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Manufacture of GMP-compliant functional adenovirus-specific T-cell therapy for treatment of post-transplant infectious complications

CLAIRE HORLOCK[1], AMANDA SKULTE[1], ARINDAM MITRA[1], ALKA STANSFIELD[1], SHRISTI BHANDARI[1], WINNIE IP[2], WASEEM QASIM[2], MARK W. LOWDELL[3], SHREENAL PATEL[1], ANKE FRIEDETZKY[1], MARCO A. PURBHOO[1] & KATY NEWTON[1]

[1]Cell Medica Ltd, London, United Kingdom, [2]Great Ormond Street Hospital, London, United Kingdom, and [3]Research Department of Haematology, Cancer Institute, UCL, Royal Free Hospital, London, United Kingdom

Abstract

Background aims. In pediatric patients, adenovirus (ADV) reactivation after allogeneic hematopoietic stem cell transplantation (allo HSCT) is a major cause of morbidity and mortality. For patients who do not respond to antiviral drug therapy, a new treatment approach using ADV-specific T cells can present a promising alternative. Here we describe the clinical scale Good Manufacturing Practice (GMP)-compliant manufacture and characterization of 40 ADV-specific T-cell products, Cytovir ADV, which are currently being tested in a multi-center phase I/IIa clinical trial. This process requires minimal intervention, is high yield, and results in a pure T-cell product that is functional. *Methods.* Mononuclear cells (2×10^7) were cultured in a closed system in the presence of GMP-grade ADV peptide pool and cytokines for 10 days. On day 10, the T-cell product was harvested, washed in a closed system, counted and assessed for purity and potency. Additional characterization was carried out where cell numbers allowed. *Results.* Thirty-eight of 40 products (95%) met all release criteria. Median purity of the cell product was 88.3% CD3+ cells with a median yield of 2.9×10^7 CD3+ cells. Potency analyses showed a median ADV-specific interferon (IFN)γ response of 5.9% of CD3+ and 2345 IFNγ spot-forming cells/million. CD4 and CD8 T cells were capable of proliferating in response to ADV (63.3 and 56.3%, respectively). These virus-specific T cells (VST) were heterogenous, containing both effector memory and central memory T cells. In an exemplar patient with ADV viremia treated in the open ASPIRE trial, ADV-specific T-cell response was detected by IFNγ enzyme-linked immunospot from 13 days post-infusion. ADV DNA levels declined following cellular therapy and were below level of detection from day 64 post-infusion onward. *Conclusions.* The clinical-scale GMP-compliant One Touch manufacturing system is feasible and yields functional ADV-specific T cells at clinically relevant doses.

Key Words: *adoptive T-cell therapy, ADV, GMP compliant, HSCT*

Introduction

Allogeneic hematopoietic stem cell transplantation (allo HSCT) is recognized as a curative treatment option available for many patients with malignant and non-malignant hematological diseases [1]. In situations in which a fully HLA-matched sibling donor is not available, there is an increasing use of alternative stem cell donor sources, such as unrelated donors, haploidentical-related donors and umbilical cord blood [2]. The broader use of unrelated and haploidentical donors may necessitate T-cell depletion strategies to prevent acute graft-versus-host disease (aGvHD) [3]. However, such T-cell depletion strategies have been recognized as a severe risk factor for post-transplant activation of latent viruses including cytomegalovirus (CMV), Epstein-Barr virus and adenovirus (ADV) [4,5]. These infectious complications pose a significant risk, particularly with regard to early and late morbidity and mortality after allogeneic HSCT. Viral reactivation is attributed to delayed immune reconstitution, where T- and B-lymphocyte function remain severely weakened for several months or years post-HSCT [6].

Antiviral drug therapy is only modestly effective in limiting ADV reactivation in HSCT patients and is associated with significant toxicity [7]. Ultimately, T-cell reconstitution may be required for sustained viral clearance [8,9]. Adoptive transfer of virus-specific T cells (VST) is an innovative approach for virus-specific immune reconstitution (VSIR) that may overcome the limitations associated with the current pharmaceutical approaches. Early exploratory clini-

Correspondence: **Claire Horlock**, PhD, Cell Medica Ltd, 1 Canal Side Studios, 8-14 St Pancras Way, London NW1 0QG, United Kingdom. E-mail: claire.horlock@cellmedica.co.uk

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cal studies demonstrated the feasibility of VSIR to prevent or treat ADV infections post–allo HSCT and treatment options are being investigated [10–14]. Although recent publications report on small-scale studies to demonstrate feasibility of ADV-specific adoptive T-cell therapy (ACT) [13–15], successful commercial therapeutic ACT requires the capacity for large-scale production of Good Manufacturing Practice (GMP)-compliant, readily accessible cellular therapy products.

Here we describe the production of 40 ADV-specific T-cell immunotherapy products, Cytovir ADV, manufactured under GMP conditions and released according to established criteria. Cytovir ADV meets the definition of an advanced therapy medicinal product (ATMP—more specifically, advanced therapy investigational medicinal products, or ATIMP) according to Annex I, part IV of Directive 2001/83/EC, as amended, and Regulation (EC) No. 1394/2007. The VST described here comprise donor-derived, naturally occurring, *ex vivo* expanded ADV-specific T cells. We demonstrate the effective translation of a research concept to a commercially viable process with high manufacturing success rate. The characteristics of ADV-specific T-cell products are summarized subsequently. The safety and tolerability of Cytovir ADV are currently being evaluated in a clinical trial (EudraCT 2011-001788-36, NCT01822093) at three pediatric transplant centers in the United Kingdom.

Methods

Starting material

Mobilized apheresis (n = 31) or whole blood (n = 9) was obtained from healthy stem cell donors already serving as the HSCT donor. Collection was carried out as per local procedures, and samples were transported in controlled temperature conditions to the GMP facility of the Centre for Cell, Gene and Tissue Therapeutics at the Royal Free Hospital London. Procurement and subsequent processing formed part of a clinical study (ASPIRE, EudraCT 2011-001788-36, NCT01822093), which was approved by the NRES Committee London Riverside. All donors gave written informed consent obtained according to the Helsinki Declaration. Consent for research and development (R&D) was optional, with 28 of 40 donors consenting to R&D analysis. All samples were screened negative for infectious disease markers (IDM) performed in compliance with EC directive 2006/17/EC within 30 days of procurement.

ATIMP manufacture

Lymphocytes were sourced from the transplant donor, either from a 100 mL whole blood donation for a bone-marrow donor or a 5 mL aliquot of the original peripheral blood mobilized stem cell donation. The donors were not screened for ADV assuming that >90% of donors have been exposed to ADV [16]. Mononuclear cells (MNC) were isolated via density gradient separation (Ficoll-Paque, GE Healthcare) in a closed process using Biosafe Sepax, eluted in RPMI Glutamax (Life Technologies) and counted using a Sysmex pocH-100i Automated Hematology Analyzer. MNC (2×10^7) were seeded in a closed G-Rex 10 cell culture device (Wilson Wolf) in 20 mL RPMI Glutamax supplemented with 10% pooled human AB serum (Centre for Clinical Transfusion Medicine, Tübingen, Germany), 200 ng/mL interleukin (IL)-4, 10 ng/mL IL-7 (IL-4 and IL-7 from CellGenix) and 5 ng/peptide/mL GMP-grade ADV peptide pool (Miltenyi Biotec) for 10 days at 37°C/5% CO_2, without additional feeding, medium change or supplementation. Note that excess starting material following setup was cryopreserved in 10% dimethyl sulfoxide (DMSO), 45% human serum albumin (HSA; Bio Products Laboratory), 45% RPMI Glutamax, and control-rate frozen in a CoolCell (Biocision) before transferring to liquid nitrogen storage.

After 10 days, cells were gently resuspended with a syringe and a 1.5 mL sample removed for in-process controls (IPC). Remaining volume was transferred into a transfer pack (Terumo) containing 4.5% HSA. Harvested cells were washed in a closed procedure with 4.5% HSA using a Sepax device (Smartwash; two washes, no dilution step). This reduces culture medium components to <0.03%. Cells were eluted in 4.5% HSA and counted using BD TruCount in combination with CD3, CD8 and CD45 staining (BD Biosciences). Cell viability is assessed using TO-PRO-3 iodide (Life Technologies) staining and analyzing by flow cytometry (BD FACSCalibur).

During formulation, two doses of product were made as follows. Equal volumes of 2×10^4 and 2×10^5 T cells per mL per kg of patient weight solutions were transferred into labeled cryocyte bags (Origen) with equal volume of freezing media (80% HSA/20% DMSO; WAK-Chemie) on ice. These final formulated ATIMP product bags then contained either 1×10^4 or the maximum target dose of 1×10^5 T cells/mL/kg of patient weight, respectively. Two milliliters of final product from each formulated dose was removed for microbial control and endotoxin testing. The final product was then labeled, double bagged, cryopreserved in a 75 min controlled-rate freeze program (Planer) and transferred for storage in vapor phase liquid nitrogen.

In most cases, an identical duplicate G-Rex10, labeled "Product QC," was cultured in parallel to each product (cell number permitting) and used to perform additional product characterization. Product QC cells

were usually cryopreserved on the day of product harvest and analyzed at a later date. Cells were cryopreserved in 10% DMSO, 45% has and 45% RPMI Glutamax and control rate frozen in a CoolCell (Biocision) before transferring to liquid nitrogen storage.

Viability testing

Cell viability was assessed using TO-PRO-3 iodide (Life Technologies) staining during TruCount cell enumeration. After cell staining for CD45, CD3 and CD8, cells were stained with 1 μmol/mL TOPRO-3 before acquiring on the BD FACSCalibur. Viable cells were determined as the percentage of cells staining negative for TOPRO-3.

Sterility testing

Each final product was subject to several sterility tests before being certified for release. Acceptable microbial control was confirmed in starting material and product by inoculation of aerobic and anaerobic Bactec culture system (BD Biosciences) and analyzed by the Microbiology Department at the Royal Free Hospital, London. Absence of mycoplasma contamination was confirmed by analysis of culture supernatant from product using polymerase chain reaction (PCR; Minerva Analytix). Microbial Bactec and mycoplasma PCR tests were subject to full matrix validation prior to introduction. In addition, endotoxin levels in the final product were measured using kinetic chromogenic limulus amebocyte lysate assay by the Scottish National Blood Transfusion Service.

Intracellular cytokine staining assay

Cells from final product were assessed for ADV-specific interferon (IFN)γ production in an intracellular staining (ICS) assay. Cells were washed and resuspended in 10% human AB serum (Biowest)/RPMI Glutamax, and 2×10^5 cells were plated per well in a round-bottom 96-well plate. Cells were stimulated with media alone (negative control), 1 μg/peptide/mL of ADV peptide pool (Miltenyi Biotec) or 1 μg/mL *Staphylococcus enterotoxin* B (SEB; Sigma Aldrich; positive control). Brefeldin A (5 μg/mL; Sigma Aldrich) was added to all wells, and cells were incubated at 37°C/5% CO_2 for 16 ± 2 h. Cells were stained extracellularly for CD3 PE Cy7 and CD8 FITC (BioLegend) before fixation and permeabilization (Intrastain, Dako) and staining intracellularly for IFNγ PE (BD Biosciences). Samples were analyzed on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) within 2 h of staining. Gating strategies are shown in Supplementary Figure S1. Note that where starting material was cryopreserved (n = 34), this was analyzed in parallel with product (n = 39) to minimize interassay variation. One product (A1-45) that failed release criteria was not analyzed.

Cell surface phenotyping

Cryopreserved peripheral blood mononuclear cells (PBMC; starting material; n = 23) or "product QC" samples (n = 28) were thawed, counted and 2×10^5 cells stained per test with a combination of anti-human CD3 PE Cy7, CD8 APC Cy7, CD8 PerCP, CD16 PE, CD19 APC Cy7, CD45RA PE Texas Red (Invitrogen), CCR7 PE, and Sytox Blue viability dye (Invitrogen). All antibodies were purchased from BioLegend unless specified. Cells were analyzed on a MACSQuant Analyzer 10 flow cytometer within 1 h of staining. Gating strategies are shown in Supplementary Figure S2.

IFNγ enzyme-linked immunospot assay

Cryopreserved PBMC (starting material, n = 23) or "product QC" (n = 20) samples were thawed, counted and analyzed in an IFNγ enzyme-linked immunospot (ELISpot) assay. Starting material and samples for quality control (QC) from the same donor were tested in parallel on the same day to minimize assay variability. Briefly, polyvinylidene fluoride plates (Millipore) were pretreated with 35% ethanol, washed with phosphate-buffered saline (PBS) (Invitrogen) and coated with 15 μg/mL anti-human IFNγ primary antibody (clone 1-D1K; Mabtech) for 2 h at 37°C/5% CO_2. The plate was washed with Dulbecco's PBS before blocking with 10% HSA/RPMI Glutamax for ≥1 h. Blocking buffer was discarded immediately before use. Cells (2×10^5) were stimulated with 1 μg/peptide/mL ADV peptide pool (Miltenyi Biotec). Negative (complete media alone) and positive (1 μg/mL SEB) controls were included in all assays. After 16 ± 2 h culture at 37°C/5% CO_2, plates were washed and incubated with 1 μg/mL biotinylated anti-human IFNγ (clone 7-B6-1; Mabtech). Cells were discarded and plate washed with PBS, and secondary antibody was added for 1 h at 37°C/5% CO_2, followed by extravidin alkaline phosphatase (Sigma). Production of IFNγ was detected using the AP conjugate substrate kit (Bio-Rad) per manufacturer's instructions. Plates were scanned and counted using an automated ELISpot reader (AID). Results are expressed as spot forming cells (SFC) per 10^6 cells.

Proliferation assay

Cryopreserved PBMC (starting material, n = 9) or "product QC" (product, n = 13) samples were thawed, counted and $\geq 5 \times 10^6$ cells were stained with 1 μM/mL of carboxyfluorescein succinimidyl ester (Sigma) in RPMI Glutamax for 10 min at 37°C/5% CO_2 before

washing with 10% HSA/RPMI Glutamax. Cells were counted and 2×10^5 cells added per well in a round-bottomed 96 well plate. Cells were stimulated with 1 µg/peptide/mL ADV peptide pool, culture media alone (negative control) or 1 µg/mL SEB (positive control) and cultured for 5 days at 37°C/5% CO_2 before washing with PBS and staining with CD3 PE Cy7 and CD8 PE (BioLegend). Cells were analyzed on a MACSQuant Analyzer 10 flow cytometer within 1 h of staining, a minimum of 1000 live events were collected, and the percentage of dividing cells (CD3+ and CD8+/–) were analyzed using FlowJo v7.6.5 (FLOWJO). Gating strategies are shown in Supplementary Figure S3.

Statistical analysis

All statistical analysis and data transformation was carried out using GraphPad Prism version 5.01. Data were compared using the non-parametric Mann-Whitney or Wilcoxon matched paired tests, as specified.

Dosing rationale and treatment regimen

Our dosing rational was based on a meta-analysis of 100 patients undergoing DLI [17]. This study suggested that a maximum dose of 1×10^6 CD3+/kg is safe for a DLI from an HLA-identical sibling but that the dose from a matched unrelated donor should be lowered to 1×10^5 CD3+/kg. Data from manufacturing runs of Cytovir ADV on material from healthy adult donors demonstrated a low, but detectable, level of alloreactivity in T-cell product (Supplementary Figure S4), similar to that observed by others [13]. To minimize the risk of GvHD, we therefore chose a low initial cell dose (1×10^4 CD3+/kg) as an appropriate starting dose for an escalation study. For this escalation study, we prepare two cell doses of Cytovir ADV, one at 1×10^4 CD3+/kg and one at 1×10^5 CD3+/kg. During the first 28 days after HSCT, patients are ineligible for treatment because of the prolonged effect of conditioning serotherapy on T-cell viability. Patients commence the study on reactivation of ADV (defined as two consecutive positive diagnostic ADV PCRs >1000 copies/mL) post-transplant, in addition to antiviral drugs that are administered as part of standard of care. Patients are only treated if they are considered stable as deemed by the attending physician. The primary dose is 1×10^4/kg total CD3+ T cells. Patients are monitored at the following approximate time points post-Cytovir ADV administration: 14, 30, 60, 90, 120, 150 and 180 days. If the patient exhibits significant levels of ADV viraemia requiring treatment ≥4 weeks post-infusion they are assessed to receive a secondary maximum target dose of 1×10^5/kg total CD3+ T cells and will be monitored for a further 180 days.

Results

We describe the production of 40 ADV-specific T-cell products for adoptive T-cell therapy. Cells were expanded for 10 days in a One Touch expansion process. All manufacturing steps were performed under full GMP conditions within the Centre for Cell, Gene and Tissue Therapeutics, Royal Free Hospital, London. All open processing steps were performed under a laminar flow biological safety cabinet (Grade A) within a Grade B cleanroom. This OneTouch expansion process required minimal manual manipulation in the laboratory (approximately 10 h hands-on laboratory time) and was simple to perform.

A total of 40 ADV-specific T-cell products were manufactured between January 2013 and December 2014 (see Table I). Thirty-eight of 40 products passed established release criteria. Two products (A1-20 and A1-45) did not meet specifications and were not released for potential infusion.

Manufacturing was initiated from 2×10^7 CD45+ cells, and the OneTouch expansion process had a median yield of 2.9×10^7 CD3+ cells (range: 1.4×10^6–8.6×10^7) VST representing a median CD3+ expansion during manufacturing of 2.7-fold (range 0.3- to 7.7-fold). The composition of the final cell product within the leukocyte population varied, largely comprising CD3+ T cells (median: 88.3%, range: 70.6–94.1%) with the bulk of these being CD8 negative T cells at 58.4% (range: 29.2–90.3%). Final T-cell product had high viability, except for one outlier that failed the viability specification (median 95.9%, range: 0.6–99.7%). Starting material and VST was characterized by flow cytometry for key cell subsets including T cells, B cells, natural killer (NK) cells and monocytes. Enrichment during manufacture was specific for T cells, with other leucocyte populations (B cells, NK cells, monocytes) reducing significantly in frequency

Table I. Final product composition.

Measurement	Median (range; SD)
CD3+ (% of CD45+)	88.3 (70.6–94.1; 4.8)
CD4+ (% of CD3+)	58.4 (29.2–90.3; 16.6)
CD8+ (% of CD3+)	41.3 (9.7–70.8; 16.5)
Viability (% of CD45+)	95.9 (0.6–99.7; 15.4)
Potency (% ADV-specific IFNγ+ of CD3+)	5.9 (1.1–42.1; 9.2)
Fold expansion (CD3+ increase during manufacture)	2.7 (0.3–7.7; 2.0)

Summary of 40 ADV-specific ATIMPs manufactured during 2013 and 2014. CD3+ numbers and cell viability were measured by flow cytometry. Potency was measured by analyzing ADV-specific IFNγ production by CD3+ cells in an IFNγ intracellular staining assay. Expansion of CD3+ cells was calculated by comparing absolute numbers of CD3+ cells in starting material and product. Should clarify CD4+ T cells (as gated on CD3+CD8–).

Table II. Cell composition.

Cell phenotype	Starting material Median (range; SD)	Product Median (range; SD)	Comparison between starting material and product *P* (Mann-Whitney)
CD45+ WBC (% of live cells)	72.4 (2.7–92.5; 24.9)	87.4 (13.7–98.0; 24.4)	0.0357
CD3+ (% of CD45+)	50.1 (19.1–87.5; 17.7)	88.3 (70.6–94.1; 4.8)	<0.0001
CD13+ (% of CD45+)	23.0 (0.2–53.5; 15.3)	1.0 (0.2–9.7; 2.5)	<0.0001
CD16+ (% of CD45+)	3.6 (1.3–22.8; 6.3)	1.2 (0.6–8.6; 2.3)	0.0058
CD19+ (% of CD45+)	5.9 (0.9–42.2; 10.7)	3.6 (0.5–12.8; 3.0)	0.0225

Summary of cell composition of starting material (n = 21) and product (n = 22). Percentages of different cell types in starting material and product are shown. CD45+ white blood cells (WBC) were measured as percentage of live cells, CD3+, CD13+, CD16+ and CD19+ cells were measured as percentage of CD45+ cells. Cell composition in starting material and product was compared using a Mann-Whitney test with *P* values as shown. Only samples that were consented for R&D analysis were assessed.

(Table II) and found in product at low levels also seen by others [13]. Of these 40 products, 31 were manufactured from mobilized apheresis, and 9 were manufactured from whole blood. Comparing CD3+ cells in the product (% and absolute numbers) and ADV-specific IFNγ production revealed no differences in product quality between these starting materials (data not shown).

The ADV-specific antiviral response in product was measured by IFNγ ELISpot (Figure 1). Product demonstrated a median frequency of 2345 (range: 190–7733) ADV-specific IFNγ spot-forming cells per million cells. In all cases where paired samples were available for comparison, the frequency of IFNγ+ spots increased in the products, relative to the starting material. Potency of the ADV-specific T-cell product was assessed by antigen-induced IFNγ production (Figure 2). IFNγ intracellular cytokine staining

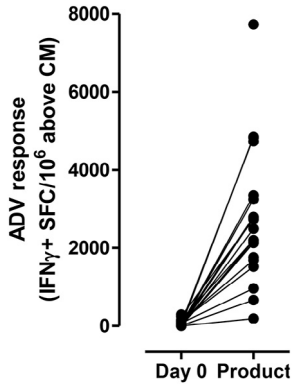

Figure 1. IFNγ ELISpot data. ADV-specific IFNγ secretion by cells before manufacture (day 0; n = 23) and after manufacture (Product; n = 20). Data are shown as ADV-specific IFNγ spot forming cells (SFC) per million cells above that seen in complete medium (CM) alone. Paired samples from individual donors are connected with a line. ADV-specific T-cell frequency was significantly higher in product ($P = 0.0003$; Wilcoxon matched pairs). Only samples that were consented for R&D analysis were assessed.

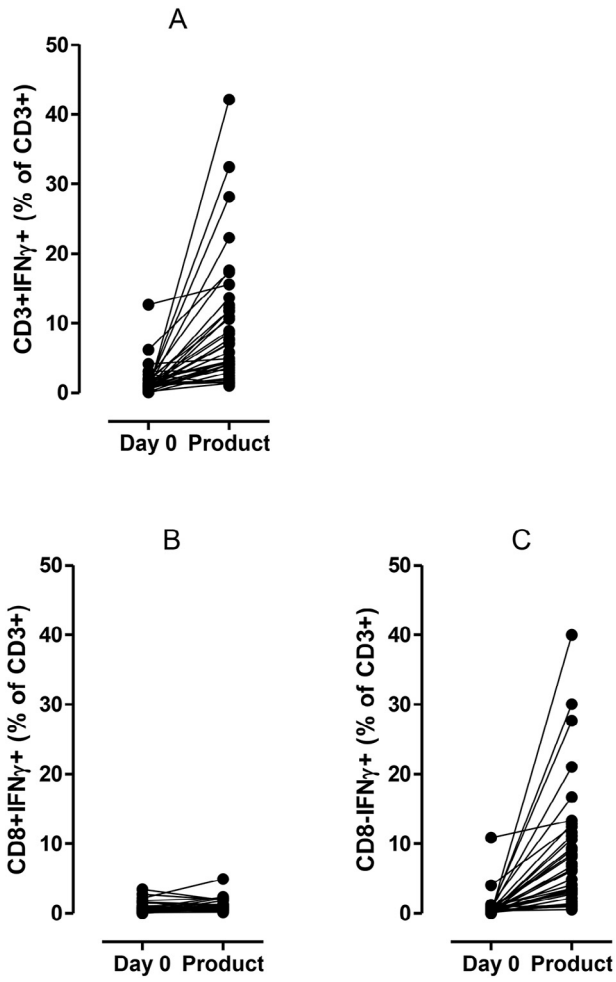

Figure 2. IFNγ intracellular cytokine stain. ADV-specific IFNγ production by (A) CD3+ T cells, (B) CD8+ T cells and (C) CD4+ T cells (gated as CD3+CD8–) before manufacture (day 0; n = 34) and after manufacture (Product; n = 39). Percentage shown is the ADV-specific IFNγ production. Paired samples from individual donors are connected with a line. One sample (A1-45) that failed release criteria was not analyzed. ADV-specific IFNγ frequency was significantly higher in product (A: $P < 0.0001$, B: $P = 0.0419$, C: $P < 0.0001$; Wilcoxon matched pairs). Only samples with excess starting material following initiation of manufacturing were assessed at day 0.

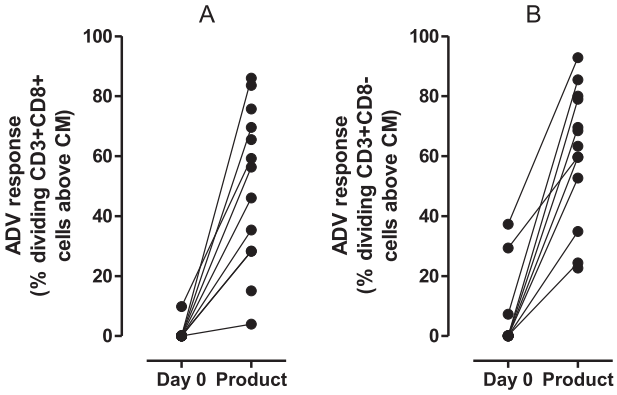

Figure 3. Proliferation data. Percentage of CD8+ T cells (A, CD3+CD8+) or CD4+ T cells (B, CD3+CD8–) dividing in response to ADV peptide (above that seen in culture medium [CM] alone). Day 0 (n = 9) and product (n = 13). Paired samples from individual donors are connected with a line. The percentage of T cells dividing in response to ADV peptide was significantly higher in product (A: $P = 0.0039$, B: $P = 0.0039$; Wilcoxon matched pairs). Only samples that were consented for R&D analysis were assessed.

demonstrated that whereas the ADV-specific IFNγ response in the starting material (PBMC) was 1.0% of CD3+ cells (range: 0–12.7%; Figure 2A), this increased to a median frequency of 5.9% of CD3+ cells in the product (range: 1.0–42.1%). Therefore, the 10-day G-Rex expansion process achieved an average of greater than 5.5-fold enrichment of the ADV-specific T cells. The majority of IFNγ-producing T cells were CD4+ (CD3+CD8–; Figure 2B,C). Of the 34 paired data, 33 showed an increase in CD3+IFNγ+ ADV-specific production compared with starting material, of these 23 had an increase in ADV-specific CD8+IFNγ+ and all showed an increase in CD8-IFNγ+ production assumed to be attributed to CD4+ cells.

We further assessed the functionality of ADV-specific VST by determining the capacity of T cells to proliferate in response to antigen (Figure 3). The median ADV-specific proliferative response observed in starting material was minimal (median 0.0% of CD3+CD8–[CD4+] and 0.0% CD3+CD8+ cells). Upon expansion, the majority of CD4+ and CD8+ T cells demonstrated an antigen-specific proliferative response (median 63.3%, range: 22.7–92.9 and 56.3%, range: 4.0–86.0, respectively), again indicating specific enrichment of ADV-specific T cells in product. All paired samples had a significant increase in ADV-specific proliferation in product compared with starting material ($P = 0.004$ as measured in a Wilcoxon signed rank test).

As a final characterization of our ADV-specific T-cell product, we determined the T-cell memory composition within the product (Figure 4). PBMC used as starting material were heterogeneous, containing a mixture of naive, effector memory, central memory and terminally differentiated effector memory cells. After ADV expansion, VST had expanded and contained largely CD4+ effector memory T cells (41.5% of T cells). Remaining T cells were a mixture of CD8+ effector memory (12.8%), CD4+ central memory (15.7%) and CD8+ naive cells (12.1%), with the remainder equally spread across remaining memory subsets.

Sterility of VST product was assessed following OneTouch expansion. No growth of aerobic or anaerobic bacteria was detected (Bactec testing). Product was confirmed mycoplasma free (mycoplasma PCR test) and endotoxin free (chromogenic limulus amebocyte lysate test).

Case report

The ADV-specific T-cell product is currently being assessed in the open phase I/IIa clinical trial (ASPIRE) with eight patients treated (May 2016). We present here as an example (Figure 5) the case of a 13-year-old girl with juvenile idiopathic arthritis/macrophage activation syndrome/haemophagocytic lymphohistiocytosis who received a fully matched unrelated stem cell transplant conditioned with fludarabine (30 mg/m^2 × 5, days −7 to −3), melphalan (140 mg/m^2, day −2) and alemtuzumab (0.2 mg/kg ×5, days −8 to −4); and cyclosporine, mycophenolate and prednisolone as GvHD prophylaxis. Full donor chimerism in peripheral blood was detected on D+13. The patient developed early adenoviremia on D+12 with a viral load of 28 712 copies/mL. Cidofovir (5 mg/kg) was started on D+15 when viral load had risen to 166 049 copies/mL, followed by a second dose 1 week later. Clinically the patient had evidence of ADV gut disease with diarrhea and vomiting and isolation of the virus in stool and endoscopic biopsies. She did not have evidence of pneumonitis or hepatitis. ADV in blood continued to rise quickly to a peak of >20 million copies/mL on D+22, where it stayed until subject received Cytovir ADV manufactured from the original HSCT donor at 1×10^4 CD3+/kg on D+28. ADV-specific T-cell response was detected by ELISpot and IFNγ capture from 13 days post-infusion until the last monitoring visit. ADV levels declined after ACT, and virus was undetectable by day 92 post-HSCT, remaining so at day 210. The patient did not receive the second, escalated dose of 1×10^5 CD3+/kg. No infusion-related side effects and no aGvHD were observed following ACT, although the patient had stage 1 skin GvHD treated early on and was quiescent at time of administration of ACT. She remained on cyclosporine as GvHD prophylaxis at time of ACT. Steroids were started before transplant for pre-existing disease and dose had been reduced since appearance of ADV in blood. At time of ACT, she was on 0.04 mg/kg/day of dexamethasone.

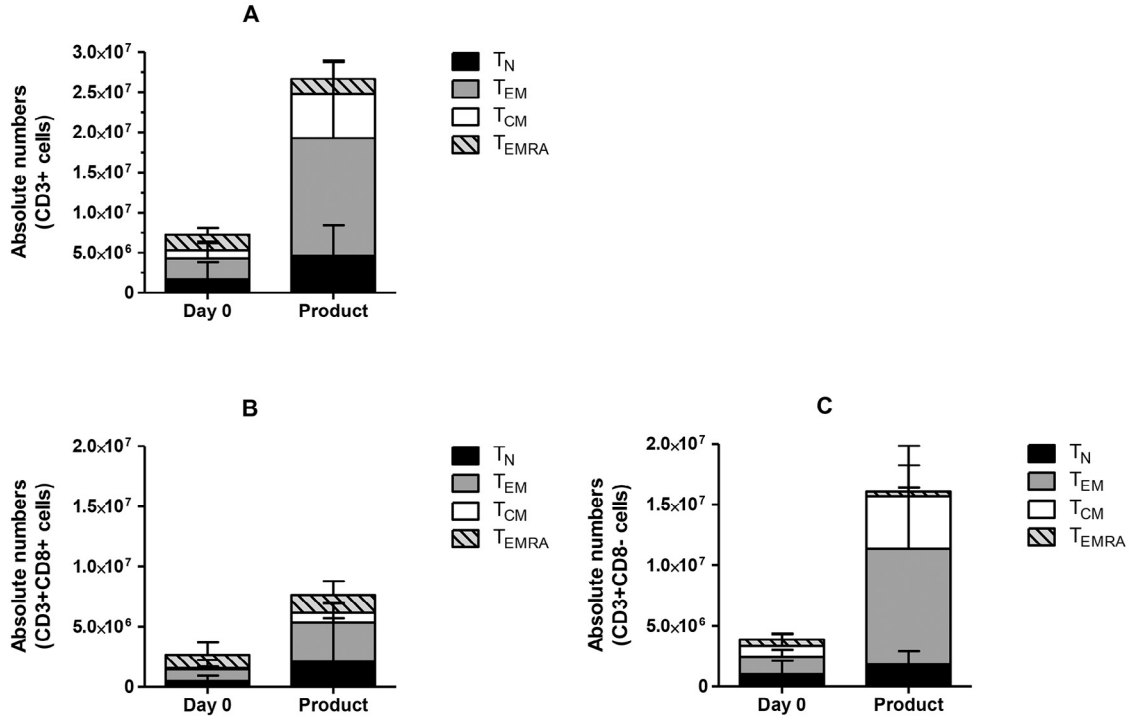

Figure 4. T-cell phenotype (Memory). Median absolute number of CD3+ (A), CD8+ T-cell (B, CD3+CD8+) and CD4+ T-cell (C, gated as CD3+CD8–) memory subsets in starting material (day 0, n = 23) and product (n = 27). Absolute numbers were calculated using flow cytometry analysis of T-cell memory subsets and transforming into absolute numbers based on cell count information relating to number of cells cultured for manufacture. "Day 0" is the number of cells seeded per GRex 10 and "Product" is the number of cells harvested in the product after 10 days of culture. Data are presented as median; error bars show interquartile range. The absolute number of T_{EM} and T_{CM} was significantly higher in product compared with starting material ($P < 0.0005$; Wilcoxon matched pairs). Only samples that were consented for R&D analysis were assessed. T_N, naive T cells (CD45RA+CCR7+); T_{EM}, effector memory T cells (CD45RA–CCR7–); T_{CM}, central memory T cells (CD45RA–CCR7+); T_{EMRA}, terminally differentiated memory cells (CD45RA+CCR7–).

Discussion

The use of adoptive T-cell therapy to enhance patient immune reconstitution holds great promise for the treatment of a viral infection and cancers, and ACTs targeting numerous diseases are in development. In particular, ACT has recently proven efficacious in the treatment or prevention of viral reactivation after HSCT [18–21]. Where frequencies of antiviral T cells are naturally high in healthy donors, such as is the case for CMV, effective ACT can often be achieved by direct isolation of antiviral T cells from donor material (using HLA-multimer or IFNγ-capture based procedures) with no further manipulation of the T cells required before administration into patients. We have previously demonstrated the safety and efficacy of HLA-multimer selected CMV-specific T cells in ACT (in two multicenter randomized, controlled trials: NCT01220895 and NCT01077908) and this anti-CMV ACT product (Cytovir CMV) is now available commercially under a manufacturing license from Cell Medica GmbH. For treatment of disease where specific antiviral T cells occur at lower frequencies in donor material, as is often the case for ADV, antiviral T cells can be grown *ex vivo* from donor material to numbers required of ACT. Several studies have demonstrated the safety of this approach, and clinical trials are currently underway investigating the use of expanded antiviral T cells for ACT in the treatment of ADV-, Epstein-Barr virus– and human papilloma virus–associated diseases. However, the routine production of expanded T-cell products under GMP conditions remains challenging.

Here we described the GMP-compliant manufacture of ADV-specific T-cell products using a One Touch expansion process lasting 10 days. This process produces an ADV-specific T-cell product with ability to produce IFNγ and proliferate in response to antigen. A total of 40 products were manufactured within a period of 24 months, with a 95% success rate in achieving release criteria (T-cell purity median 88.3%). This robust, high-throughput manufacturing process requires only modest hands-on laboratory time (approximately 10 h). This compares favorably to methods that require increased manipulation and laboratory time [14,15,22,23] and is comparable to the expansion method published by Geyeregger *et al.* [13]. The T-cell yield and ADV-specificity of our ex-

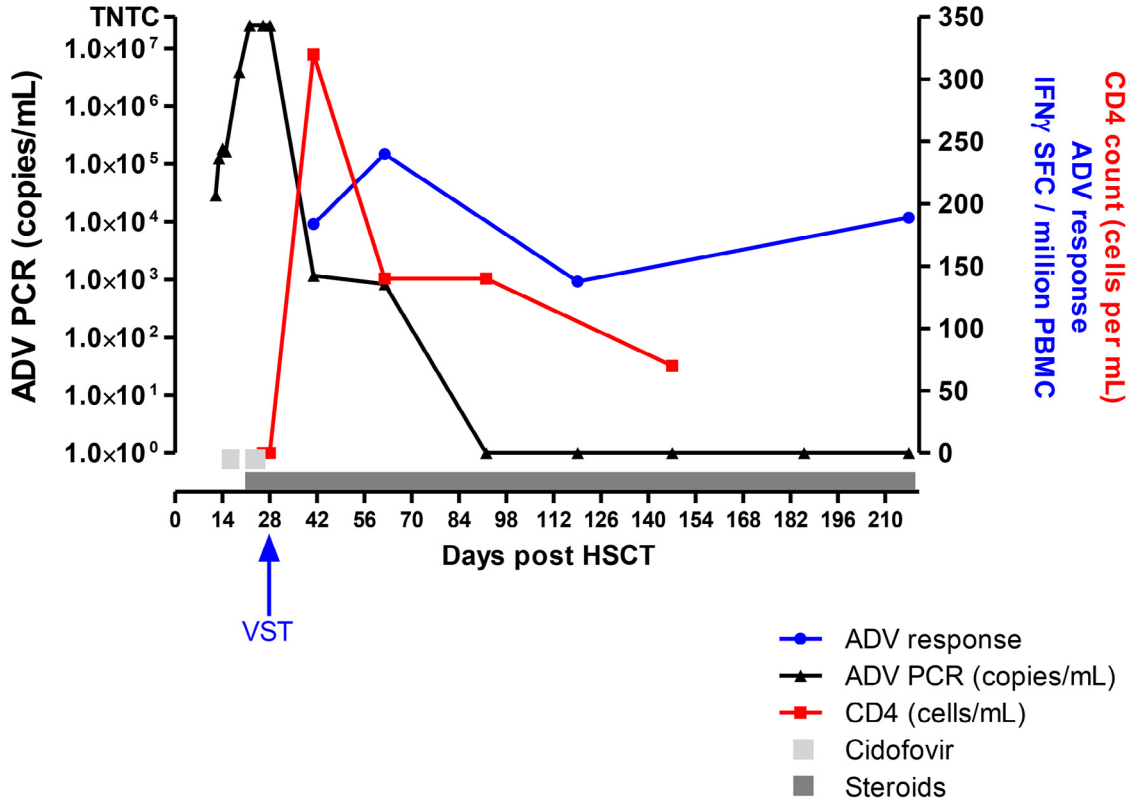

Figure 5. Exemplar patient data. Exemplar patient data from the open ASPIRE trial. A summary over time from point of HSCT showing ADV viral load (copy number/mL), total CD4+ T-cell count (cells/mL whole blood) and IFNγ ELISpot for ADV-specific response (spot-forming cells/million PBMC) is depicted. Concomitant treatments with cidofovir and corticosteroids are highlighted in light and dark gray, respectively. TNTC, too numerous to count; VST, virus specific T-cell infusion.

panded T-cell product is comparable to that achieved through expansion-based methodologies published by other groups [13,15,23].

Our expanded ADV-specific T-cell product demonstrates IFNγ production predominantly in CD4+ cells, as has also been observed by others [13,15,23,24]. CD4+ T cells are likely to be critical for protective immunity against ADV because ADV-specific memory CD4+ T cells (but not necessarily CD8+ T cells) are readily detected in nearly all healthy individuals [16]. Moreover, the presence of ADV-specific CD4+ T cells correlates with protection from ADV-disease in patients following HSCT [25]. The largest fraction of T cells within our expanded T-cell product are of effector memory phenotype (T_{EM}), followed by central memory T cells (T_{CM}) and naive cells (T_N). Terminally differentiated effector cells (T_{EMRA}) constituted only a minor fraction of our T-cell product. A similar composition of memory T-cell phenotypes following ADV-specific T-cell expansion has been observed in other studies [13,15]. T_{CM} cells have superior *in vivo* expansion and persistence profiles compared with T_{EM} cells and are thought to be critical for the establishment of long-term protective immunity following ACT [26]. T_{EM} cells, in turn, rapidly locate to peripheral tissue after ACT where they execute potent antiviral effector functions and could therefore act as a first line of attack. This may be of particular importance for ACT targeting active, uncontrolled viremia such as during ADV reactivation following HSCT. It is therefore likely that the most efficient suppression of viral replication by ACT in the immediate and long-term can be achieved if the expanded T-cell product contains large fractions of both T_{CM} and T_{EM} cells, as can be generated by the manufacturing protocols described here and by others.

In summary, we have described a robust and highly standardized One Touch T-cell manufacturing process. The ADV-specific T-cell product is currently being assessed in a phase I/IIa clinical trial (ASPIRE), and data reported to date suggest a good clinical efficacy and safety profile. In the trial, the VST product is manufactured for all children identified as being at high risk of viral complications after allogeneic HSCT, enabling early intervention soon after ADV reactivation is detected.

We present one case study demonstrating a clinical outcome comparable to other studies and protocols [10,11,13,14] highlighting the validity of our approach for the treatment of ADV reactivation in HSCT.

Our straightforward OneTouch expansion methodology can be adopted for other antigen-specific T cells (e.g., CMV), and current work is being be carried out to assess broad application of this approach in generating antigen-specific T cells.

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AM, ASk, ASt, KN, and SB performed T-cell manufacturing and release assays. SP performed patient monitoring. KN designed the manufacturing process. WQ designed and leads the ASPIRE trial. WI was a co-investigator of the ASPIRE trial. MWL released the product as qualified person. AF, ASk, CH, MAP analyzed data and wrote the manuscript.

Disclosure of interest: AM, AF, ASk, ASt, KN, SB, SP, CH and MAP are current or former employees of Cell Medica Ltd. WQ received research funding from Miltenyi Biotec GmbH and Cell Medica Ltd. MWL is a shareholder of Cell Medica Ltd and a member of the Scientific Advisory Board. The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

[1] Gyurkocza B, Rezvani A, Storb RF. Allogeneic hematopoietic cell transplantation: the state of the art. Expert Rev Hematol 2010;3:285–99.

[2] Sureda A, Bader P, Cesaro S, Dreger P, Duarte RF, Dufour C, et al. Indications for allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015. Bone Marrow Transplant 2015;50:1037–56.

[3] Choi SW, Reddy P. Current and emerging strategies for the prevention of graft versus host disease. Nat Rev Clin Oncol 2014;11:536–47.

[4] Dong L, Gao Z, Chang L, Liang Y, Tan X, Liu J, et al. Adoptive transfer of cytomegalovirus/Epstein-Barr virus-specific immune effector cells for therapeutic and preventive/preemptive treatment of pediatric allogeneic cell transplant recipients. J Pediatr Hematol Oncol 2010;32:e31–7.

[5] Saglio F, Hanley PJ, Bollard CM. The time is now: moving toward virus-specific T cells after allogeneic hematopoietic stem cell transplantation as the standard of care. Cytotherapy 2014;16:149–59.

[6] Leung AYH, Kwong YL. Haematopoietic stem cell transplantation: current concepts and novel therapeutic strategies. Br Med Bull 2010;93:85–103.

[7] Florescu DF, Hoffman JA. Adenovirus in solid organ transplantation. Am J Transplant 2013;13:206–11.

[8] Leen AM. T-cell therapy: the next generation. Expert Rev Hematol 2010;3:123–5.

[9] Rutella S, Locatelli F. Strategies to harness immunity against infectious pathogens after haploidentical stem cell transplantation. Am J Transl Res 2011;3:404–21.

[10] Feuchtinger T, Matthes-Martin S, Richard C, Lion T, Fuhrer M, Hamprecht K, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 2006;134:64–76.

[11] Leen AM, Christin A, Myers GD, Liu H, Cruz CR, Hanley PJ, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. Blood 2009;114:4283–92.

[12] Zandvliet ML, Falkenburg JHF, van Liempt E, Veltrop-Duits LA, Lankester AC, Kalpoe JS, et al. Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection. Haematologica 2010;95:1943–51.

[13] Geyeregger R, Freimüller C, Stemberger J, Artwohl M, Witt V, Lion T, et al. First-in-man clinical results with Good Manufacturing Practice (GMP)-compliant polypeptide-expanded adenovirus-specific T cells after haploidentical hematopoietic stem cell transplantation. J Immunother 2014;37:245–9.

[14] Feucht J, Opherk K, Lang P, Kayser S, Hartl L, Bethge W, et al. Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. Blood 2015;125:1986–94.

[15] Freimüller C, Stemberger J, Artwohl M, Germeroth L, Witt V, Fischer G, et al. Selection of adenovirus-specific and Epstein-Barr virus-specific T cells with major histocompatibility class I streptamers under Good Manufacturing Practice (GMP)-compliant conditions. Cytotherapy 2015;17:989–1007.

[16] Onion D, Crompton LJ, Milligan DW, Moss PAH, Lee SP, Mautner V. The CD4+ T-cell response to adenovirus is focused against conserved residues within the hexon protein. J Gen Virol 2007;88:2417–25.

[17] Raiola AM, Van Lint MT, Valbonesi M, Lamparelli T, Gualandi D, Occhini D, et al. Factors predicting response and graft-versus-host disease after donor lymphocyte infusions: a study on 593 infusions. Bone Marrow Transplant 2003;31:687–93.

[18] Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood 2010;116:4360–7.

[19] Peggs KS, Thomson K, Samuel E, Dyer G, Armoogum J, Chakraverty R, et al. Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. Clin Infect Dis 2011;52:49–57.

[20] Blyth E, Clancy L, Simms R, Ma CKK, Burgess J, Deo S, et al. Donor-derived CMV-specific T cells reduce the requirement for CMV-directed pharmacotherapy after allogeneic stem cell transplantation. Blood 2013;121:3745–58.

[21] Peggs KS, Tholouli E, Chakraverty R, Nikolousis E, Bloor A, Devereux S, et al. CMV~IMPACT: results of a randomized controlled trial of immuno-prophylactic adoptive cellular therapy following sibling donor allogeneic HSCT. Blood 2014;21:124.

[22] Qasim W, Gilmour K, Zhan H, Derniame S, McNicol AM, Ip W, et al. Interferon-γ capture T cell therapy for persistent adenoviraemia following allogeneic haematopoietic stem cell transplantation. Br J Haematol 2013;161:449–52.

[23] Chatziandreou I, Gilmour KC, McNicol AM, Costabile M, Sinclair J, Cubitt D, et al. Capture and generation of adenovirus specific T cells for adoptive immunotherapy. Br J Haematol 2007;136:117–26.

[24] Feuchtinger T, Lücke J, Hamprecht K, Richard C, Handgretinger R, Schumm M, et al. Detection of adenovirus-specific T cells in children with adenovirus infection after

allogeneic stem cell transplantation. Br J Haematol 2005;128:503–9.

[25] Heemskerk B, Lankester AC, van Vreeswijk T, Beersma MFC, Claas ECJ, Veltrop-Duits LA, et al. Immune reconstitution and clearance of human adenovirus viremia in pediatric stem-cell recipients. J Infect Dis 2005;191:520–30.

[26] Wherry EJ, Teichgräber V, Becker TC, Masopust D, Kaech SM, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003;4:225–34.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.06.009.