6.9%-36.5%) or the 3 HLA B*3501 (median 0.4%, range 0.2%-3.8%). Culture from 1 HLA A*2401 donor had 0.7% tetramer^+ cells. Tetramer^+ binding for the single culture from an HLA B*0801^+ donor was 0.05%

In 2 cultures, specificity for the HLA A*0101 restricted epitope of the adenoviral hexon protein (TDLGQNLLY) was assessed by pentamer staining (Prolimmune, Oxford, UK). In these cultures, 5.3% and 15% of the cells were specific for this
epitope. All cultures lysed pp65 pulsed target cells (median specific lysis 65%, range 42.6%-82.5%) with lower lysis of adenovirus hexon protein pulsed targets (median specific lysis 4.8%, range 0%-71%). All cultures had less than 5% lysis of unpulsed allogeneic target cells (median specific lysis 0.1%, range 0%-3.6%). Phenotypic and functional characteristics of the T-cell products infused can be seen in Figure 1. Patient characteristics can be found in Table 1. At the time of infusion, all patients were receiving prophylactic cyclosporin. Five patients were taking oral prednisolone at a dose of less than 1mg/kg/day (0702 0.25mg/kg/day for gout; 06205 0.5mg/kg/day for prior GVHD; 0775 0.3mg/kg/day for prior GVHD; 07110 0.6mg/kg/day for red cell aplasia post-transplant; 07138 0.25mg/kg/day for prior GVHD). All patients except for 2 were receiving standard prophylactic oral dose valaciclovir of 500mg daily. 07110 was taking oral valaciclovir 2g four times per day for genital warts and 07143 was receiving intravenous foscarnet 6g two times per day for multiple prior episodes of CMV reactivation. This was ceased 2 days post-T-cell infusion.

T-cells were given at a median of 55 (range 31-101) days post-HSCT. Reason for delay included 3 with GVHD (06205, 0775, 07138), 1 due to corticosteroids for red cell aplasia (07110), 2 with transiently elevated creatinine (06213, 07169), 1 with intractable nausea and vomiting (0786), 1 with persistent fevers (07143), 3 due to cell product or patient availability (0702, 0735, 0743- all less than 2 weeks) and 1 due to patient preference (07141). There were no adverse reactions to the infusion within the first 24 hours; however, 1 recipient (0743) had a self limiting episode of dizziness and weakness 24 hours post-infusion, not
associated with any evidence of focal neurological deficits, haemodynamic changes or sepsis. This was consistent with multiple prior episodes and was not felt to be related to the T-cells. A summary of the incidence of CMV reactivation, GVHD, infections and other adverse events can be found in Table 2.

INCIDENCE OF CMV REACTIVATION AND REQUIREMENT FOR ANTIVIRALS POST-T-CELL INFUSION

After a median follow-up of 218 (range 94-354) days post-T-cell infusion (median 246, range 135-453 days post-transplant), 6 episodes of CMV reactivation as detected by qualitative and quantitative plasma PCR occurred in 4 patients (Table 2). Four of these episodes consisted of a single positive PCR with a level of less than 600 copies/ml. Three occurred within the first week post-infusion (06213, 0743, and 07143). One reactivation occurred in the context of grade III acute GVHD treated with 1mg/kg/day prednisolone (06213) 101 days post-infusion. In one recipient (0702), there were persistent low levels (maximum 880 copies/ml) of CMV detectable over a 6 week period. This was associated with low dose (<0.5mg/kg/day) oral prednisolone for acute gout. Reactivation was followed by immune reconstitution to CMV which was predominantly directed at pp65 (when compared to IE1) (Figure 2A).

07143 had developed CMV reactivation during induction chemotherapy for AML and had documented reactivation early post-HSCT (day +10). Though promptly suppressed by foscarinet, CMV levels rose rapidly with cessation of pharmacotherapy on day +34 with a maximum level of 2810 copies/ml on day +38. Five days post-CMV-specific T-cell infusion, a single positive CMV PCR with
copy levels less than 600/ml was recorded, followed by persistently negative results despite absence of foscarnet therapy. A second positive PCR with a level of less than 600 copies/ml was detected 56 days post-T-cell infusion associated with corticosteroid therapy (prednisolone 0.5mg/kg/day) for grade I acute GVHD. This low level DNAemia persisted until prednisolone therapy was reduced to less than 0.1 mg/kg/day.

No patient required foscarnet or ganciclovir therapy. Two patients received high dose valaciclovir. 0702 was prescribed valaciclovir 1g four times per day 2 weeks post-infusion with the intent of suppressing CMV levels. This was continued until 9 weeks post-infusion when the dose was reduced to 500mg per day. 07143 started valaciclovir 2g four times per day 2 weeks post-infusion concurrent with immunoglobulin for prevention of acute varicella zoster after exposure to an individual with active infection. This continued for 4 weeks after which the dose was reduced to 500mg twice per day. Valaciclovir was again briefly increased to 2g four times per day by an independent treating physician when a positive CMV PCR was detected 56 days post-T-cell infusion.

CMV-SPECIFIC IMMUNE RECONSTITUTION POST-T-CELL INFUSION

Functional immune reconstitution to whole CMV and adenoviral hexon protein antigens was determined by ELISPOT analysis of samples taken during the first 3 months post-T-cell infusion (Figure 2A and B). Epitope specific reconstitution was also examined using the tetramer analysis (Figure 2C). All 12 demonstrated evidence of CMV-specific immunity at at least one time-point when examined by
ELISPOT. In 5 of the 12 participants, rapid reconstitution to pp65 occurred with minimal (0702, 06205 and 0743) or no (0775 and 07169) reconstitution of IE1-specific immunity (Figure 2A). In 3 patients, IE1-specific reconstitution either matched pp65 (06213 and 07143) or dominated the immune response (0786) (Figure 2B). In the 4 remaining participants, CMV-specific immunity remained unchanged or decreased post-T-cell infusion (data not shown). Of these 4 patients, 2 were CMV seronegative (07141 and 0735) and 2 were receiving corticosteroids at the time of infusion (07110 and 07138). 07138 already had immunity present prior to infusion with 735 spot forming cells per $10^5$ cells. This was similar to peak levels reached in other patients with reconstitution in Figure 2A. There was no reconstitution of adenovirus-specific immunity in any of the 12 patients infused.

Tetramer$^+$ T-cell numbers were seen to rise in 5 of the 12 participants (Figure 2C). Two of these were HLA-A0201$^+$ (06205 and 07169) and 3 were HLA-B0702$^+$ (0702, 07143 and 0786). 4 of these patients demonstrated functional reconstitution to pp65 (06205, 07169, 0702 and 07143) and 1 to IE1 (0786) on ELISPOT (Figure 2A and B).

GVHD POST-T-CELL INFUSION

Four patients developed GVHD a median of 82 (range 42 to 133) days post-T-cell infusion (Table 2). All were associated with subtherapeutic levels of cyclosporin (06213 and 07143) or intentional weaning of immunosuppression (06205 and 0743). 06213 developed grade III GVHD affecting the skin and liver
45 days post-infusion. This did not resolve with oral prednisolone 1mg/kg/day but responded to five 50mg doses of daclizumab over 4 weeks. Another episode of grade III GVHD affecting the gastrointestinal tract occurred 149 days post-T-cell infusion. This second episode resolved with intravenous methylprednisolone 2mg/kg/day. In all other patients, GVHD consisted of skin involvement no greater than grade II in severity and resolved with oral prednisolone therapy 1mg/kg/day or less.

OTHER ADVERSE EVENTS POST-T-CELL INFUSION

One patient (0702) developed graft failure 94 days post-infusion (125 days post-transplant) associated with trimethoprim/sulphamethoxazole therapy. This patient had developed neutropenia and thrombocytopenia 1 week after initiating trimethoprim/sulphamethoxazole for pneumocystis carinii pneumonia prophylaxis early post-HSCT. Trimethoprim/sulphamethoxazole was restarted day +94 post-HSCT, mild thrombocytopenia (139x10^9/L) was noted 2 weeks later and profound pancytopenia noted day +125 (white cell count 0.6x10^9/L, haemoglobin 75g/L, platelet count 9x10^9/L). This necessitated a second transplant with subsequent uncontrolled GVHD leading to death 177 days post-T-cell infusion (208 days after the initial transplant). We considered the possibility that the cells infused contained an auto-reactive clone which led to this graft failure. We therefore assessed the CMV-specific T-cells infused by co-culturing with irradiated donor stem cells at a ratio of 1:1 in an interferon-γ ELISPOT assay as described. This
showed no interferon production elicited in response to the donor stem cell product.

Three patients had malignant relapse post-T-cell infusion (Table 2). 0735 who received a matched sibling transplant for relapsed ALL involving the central nervous system, was diagnosed with relapse at the same site 136 days post-infusion (174 days post-transplant) and subsequently died of complications of relapse 321 days post-T-cell infusion (day 359 post-transplant). 0775 relapsed with AML 107 days post-infusion (166 days post-transplant). 07138 was diagnosed with relapsed AML 64 days post-T-cell infusion (day 137 post-transplant). Both 0775 and 07138 subsequently received re-induction chemotherapy and donor lymphocyte infusions and are alive in remission 252 and 140 days post-T-cell infusion (day 311 and 213 post-transplant) respectively.

Non-CMV related infection occurred in 3 patients (Table 2). 06205 developed type II herpes simplex upon cessation of valaciclovir prophylaxis 112 days post-T-cell infusion (213 days post-transplant). 07143 had haemorrhagic cystitis associated with polyomavirus isolated from the urine at the time of T-cell infusion. Cystitis improved over the subsequent week with conservative management. This patient also had two episodes of central venous catheter related septicaemia 47 and 97 days post-infusion (94 and 144 days post-transplant respectively) which resolved with antibiotics and line removal. 0743 was admitted to hospital 243 days post-T-cell infusion (281 days post-transplant) with hypoxia after symptoms suggestive of a viral respiratory tract infection. No aetiology was
identified and the episode resolved with supportive care and prednisolone 1.2 mg/kg/day.

There were 2 deaths as described above, one from GVHD post-second transplant and another from relapsed ALL.

**DISCUSSION**

Immunotherapy for the prevention and treatment of CMV post-HSCT is a promising alternative to pharmacotherapy. Varying in-vitro methods to generate CMV-specific T-cells have been described, but the number of clinical trials is still relatively small. Different sources of antigen have been employed to generate the T-cell product infused 1-4,6,7. Here we have confirmed the safety and efficacy of the approach used by Leen *et al* 5, generating CMV-pp65-specific T-cells for prophylactic infusion in 12 HSCT recipients.

CMV reactivation occurred in 4 of the 12 patients but was of low titre in each case. In these patients there was a notable lack of need for antivirals including high dose valaciclovir, foscarnet and ganciclovir. Only 2 trial patients received high dose valaciclovir and none required foscarnet or ganciclovir. In contrast, during a similar time period to our ongoing immunotherapy trials, 82% of 28 CMV PCR positive non-trial patients with CMV seropositive donors received antivirals and 50% required foscarnet or ganciclovir. 30% (4 of 13) of non-trial patients with CMV reactivation who were initially treated with high dose valaciclovir went on to receive ganciclovir or foscarnet.
Dominant and rapid pp65 directed immune reconstitution in the presence of minimal or no IE1 directed reconstitution was clearly evident in 5 participants. This is suggestive of the efficacy of the adoptive transfer of CMV-specific immunity in this cohort of patients. In 3 recipients, rapid IE1-directed reconstitution matched that of pp65 or dominated the immune response. This may reflect a normal process post-HSCT. However, it is possible that the rapidity of reconstitution may have been due to CD4\(^+\) T-cell help provided by the infused cells. Of the 4 recipients who did not show significant changes to immunity post-infusion, 2 were CMV-seronegative and 2 were taking corticosteroids for GVHD. There were no adverse events related to the product within the 24 hours post-infusion. Rates of GVHD, infection and death were not increased compared to expected rates. This suggests the safety of the currently used cell dose and the possibility of infusing higher cell doses in order to establish a correlation between cell dose, CMV-specific immune reconstitution and CMV prevention. Our study did not do this because of ethical concerns about the risk of infusing higher cell doses as prophylaxis in patients without either CMV reactivation or disease. Significant delays in the planned day of administration were mostly due to GVHD, corticosteroid use or cyclosporin toxicity. Since prior GVHD or steroid usage if anything increases the risk of post infusion events such as GVHD or poor CMV immune reconstitution, the delay in administration is unlikely to account for the good safety profile, low CMV PCR titres or absence of antiviral use seen in this cohort. These positive benefits could be attributed to low rates of grade III and IV GVHD and steroid dosage in the patient group, but this is unlikely to account for
all of the benefit seen. Although numerous studies have highlighted the importance of GVHD as a predisposing factor for CMV reactivation \(^{16-18}\), the role of steroid dosage is considered to be less significant with at least one early study showing that use of topical, low or high dose systemic steroids in GVHD did not impact the rate of reactivation \(^{17}\). Taken together with the 9 patients in our earlier trial that received T-cells stimulated with NLV peptide, a total of 21 patients have received prophylactic CMV specific T-cells at our institution \(^{8}\). None have required therapeutic foscarnet or ganciclovir, further strengthening the above results.

There are several differences between this trial and our previous experience with CMV peptide-specific T-cell infusions in HSCT recipients \(^{8}\). Firstly, despite all 12 Ad5f35pp65 trial recipients demonstrating some degree of CMV-specific immunity by ELISPOT, single epitope tetramers were only useful for assessing reconstitution in 5 (2 HLA-A0201\(^+\) and 3 HLA-B0702\(^+\)) of the 12 Ad5f35pp65 trial recipients; 6 of the 9 patients receiving CMV peptide-specific T-cells showed a rise in the tetramer\(^+\) population. This difference is not surprising given the polyepitope specific nature of cells infused in this current trial and the sensitivity of tetramers used. The second difference is in regards to the persistence of functional immunity in those with CMV-specific reconstitution. In 2 patients receiving CMV peptide-specific T-cells, ELISPOT analysis showed a transient rise in functional response to the individual epitope which mirrored the temporary rise in tetramer\(^+\) cell numbers post-infusion. In contrast, the 5 recipients in the current trial who had predominantly pp65-specific reconstitution had persistently
elevated levels of immunity compared to baseline. This was apparent even in those on corticosteroids. This may reflect the presence of CMV-specific CD4+ T-cell help in these cultures in contrast to the predominantly CD8+ cytotoxic T-cells seen in peptide stimulated cultures.

Although the results of this trial confirm those of the Houston group in regards to prevention of CMV disease, significant differences exist regarding the patient mix, culture technique and adenovirus specific immune reconstitution. The Houston patient group differed from this one, specifically in terms of younger age (mixed child and adult compared with only adults), higher proportion of transplants from matched unrelated donors (6 of 11 compared to 4 of 12) and use of in-vivo T-cell depletion (9 of 11 compared to 4 of 12 in our study). The efficacy seen amongst these differing populations demonstrates the applicability of this technique to a broad range of patients.

Unlike the cells raised by the Houston group, generation of EBV specific T-cells was not incorporated into this protocol. Our institution has had few problems with post-transplant EBV infections and the majority of our transplant recipients do not receive T-cell depletion. The advantage of this omission is that it avoids the need for the production of EBV infected lymphoblastoid cell lines, thus shortening the overall T-cell generation process by several weeks. The simplicity of our culture system makes it easier to implement in smaller yet busy transplant centres with limited resources who may have a similar case mix to ours.

Like the Houston group small populations of adenovirus-specific T-cells were seen in the infusional product of 2 patients examined with adenovirus-specific
pentamers. There was also killing of adenovirus pepmix pulsed target cells above background in 10 of the cultures (with 3 having a specific lysis greater than 10% and 2 of these greater than 50%). In contrast to the Houston trial, none of the 12 recipients had prominent reconstitution to adenovirus post-infusion. This may be due to the small number of adenovirus-specific cells present in the infusional product. This could be attributed to the dominance of a CMV response in the culture system in CMV seropositive donors (personal communication from C. Bollard, Centre for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, USA) and might be overcome by addition of a culture well comprising dendritic cells transduced with an empty adenoviral vector. Alternatively, lack of adenovirus-specific reconstitution in-vivo could reflect an absence of adenoviral infections in the current trial participants (all adults) compared to the 5 documented infections amongst the patients in the Houston trial.

The positive results of this trial add to the mounting evidence in favour of incorporating immunotherapy routinely into HSCT. Much work still remains to be done however. Optimisation of techniques is an ongoing process. Recent advances, such as direct isolation of antigen specific T-cells through cytokine secretion or markers of activation may lead to less manipulation and improved survival of cells infused. CMV is just one of many organisms that may be amenable to this form of therapy. We are currently examining the incorporation of generation of varicella-zoster virus specific and Aspergillus specific T-cells into routine cultures.
Administration of \textit{ex-vivo} generated antigen specific cells remains an experimental approach requiring adherence to principles of good manufacturing practice, clean room facilities and satisfaction of local regulatory requirements. As laboratories supporting clinical stem cell transplantation conform to regulatory requirements, this form of therapy will become widely available if it can be demonstrated to be clinically beneficial. Its complexity should not be exaggerated. In our trial, cell culture was performed using one stem cell transplant scientist (LC). Alternatively, the outsourcing of tissue culture procedures to a few large centres with concentrated facilities and expertise may be the more cost effective and rational means of expanding this therapy into the mainstream.

In conclusion, prophylactic infusion of CMV-specific T-cells generated using the Ad5f35pp65 vector is safe and appears to be effective in preventing CMV and avoiding the need for anti-CMV pharmacotherapy in HSCT recipients. This trial provides evidence of the efficacy of this technique in a broader range of patients than previously studied.

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College of Medicine, Houston for supplying the Ad5I35pp65 vector and technical advice.

**AUTHORSHIP**

KM enrolled trial participants, infused the CMV-specific T-cells, performed clinical and laboratory post-infusion monitoring and wrote the paper. LC and US generated the CMV-pp65 specific T-cells for infusion. AH optimised culture conditions and wrote the protocols for the generation of CMV-specific T-cell product. EB assisted with the clinical followup and laboratory monitoring. VA assisted with clinical grade cell generation and freezing. MMS assisted with technical advice on flow cytometry. KFB cared for transplant patients and reviewed the manuscript. DJG designed and established the protocol, oversaw the trial, assisted with post-infusion clinical monitoring and assisted KM in writing the paper.

None of the authors have any conflict of interest to declare.
REFERENCES


Table 1 Patient Characteristics.

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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Disease Status</th>
<th>Conditioning</th>
<th>MUD vs MSD</th>
<th>CD34+ Dose (^{(*)}10^6/kg)</th>
<th>Day to Engraftment</th>
<th>CMV Status</th>
<th>Immunosuppression</th>
<th>Antivirals</th>
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Table 1 Patient Characteristics.

ALL= acute lymphoblastic leukaemia, AML= acute myeloid leukaemia, ATG= antithymocyte globulin, Bu= busulphan, CML =chronic myeloid leukaemia, CP2= second chronic phase, CR= complete remission, CSA= cyclosporin, Cy= cyclophosphamide, Flu= fludarabine, MDS= myelodysplastic syndrome, Mel= melphalan, MSD= matched sibling donor, MUD= matched unrelated donor, NEG= negative, POS= positive, PR= partial remission, Pred= prednisolone, TBI= total body irradiation, Val= valaciclovir. * second transplant after prior allogeneic transplant for CML. # In-vivo T-cell depletion included antithymocyte globulin or alemtuzumab as part of the conditioning regimen in 4 patients. “Days to Engraftment” indicates the days to neutrophil engraftment and is defined as a peripheral blood neutrophil count greater than 1x10^9/L on 2 or more successive days. “Antivirals” refers to therapeutic antivirals excluding standard prophylactic valaciclovir 500mg/day given from day +1 post-HSCT until immune reconstitution. All patients were receiving prophylactic valaciclovir at the time of infusion except 07110 and 07143 who were receiving valaciclovir 2g four times per day and therapeutic foscarnet respectively as described in the text.
Table 2 Post-Infusion Progress of Patients Receiving CMV-Specific T-cells.

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<th>Day of Infusion</th>
<th>Dose</th>
<th>Infusional Adverse Events</th>
<th>Last Day of Followup</th>
<th>CMV Reactivation</th>
<th>GVHD</th>
<th>Non-CMV Infection</th>
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</table>
Table 2 Post-Infusion Progress of Patients Receiving CMV-Specific T-cells.

All “days” indicated refer to the day post-haemopoietic stem cell transplant. “CMV Reactivation” refers to any positive peripheral blood CMV PCR. “GVHD” refers to episodes of acute GVHD post-T-cell infusion. “Non-CMV Infection” refers to infectious events not associated with any evidence of CMV reactivation. “Other” includes bone marrow failure day 125 in 0702 likely secondary to trimethoprim/sulphamethoxazole, necessitating a second transplant with death from uncontrolled GVHD day 239; death from relapse in 0735 day 359; and an episode of hypoxia with uncertain aetiology in 0743 day 281. Px= patient, Val= valaciclovir.
FIGURE LEGENDS

Figure 1 Phenotypic and Functional Characteristics of the T-cell Products Infused

A - Phenotypic characteristics of T-cells infused. CD3⁺, CD56⁺ and Tetramer⁺ cells are expressed as a percentage of total cells. CD8⁺, CD4⁺, CD62L⁺, and CD45RO⁺ cells are expressed as a percentage of CD3⁺ cells. B - Function of T-cells infused as assessed by chromium release assay. Top-left plot shows an example of killing of recipient PHA blasts pulsed with CMV-pp65 pepmix (diamonds), HLA-B*0702 restricted (circles) or HLA-B*3501 (triangles) restricted CMV-peptides as well as adenoviral hexon protein pepmix (squares). Top-right and lower-left plots show examples of killing of recipient PHA blasts pulsed with CMV-pp65 pepmix or HLA-B7 restricted CMV-peptide with more typical low level killing of blasts pulsed with adenoviral hexon protein pepmix. In all 3 plots, typical absence of killing of unpulsed allo-PHA blasts (crosses) is seen.
Figure 2 CMV-Specific Immune Reconstitution Post-T-Cell Infusion.

ELISPOT (A and B) and tetramer (C) analysis of response to CMV-pp65 (triangles), CMV-IE1 (squares) and Adenoviral-hexon protein (circles) in the trial participants over 3 months post-T-cell infusion. Total white cell count (diamonds) and CMV PCR titre (crosses) are also shown. Presence of GVHD and corticosteroids is indicated by striped and spotted bars respectively. Note the individual variation in scale of the y axis. A- 5 participants with predominantly pp65 immune reconstitution. B- 3 participants with IE1 immune reconstitution matching that of pp65 (2 left panels) or dominating the response (right panel). C- CMV-pp65 epitope specific immune reconstitution over 3 months post-T-cell infusion as assessed by tetramer binding in 5 patients. Total white cell count (x10^6/ml) is represented by diamonds; tetramer^+ cell count (x10^4/ml) is shown as triangles. CMV PCR titre (x10^2 copies/ml) is represented by crosses. The HLA restriction of the tetramers used included HLA A*0201 (06205 and 07169), HLA B*0702 (0702 and 07143)) and HLA B*0801 (0786).
Prophylactic infusion of cytomegalovirus specific cytotoxic T-lymphocytes stimulated with Ad5f35pp65 gene modified dendritic cells following allogeneic haemopoietic stem cell transplantation

Kenneth P Micklethwaite, Leighton Clancy, Upinder Sandher, Anna M Hansen, Emily Blyth, Vicki Antonenas, Mary M Sartor, Kenneth F Bradstock and David J Gottlieb