Paediatric Immune Reconstitution with Adenovirus Adoptive Immunotherapy post Haematopoietic Stem Cell Transplant

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‘ I, Wing Yue Winnie Ip, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

ABSTRACT

Adenovirus (ADV) can cause significant morbidity and mortality in children following allogeneic haemtopoietic stem cell transplantation (HSCT) when immunity is compromised. HSCT can offer a cure for many haematological diseases, primary immunodeficiencies, and inborn errors of metabolism. However not all transplant recipients have fully matched sibling donors and alternative donor sources have to be sought. In HLA-matched or mismatched unrelated donor setting, conditioning regimens will often include serotherapy such as Alemtuzumab (monoclonal anti-CD52 antibody) or thymoglobulin (polyclonal rabbit thymocyte globulin [ATG]) to remove donor alloreactive T cells that can cause acute Graft versus Host Disease (GVHD). During the post-transplant period of reduced T-cell immunity when reconstitution of donor-derived immune system is slow and the use of immunosuppressive agents is necessary, transplant recipients are vulnerable to viral reactivations and/or infections with CMV, EBV, or ADV (Hiwarkar et al., 2012) and other viruses.

Whilst antivirals such as Ribavirin and Cidofovir are available for the treatment of ADV, they are associated with toxicity and have variable efficacy. It has been demonstrated that reconstitution of virus-specific immunity is essential to control viral infection after allo-HSCT (Feuchtinger et al., 2005a; Heemskerk et al., 2005). Over the past 2 decades, adoptive transfer of donor-derived virus specific T cells has been explored extensively as an alternative method to prevent and treat ADV and other viral infections post-HSCT. This thesis examines recent pre-clinical and clinical studies on T-cell Immunotherapy for ADV and presents data from a phase1/2 first-in-man trial of using donor-derived ADV CTLs in high-risk paediatric HSCT patients (ASPIRE trial).

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CHAPTER 1

INTRODUCTION

Introduction

Adenovirus (ADV) causes mild illnesses in immunocompetent hosts and infections are usually benign and short-lived. The virus is endemic in paediatric populations, with 80% of children between the ages of 1 and 5 years having antibody to one or more serotypes. They most commonly manifest as upper respiratory tract infections, ranging from pharyngitis to pneumonia, particularly with serotypes 1, 2, 5 and 6 (Baldwin et al., 2000; Hale et al., 1999). Infections can also result in gastroenteritis (mainly with the non-cultivable serotypes 40 and 41), cystitis, myocarditis, and keratoconjunctivits. The virus is responsible for 5-11% of cases of viral pneumonia and bronchiolitis in infants and children; and illness typically lasts less than 2 weeks, but the virus remains latent in lymphoid or renal disease (Hale et al., 1999; Munoz et al., 1998). Once infected adenovirus persists within lymphoreticular tissue including tonsils, adenoids, and intestines; and viral shedding can persist for months or years in healthy children. However, in the immunocompromised hosts, for example, children in the post-haematopoietic stem cell transplant setting, ADV can cause significant morbidity and mortality. Haematopoietic stem cell transplantation (HSCT) can offer a cure for many haematological diseases, primary immunodefiencies, and inborn errors of metabolism. Not all transplant recipients have fully matched sibling donors and alternative donor sources have to be sought. In HLA-matched or mismatched unrelated donor setting, conditioning regimens will often include serotherapy such as Alemtuzumab (monoclonal anti-CD52 antibody) or thymoglobulin (polyclonal rabbit thymocyte globulin [ATG]) to remove alloreactive T cells in the recipient that can cause acute Graft versus Host

Disease (GVHD). During the post-transplant period of reduced T-cell immunity when reconstitution of donor-derived immune system is slow and the use of immunosuppressive agents is necessary, transplant recipients are vulnerable to viral reactivations and/or infections.

Whilst antivirals such as Ribavirin and Cidofovir are available for the treatment of ADV, they are associated with toxicity and have variable efficacy. Over the past decade or so, adoptive transfer of donor-derived virus specific T cells has been explored extensively as an alternative method to prevent and treat ADV and other viral infections post-HSCT. This review examines recent pre-clinical and clinical studies on T-cell Immunotherapy for ADV and provides a strategy for monitoring and management of ADV in children after allo-HSCT.

1.1 ADENOVIRUS

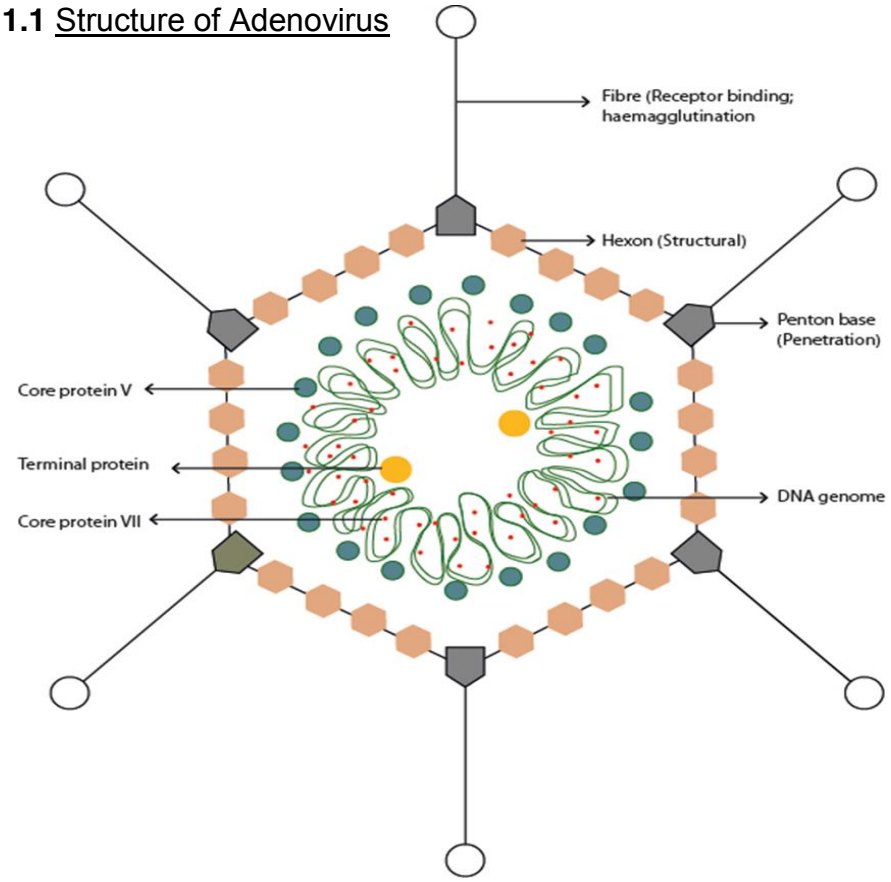
1.1.1 Classification and Structure

Adenoviruses (ADV) were first isolated in 1953 from human adenoid tissues obtained during adenoidectomy (Rowe et al., 1953). They are non-enveloped, double stranded DNA viruses that range in size from 65 to 80nm in diameter (Walls et al., 2003). To date, over 60 ADV serotypes have been identified based on their specific neutralizing abilities, and can be further classified into seven subgroups, A-G, on the basis of their haemagglutination properties, their oncogenic potential in rodents and DNA homology or GC content of their DNA (Table 1.1) (Heim, 2011; Kojaoghlanian et al., 2003; Lion et al., 2003; Robinson et al., 2013). The virion is composed of 252 capsomers: 240 hexons and 12 pentons arranged in an icosahedral shape; and a nucleoprotein core that contains the DNA viral genome and internal proteins. The linear, double stranded DNA genome is 34-36 kb in size and encodes for more than 30 structural and non-structural proteins (Kojaoghlanian et al., 2003; Robinson et al., 2013). The genome has a terminal protein (TP) attached covalently to the 5' termini, which have inverted terminal repeats (ITRs). The virus DNA is intimately associated with the basic protein VII. Another protein, V, is packaged with this DNA-protein complex and appears to provide a structural link to the capsid (Russell, 2000). Each penton in the capsid comprises a base and a rod-like outward fibre projection of variable length depending on serotypes (Hierholzer, 1992; Walls et al., 2003). The hexon contains group-specific antigenic determinants in addition to subgroup-specific determinants and type-specific neutralising epitopes (Hierholzer, 1992) (Figure 1.1).

Table 1.1 Classification of human adenoviruses and their sites of infection

Subgroup	Serotype	Sites of infection
A	12,18,31	Gastrointestinal
B1	3,7,16,21,50	Respiratory
B2	11,14,34,35	Urinary tract/Renal
C	1,2,5,6	Respiratory
D	8,9,10,13,15,17,19,20,22-30,32,33,36,37,38,39,42-48,49,51	Eye
E	4	Respiratory
F	40, 41	Gastrointestinal

Figure 1.1 Structure of Adenovirus



1.1.2 Tropism and replication cycle of Adenovirus

Tissue tropism of ADV differs among the different serotypes but generally corresponds to the subgroups (Table 1.1). Subgroups C, E and some B viruses typically infect the respiratory tract, other B serotypes infect the urinary tract (B11, B34, B35); serotypes from subgroups A and F target the gastrointestinal tract and serotypes from species D the eyes (Kojaoghlanian et al., 2003; Lenaerts et al., 2008). All the human ADV subgroups, except for B, use the coxsackie-adenovirus receptor (CAR) for entry into cells via interaction with the fibre knobs (Kojaoghlanian et al., 2003). CAR is a plasma membrane protein of 46 kDa belonging to the immunoglobulin superfamily. It plays a role in maintaining the integrity of tight junctions in polarized epithelial cells and is normally sequestered on the basolateral surface of these cells. It contains extracellular, transmembrane, and cytoplasmic domains, with the extracellular domain being sufficient for attachment (Russell, 2000; Walters et al., 2002; Wiethoff and Nemerow, 2015). It is expressed in heart, brain, pancreas, intestine and at low levels in liver and lung (Fechner et al., 1999; Wolfrum and Greber, 2013). Other receptors include the histocompatibility class I molecule (for subgroup C viruses), sialoglycoprotein receptors (for Ad37 in subgroup D) and desmoglein-2 (DSG-2) (Wiethoff and Nemerow, 2015) (Russell, 2000). ADV-B subgroup and ADV-37 (subgroup D) bind to blood and epithelial cells via the membrane cofactor CD46 (a complement regulatory protein family member) via their fibre knobs which recognize the extracellular domain of CD46 (Wolfrum and Greber, 2013).

The adenovirus infection cycle is divided into an early (E) and late (L) phase. The early phase (6-8h) covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early genes. The phase starts with high-affinity binding of the virus to cell receptors via the knob portion of the fibre. Entry into cells is then mediated by binding of the virus penton base to cell surface integrins (αvβ3 and αvβ5) (Lenaerts et al., 2008; Russell, 2000). Following endosomal acidification, conformational changes occur in the ADV capsid that ultimately provokes endosomal lysis and input of virions into the cytoplasm. ADV genome is transferred into the nucleus when the partially dismantled capsid is translocated to the nucleopore complex (Lenaerts et al., 2008). The early phase ends with activation of the early viral transcription units (*E1A, E1B, E2A, E2B, E3 and E4)* that facilitate replication of the virus DNA and the resultant transcription and translation of the late genes; eventually leading to the assembly in the nucleus of the structural proteins and virus maturation in the late phase of infection (Lenaerts et al., 2008; Russell, 2000).

1.1.3 Adaptive Immunity against ADV

1.1.3.1 Humoral immunity against ADV

Hexon, fiber and to a lesser extent penton have been shown to be the major targets for ADV neutralising antibodies (Nab) (Gahery-Segard et al., 1998; Sumida et al., 2004; Sumida et al., 2005; Toogood et al., 1992). Hexon-specific Nab titers are 4- to 10- fold higher than fiber-specific Nab titers in humans

(Sumida et al., 2005). ADV fiber induces neutralising antibodies, which function by aggregating virus particles and thereby inhibiting absorption. Hexon and penton induce antibodies which inhibit low pH induced conformational changes in the virus (Toogood et al., 1992).

After HSCT, subjects who developed ADV viraemia but subsequently cleared the infection have humoral immune responses after a period of several weeks to months after viral clearance, with titers of serotype-specific neutralising antibodies increasing by 8-16 fold. Interestingly in some cases, pre-existing, high titers of ADV-specific Nabs in serum did not prevent progression to viraemia (Heemskerk et al., 2005).

Until recently, antibodies were thought to mediate anti-viral effects in the extracellular environment (Murphy et al., 2008). However, it has now been shown that ADV specific antibodies can also be carried into cells in association with virion particles (Mallery et al., 2010) and once in the cytosol, antibody-coated virus particles are targeted via the Fc region by the cytosolic immunoglobulin receptor TRIM21 (Tripartite motif-containing 21) (McEwan et al., 2013). This process leads to antibody-dependent intracellular neutralisation as well as activation of the innate immune pathways leading to production of proinflammatory cytokines (Watkinson et al., 2013). Whilst humoral immunity clearly plays a critical role in anti-ADV immunity, administration of immunoglobulin therapy has not been shown to be effective in preventing ADV

reactivation or of proven benefit of management of established viraemia or organ specific infection (Legrand et al., 2001; Ljungman, 2004).

1.1.3.2 Cell-mediated Immunity against ADV

Cellular immunity towards adenoviruses has been extensively studied over the past two decades. In humans the response is predominantly in CD4+ T cells specific for capsid derived antigens (Flomenberg et al., 1995; Heemskerk et al., 2003; Olive et al., 2002). These ADV-specific CD4 T cells have been shown to be of CD45RO memory phenotype with expression of Th1 cytokines such as IFN-γ, Il-2, and TNF-α (Hutnick et al., 2010; Sester et al., 2002). CD8+ T cell cytotoxic responses against ADV have also been identified (Flomenberg et al., 1996; Smith et al., 1996; Tang et al., 2006) with both viral structural proteins and regulatory proteins, such as E1A (early region 1A) predicted as targets (Flomenberg et al., 1995). In generated ADV-specific T-cell (CTL) lines, cytotoxic activity has been documented to be MHC class I restricted and mediated by CD8+ T cells (Flomenberg et al., 1996; Smith et al., 1996). In contrast to ADV-specific CD4+ T cells, CD8+ T cells were found to express effector-like combinations of IFN-γ, MIP1α and perforin activity (Hutnick et al., 2010). Using peptide-MHC tetramer staining and intracellular cytokine staining following stimulation with ADV hexon peptide pool, low frequencies of ADV hexon-specific CD8+ and CD4+ have been detected in peripheral blood from 38% and 81% of healthy adults, respectively (Zandvliet et al., 2010).

The cellular immune response to adenovirus is cross-reactive across serotypes, both in CD4+ and CD8+ T cells, confirming the presence of conserved antigens (Flomenberg et al., 1995; Heemskerk et al., 2003; Leen et al., 2004b; Olive et al., 2002; Smith et al., 1996; Smith et al., 1998). Peripheral blood mononuclear cells (PBMCs) taken from a group of eight healthy subjects with no serologic evidence of prior exposure to the uncommon group B Ad35, showed specific proliferative responses to both Ad2- and Ad35-infected cell lysates (all subjects had Ad2-specific neutralising antibody). The responder T cells were CD4+ T cells (Flomenberg et al., 1995). Similarly Smith and colleagues showed that CTL lines (consisting of both CD4+ and CD8+ cells) generated against Ad5 (subgroup C) readily killed fibroblasts infected with Ad11 (subgroup B) (Smith et al., 1996); as well as to viruses from 3 other subgroups (Ad4 subgroup E, Ad18, subgroup A, Ad26 subgroup D) (Smith et al., 1998). The recognition pattern of different ADV serotypes has been investigated at the clonal level. Eleven independent T-cell clones from four different donors were tested for cross-reactivity against ADV belonging to subgroup C (Ad1, Ad5, Ad6), subgroup B (Ad3, Ad7, Ad11, Ad34, Ad35) and subgroup A (Ad12, Ad18, Ad31). These clones contained IFN-γ producing CD4+ T cells and five of the eleven clones recognised ADV from all three subgroups tested. Using blocking antibodies to HLA-DR and HLA-DP, the Ad5-specific clones have been shown to use either HLA-DR or HLA-DP as restriction element for antigen recognition (Heemskerk et al., 2003).

1.1.4 Dominant Adenovirus-derived T cell Epitopes (Table 1.2)

Recently a number of immunodominant CD4+ and CD8+ epitopes of ADV hexon have been defined ((Joshi et al., 2009; Leen et al., 2008; Leen et al., 2004b; Olive et al., 2002; Tang et al., 2004; Tang et al., 2006; Veltrop-Duits et al., 2006). Olive and colleagues identified the first human T-cell epitope from ADV, located in the major capsid protein hexon (Olive et al., 2002). Four viral protein sequences were screened for the HLA-A2 binding motif using PBMCs from HLA-A2-positive and HLA-A2-negative healthy donors. A 15-mer peptide, H910-924, was identified as the optimal CD4+ T-cell epitope with majority (9/12) of donors exhibiting memory/effector CD4+ T-cell responses to this peptide in IFN-γ ELISPOT assay (Olive et al., 2002). This epitope is located near the C-terminus within a β-pleated sheet 'viral jellyroll' structure that helps form the base of the hexon molecule. The HLA class II molecule DP4 (the most common class II allele with antigenic frequency of 75% in general population) has been identified as the restricting allele (Tang et al., 2004).

Heemskerk's group generated ADV-specific CD4+ T cell clones against ADV5 and determined the HLA restriction, protein, and peptide specificity of these clones. Most of these clones exhibited a broad cross-reactivity pattern towards serotypes from subgroups A, B and C; recognising peptides derived from structural viral proteins and appeared to have a Th1-like phenotype based on secreted cytokines. Two clones recognised II64, which contains a conserved epitope $II_{910\text{-}924}$ that was described by Olive's group above (Heemskerk et al., 2006). Since then further class II restricted epitopes have been identified, all

derived from the conserved region of the hexon protein (Leen et al., 2008; Veltrop-Duits et al., 2006).

Five CD8+ T cell clones isolated from adenovirus-specific CTL lines have had epitopes identified in the conserved regions of the capsid protein hexon. Four of these epitopes restricted by HLA-A*1, HLA-A*2, and HLA-B*7 were processed and presented in a species cross-reactive manner, while the remaining epitope, recognised in the context of HLA-A*24, was specific for viruses within subgroups C and D (Leen et al., 2004b). A further 12 class I restricted hexon epitopes have been identified since (Leen et al., 2008; Tang et al., 2006; Zandvliet et al., 2010). In addition five CD8+ T-cell epitopes (HLA-A24 TYF, HLA-B35 MPN, HLA-B35 LPG, HLA-B35/53 IPY, and HLA-A2 YVL) have been found to overlap directly with CD4+ T-cell epitopes (aa 31 to 55, FAR; aa 321 to 335, PNP; aa 56 to 590, FFA; aa 691 to 715, LGS; and aa 906 to 930, EVD) (Leen et al., 2008).

Although the ADV capsid protein hexon has been found to be the dominant T-cell target, CTLs targeting early viral proteins (early region 2 proteins DNA polymerase [Pol] and DNA-binding protein [DBP]) have also been identified (Joshi et al., 2009) (Table 1.3). Pol-epitope-specific T cells were detected at similar or higher frequencies than hexon and DBP in three of three HSCT recipients recovering from invasive ADV disease. In particular, Pol contains T-cell epitopes that were well conserved among different serotypes (Joshi et al., 2009).

Table 1.2 HLA restricted class I epitopes from human Ad5 hexon

HLA restriction	Epitope sequence	Ad5 hexon amino acids	References
A*24	TYFSLNNKF	37-45	(Leen et al., 2004b)
B*7	KPYSGTAYNSL	114-124	(Leen et al., 2004b)
B*7	MPNRPNYIAF	320-329	(Leen et al., 2004b)
A*2	TFYLNHTFKK	711-721	Leen et al., 2004b)
A*1	TDLGQNLLY	886-894	Leen et al., 2004b)
B*13/49	LFEVFDVVRV	918-927	(Tang et al., 2006)
A*2	YVLFEVFDVV	916-925	Tang et al., 2006)
A*2	LLYANSAHAL	892-901	Tang et al., 2006)
B*53	LPGSYTYEW	575-583	(Leen et al., 2008)
A*2	GLRYRSMLL	542-550	(Leen et al., 2008)
B*35/53	IPYLDGTFY	705-713	(Leen et al., 2008)
B*35	MPNRPNYIAF	320-329	(Leen et al., 2008)
B7	FRKDVNMVL	585-593	(Zandvliet et al., 2010)
B35	IPFSSNFMSM	873-882	(Zandvliet et al., 2010)
B52	ETYFSLNNKF	36-45	(Zandvliet et al., 2010)
B63	YSYKARFTL	78-86	(Zandvliet et al., 2010)

Table 1.3 HLA-restricted class I epitopes targeting ADV early region 2 proteins

HLA restriction	Epitope sequence	Ad5 Pol amino acids	References
A*02	GLTDASFNV	608-617	(Joshi et al., 2009)
A*02	TLNHRGWRV	779-788	(Joshi et al., 2009)
A*02	VLAWTRAFV	977-986	(Joshi et al., 2009)
HLA restriction	**Epitope sequence**	**Ad5 DBP amino acids**	**References**
A*02	MMGRFLQAYL	206-215	(Joshi et al., 2009)
A*02	KLLPDQVEAL	243-252	(Joshi et al., 2009)
A*02	FLGRQLPKL	407-416	(Joshi et al., 2009)

1.2 ADENOVIRAL INFECTION IN IMMUNOCOMPROMISED HOSTS

1.2.1 Epidemiology

Adenovirus is endemic in paediatric populations with 80% of children between 1 and 5 years of age having antibody to one or more serotypes (Hale et al., 1999). Infections in immunocompetent hosts are usually benign and short-lived, and most commonly manifest as upper respiratory tract infections (Hale et al., 1999). ADV causes 2-7% of respiratory tract infections in children in the first 5 years of life (Munoz et al., 1998). Infections can also result in lower respiratory infection, gastroenteritis, cystitis, myocarditis, and keratoconjunctivits. The virus is responsible for 5-11% of cases of viral pneumonia and bronchiolitis in infants and children. Illness typically lasts less than 2 weeks, but the virus remains latent in lymphoid or renal disease (Hale et al., 1999; Munoz et al., 1998). Once infected adenovirus persists within lymphoreticular tissue including tonsils, adenoids, and intestines. In healthy children viral shedding can persist for months or years and in children with congenital immunodeficiency disorders, ADV has been recognised as a cause fatal infections since 1960 (Wigger and Blanc, 1966) . The immunosuppressed paediatric host is particularly susceptible, most notably in the allogeneic transplant setting where cellular immunity is compromised and ADV can cause severe and protracted systemic illnesses such as hepatitis, pneumonitis, colitis, haemorrhagic cystitis, and encephalitis (Carrigan, 1997; Hale et al., 1999). The incidence of ADV infection reported in bone marrow transplant recipients ranges from 5% to 29% (Baldwin et al., 2000; Flomenberg et al., 1994; Hale et al., 1999; Hierholzer, 1992; Howard et al.,

1999; Runde et al., 2001; Shields et al., 1985). In these earlier studies ADV was detected via routine weekly surveillance cultures usually up to first 100 days post-transplant. With the advent of robust PCR based detection, serial ADV PCR has become the mainstay of routine surveillance. Using serial real-time PCR to identify ADV, the incidence of viral isolation is between 17-27% in paediatric transplant recipients (Kampmann et al., 2005; Lion et al., 2003).

1.2.2 Risk factors and mortality

These retrospective and prospective studies have facilitated the identification of several risk factors that are predictive for the development of ADV infection and/or disease in transplant recipients. One risk factor identified is T-cell depletion either ex vivo by CD34+ positive selection, or in vivo with Alemtuzumab or Antithymocyte globulin (ATG). In a group of 153 children receiving HSCT, adenoviraemia occurred in 26 children (17%), all of whom had received T-cell-depleted grafts (Kampmann et al., 2005). Similarly Lion and colleagues found a significant increase (*p* value = 0.014) in incidence of ADV infection in a group of 132 paediatric patients transplanted with T-cell depleted grafts (*in vivo* ATG combined with *ex vivo* CD34+ positive selection) where 27% of patients developed adenovriaemia and all who died from disseminated adenoviraemia had received heavily T-cell depleted grafts (Lion et al., 2003). In an adult cohort of 76 allograft recipients, adenovirus was isolated exclusively (15 of 76 patients, 20%) in recipients of T-cell depleted grafts. Patients who received 50 or 100 mg of alemtuzumab had 45% probability of developing adenovirus infection, regardless of donor type (Chakrabarti et al., 2002).

Other identified risk factors for ADV infection include type of transplant and age of recipient. In a mixed population (adults and children) of 532 HSCT recipients, those who received allogeneic stem cells were more likely to have a ADV positive culture compared to group who received autologous stem cells (16% vs 3%, p <0.0001). And paediatric recipients of partially matched related donors were at significantly greater risk of infection compared to their adult counterparts (p <0.001) (Howard et al., 1999). Similar findings have been reported – in a group of 572 transplanted patients the incidence of ADV infection was higher in children than adults (21% vs 9%, p<0.001); and in unrelated donor vs matched sibling donor transplants (26% vs 9%, p<0.001) (Baldwin et al., 2000). In Kampmann's group of 153 children, none of the children transplanted from a matched sibling donor developed adenoviraemia (p=0.05) (Kampmann et al., 2005). Detection of ADV infection at multiple sites has also been correlated with increased risk for invasive disease in children (Baldwin et al., 2000; Howard et al., 1999; Lion et al., 2003) but not in adults (Chakrabarti et al., 2002).

However, the most significant predictor of adenovirus infection identified in the majority of studies was lympopaenia, with all patients who developed adenovirus disease or with persistent adenoviraemia having an absolute lymphocyte count (ALC) of less than 300/μL (Chakrabarti et al., 2002; Kampmann et al., 2005). In patients with established adenoviraemia, an increase in lymphocyte counts correlated with clearance of infection and survival of the host whereas those who died of adenoviraemia had continuously increasing ADV DNA loads in plasma with no lymphocyte recovery (Heemskerk et al., 2005). To further

illustrate the importance of immune reconstitution in clearance of adenoviraemia, 46 children post HSCT were prospectively studied. Children who died (7/21) of ADV infection had no adenovirus-specific T cells and had significantly reduced T-cell reconstitution, although absolute lymphocyte count was above $0.3x10^9$/L at 30 days post-transplant. 93% of patients who successfully cleared ADV infection had presence of virus specific T cells, compared to 54% of children without any ADV infection. They also had good T-cell reconstitution, especially CD8+ T cells (>$0.4x10^9$/L) at 60 days post-transplant (Feuchtinger et al., 2005a). In the current era of prospective monitoring, Hiwarkar and colleagues reported the impact of ADV reactivation in 291 paediatric HSCT procedures and again found reduced CD4 counts of less than $0.15x10^9$/L in the first 3 months after transplantation as a significant risk factor for developing adenoviraemia (Hiwarkar et al., 2012).

Overall mortality from ADV infection after HSCT ranges from 6%-60% in studies of mixed populations with adults and children (Flomenberg et al., 1994; Hierholzer, 1992; Howard et al., 1999; Runde et al., 2001); and between 19% to 83% amongst paediatric patients (Hale et al., 1999; Kampmann et al., 2005; Munoz et al., 1998). There is significant association of ADV positivity in peripheral blood with transplant related mortality (relative risk, 5.8; P <0.001). The median days adenoviral DNA was detected in peripheral blood before death was 29 days in Lions' study; and Kampmann reports a median of 21 days post-transplant before adenoviraemia was evident (Kampmann et al., 2005; Lion et al., 2003). In a prospective study that identified 21 paediatric HSCT patients

with ADV infection, 90% of infections occurred during the first 3 months post-transplant, with more than 50% of patients having ADV infection within 30 days post HSCT (Feuchtinger et al., 2005a). Most prevalent group of Adenovirus identified had been subgroup C (Chakrabarti et al., 2002; Kampmann et al., 2005; Lion et al., 2003), and subtypes 2, 5, 1, 6, 31, and 4 (in decreasing frequency) are the most prevalent (Feuchtinger et al., 2005b).

1.2.3 Diagnosis and Monitoring

The American Society of Transplantation 2013 guidelines define asymptomatic ADV infection as detection of ADV from stool, blood, urine, or upper airway specimens in the absence of signs and symptoms. ADV disease is defined as presence of attributable organ signs and symptoms combined with virus detection in biopsy specimens. Disseminated ADV disease requires involvement of two or more organs, not including viraemia (Florescu et al., 2013).

Historically the standard method to detect adenovirus in clinical samples was by cellular viral culture (Walls et al., 2003). Samples were inoculated into a range of different cell lines including A549, human foreskin fibroblast, Hep 2, monkey kidney (RhMK) and human embryo kidney (HEK) cell lines, and cultured for several weeks. Cultures were observed for evidence of characteristic adenovirus cytopathic effect (rounding and clustering of infected cells) generally seen by the 10^{th} day, but this can range from 3-21 days (Baldwin et al., 2000; Flomenberg et al., 1994; Hale et al., 1999; Howard et al., 1999; Munoz et al., 1998; Walls et al., 2003). Positive cultures were confirmed by indirect immunofluorescence

antibody assays and serotyping performed by neutralisation tests with hyperimmune type-specific animal antisera (Baldwin et al., 2000; Hierholzer, 1992). Virus can also be identified in stool and urine samples by electron microscopy which shows the typical adenoviral particles of 70-80 nm diameter within the nuclei and cytoplasm of tubular epithelial cells (Baldwin et al., 2000; Chakrabarti et al., 2000) and in respiratory secretions by immunofluorescence and ELISA (Walls et al., 2003). Histopathology remains the gold standard method to diagnose invasive disease. The presence of ADV in biopsy samples is suspected by the identification of “smudge cells,” which are cells with large basophilic nuclei surrounded by a thin rim of cytoplasm, and confirmed through immunoperoxidase and/or in situ hybridization staining (Echavarria, 2008; Sandkovsky et al., 2014).

However, culture method is impractical from the clinical viewpoint due to its labor intensiveness and possibility of longer turnaround times and potential bacterial contamination in non-sterile sites (Sandkovsky et al., 2014). More recently, using qualitative PCR method, it has been shown that detection of adenoviral DNA in serum preceded the development of severe or fatal adenovirus infection in 14 recipients of haematopoietic stem-cell grafts (Echavarria et al., 2001). The sensitivity of PCR is very high, usually detecting quantities as low as 100 copies/mL, regardless of the ADV serotype (Sandkovsky et al., 2014). Since then, the development of real-time quantitative PCR assays has allowed for accurate detection of ADV in a variety of tissues, including blood, stool, and urine (Walls et al., 2003); and allows for prospective

monitoring of adenoviraemia in the post-transplantation setting. Several studies have described a correlation between high viral load and a fatal outcome (Erard et al., 2007; Schilham et al., 2002), as well as between onset of ADV-related disease and mortality. For example, in a study of 132 consecutive paediatric patients undergoing SCT, adenoviral DNA was detected in peripheral blood at a median of 29 days before death and in those who developed disseminated ADV disease, virus was detected in blood by a median of more than 3 weeks before onset of clinical symptoms. 82% of patients who had detectable ADV in peripheral blood died from infectious complications (Lion et al., 2003). These earlier studies all suggest that high viral load precedes symptoms of disseminated disease, therefore prospective monitoring of ADV load is now implemented in many of the paediatric transplant centers.

1.2.4 Current Management of Adenoviral Infections in Immunocompromised Children

1.2.4.1 Antiviral drugs

1.2.4.1.1 Cidofovir (CDV*)*

Cidofovir is an acyclic nucleoside phosphate derivative of cytosine which is converted to an active intracellular metabolite, cidofovir diphosphate, by cellular kinases (Legrand et al., 2001; Sellar and Peggs, 2012). The active intracellular diphosphate form of the drug exerts its mechanism of action as both a competitive inhibitor and an alternative substrate for 2'-deoxycytidine 5'-triphosphate in the viral DNA polymerase reaction (Hoffman et al., 2001), thus inhibiting viral replication. Antiviral selectivity results from the higher affinity for

the viral DNA polymerase compared to cellular DNA polymerases (Lenaerts et al., 2008). Cidofovir-resistant Ad mutants, discovered through serial passages *in vitro*, have been found to contain sequence changes in a conserved region of the viral DNA polymerase that is involved in nucleotide binding (Kinchington et al., 2002).

Several studies have reported on the success of CDV in the treatment of ADV infection in immunocompromised hosts after HSCT (Hoffman et al., 2001; Legrand et al., 2001), especially when given early (Bordigoni et al., 2001; Ljungman et al., 2003c; Neofytos et al., 2007; Yusuf et al., 2006) and combined with withdrawal of immunosuppression (Kampmann et al., 2005). At our center, if blood ADV reaches > 1000 copies per mL on two consecutive occasions CDV is started at 5mg/kg once every week for 2 weeks, followed by maintenance dose of 5 mg/kg once every fortnight. Notable side-effects of Cidofovir include nephrotoxicity, especially when used in combination with other nephrotoxic drugs such as cyclosporine or tacrolimus (Ljungman, 2004). CDV is a dianion that is taken up into the proximal renal tubular cells by an organic anion transporter at the antiluminal membrane. Once taken up into the cells, a slow diffusion rate into the tubule lumen as well as CDV's long intracellular half-life, can lead to toxic intracellular accumulation and subsequent tubular necrosis. Toxicity can be reduced by concomitant use of oral probenecid and intravenous hyperhydration (Sellar and Peggs, 2012). Probenecid competes for the kidney anion transporter, and along with hyperhydration can help protect tubular cells by decreasing plasma clearance rate of CDV

(Lacy et al., 1998; Lindemans et al., 2010). Two studies examined the use of CDV as therapy for ADV and CMV infection in 126 stem cell transplant patients combined (Ljungman et al., 2001; Ljungman et al., 2003b). The risk of renal toxicity in both studies was 26% and most of the renal toxicity was mild (low-degree proteinuria or mild elevation of serum creatinine), but approximately half had remaining signs of renal impairment after discontinuation of CDV (Ljungman et al., 2003c). It is recognized that ADV clearance requires T-cell immunity. The impact of CDV in the absence of T cell reconstitution post-transplant was investigated in a group of 36 paediatric allogeneic HSCT recipients. ADV viraemia was controlled in 75% of Cidofovir treatments, but the role of concurrent NK cell reconstitution could not be ruled out. In 17% (7 of 42 CDV treatments), CDV treatment could not prevent the progression of ADV viraemia, all in the absence of T cell reconstitution or during high-dose steroid treatment (Lugthart et al., 2015). These findings suggest that whilst CDV can help stabilise viral load, T cell reconstitution remains essential for viral clearance.

1.2.4.1.2 Ribavirin/Ganciclovir/Immunoglobulin

Ribavirin is a nucleoside analogue for which in vitro anti-ADV activity has been reported but it differs against different subtypes. It is active on most ADV isolates from species A, B and D, and in all isolates from species C (Morfin et al., 2009). There is anecdotal evidence of successful treatment of ADV in immunocompromised patients but larger studies have not been as supportive (Bordigoni et al., 2001; Lankester et al., 2004) (reviewed in (Ljungman, 2004).

There is no provable role for ganciclovir or immunoglobulin therapy in immunocompromised patients (Legrand et al., 2001; Ljungman, 2004).

1.2.4.1.3 Brincidofovir

More recently a new oral therapy has been trialed for the treatment of Adenovirus infections in immunocompromised patients. CMX001 (hexadecyloxypropyl cidofovir, Brincidofovir) is an orally bioavailable lipid conjugate of cidofovir with good oral bioavailability. It can achieve higher intracellular levels of active drug compared with cidofovir due to rapid transport across target cell plasma membranes, and has 5 to >2,500-fold greater potency against different ADV serotypes (Painter et al., 2012; Sandkovsky et al., 2014). It may also have a better safety profile relative to cidofovir as it is not associated with myelosuppression or nephrotoxicity. The most common side effect is diarrhoea, especially at doses of 200 mg weekly or biweekly (Marty et al., 2013) (Florescu et al., 2012). Of 13 immunocompromised patients who received CMX001, nearly two-thirds had a ≥ 10-fold drop in viral load after 1 week of therapy (Florescu et al., 2012). A Phase III, multicenter trial evaluating safety and efficacy of CMX001 for the treatment of disseminated ADV infection is currently underway (CMX001-304, AdVise Study; NCT02087306). Preliminary results from the study are available on 26 subjects, 20 of whom had allo HSCT (16 had disseminated ADV disease) (Grimley et al., 2014). Dosing schedule was BCV 100mg for ≥50kg or 2mg/kg for <50kg twice a week. Median viral load in plasma at baseline was 4.8 $\log_{10}$ copies/mL and median duration of treatment was 54 days. Suppression of plasma ADV viral load to < level of detection by

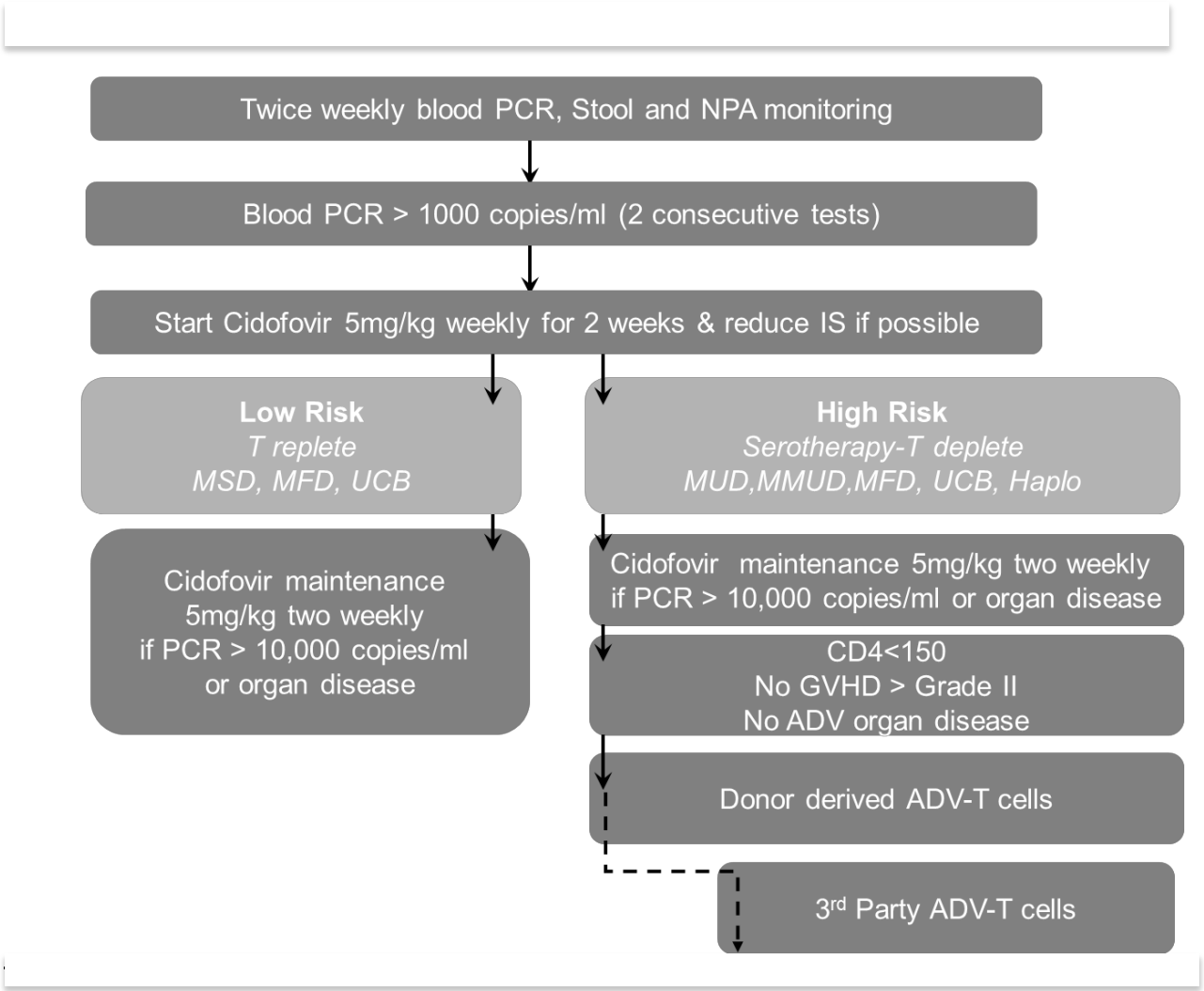
quantitative PCR was 61% (14/23) at any time on treatment and 52% (12/23) at last on-treatment value. Two subjects had severe diarrhoea requiring early cessation of treatment. The overall mortality rate was < 40% across subjects with disseminated and limited ADV and across populations with varying identified risk factors (HCT, Solid organ transplant) which is lower than those reported with disseminated disease.

1.2.4.2. Withdrawal of Immunosuppression

Immunosuppression is required post-HSCT for the prevention of Graft-versus-Host-Disease (GvHD). However the trade-off is viral reactivation/infection owing to delayed reconstitution of immunity. Preemptive reduction or withdrawal of immunosuppressive therapy at first detection of adenovirus in this setting has been reported to be successful. In a group of 76 adult allograft recipients, 15 developed adenovirus disease/infection. 12 patients had immunosuppression withdrawn or reduced and only 3 developed adenovirus disease; whereas all 3 patients in whom immunosuppression had to be continued succumbed to adenovirus disease (Chakrabarti et al., 2002).

Similarly in paediatric transplant recipients withdrawal of immunosuppression together with early antiviral therapy led to the resolution of adenoviraemia in 19/26 (86%) patients (Kampmann et al., 2005). Hence in the post-transplant setting we would recommend the following algorithm for the treatment of ADV (see figure 1.2).

Figure 1.2 Algorithm for the management of ADV reactivation in children after allogeneic stem cell transplantation



1.3 VIRAL INFECTIONS POST ALLOGENEIC STEM CELL TRANSPLANT AND TREATMENT WITH ADOPTIVE CELLULAR THERAPY

1.3.1 Viral infections post allogeneic stem cell transplant

Viral infections continue to be a major cause of morbidity and mortality in immunocompromised patients, particularly after allogeneic haematopoietic stem cell transplantation. Reactivations of adenovirus, along with herpes viruses such as human cytomegalovirus (CMV), Epstein-Barr virus (EBV) are amongst the most challenging complications; and viral clearance requires cellular immune reconstitution. In a 5-year period at a large Paediatric transplant center in London, the incidence of viraemia in 291 transplant procedures was 16% for CMV, 15% for ADV and 11% for EBV. The median time to onset of ADV and CMV reactivation was 15 and 20 days, respectively. Median time to EBV reactivation was 74 days after transplant (Hiwarkar et al., 2013). Although antiviral pharmacological agents are available, their use is costly, associated with side-effects and emergence of drug-resistant mutants. Therefore adoptive immunotherapy using both unmanipulated T cells and virus-specific T cells has been evaluated as an attractive approach to reconstitute antiviral immunity.

1.3.2 Adoptive Cellular Therapy

The use of adoptive cellular therapy where T-cells with specificity for diseased cells, virus or tumor can be engineered for the purposes of the targeted therapy of human viral and malignant diseases was first described in 1988. Autologous tumor-infiltrating lymphocytes (TIL) were administered to patients with metastatic

melanoma with 34% clinical response rate (Rosenberg et al., 1988). The process of adoptive cellular therapy includes the isolation, with or without ex vivo activation and proliferation of antigen-specific T cells, and reinfusion into the recipient. There are broadly three main therapeutic strategies in the use of adoptive cellular therapy in viral infections post HSCT: administration of cells before active viral infection is detected to prevent its occurrence (prophylaxis), targeted therapy in groups with highest risk of viral disease based on rapid surveillance PCR-based methods to diagnose viraemia (pre-emptive), or treatment of established disease.

Over the last decade, advances have been made in the field of adoptive cellular therapy for the treatment of viral reactivations/infection post HSCT; from proof-of-concept studies to adoption in clinical safety trials. The next section will examine the progress made so far in cellular therapy for EBV, CMV and ADV infections post HSCT.

1.3.3 Unmanipulated donor lymphocytes

Unmanipulated donor lymphocyte infusions (DLI) contain virus-specific T cells with particularly high frequencies for latent viruses such as CMV and EBV (Gillespie et al., 2000; Papadopoulos et al., 1994; Walter et al., 1995). Five patients with EBV-associated lymphoproliferative disorders (LPD) after T-cell depleted allogeneic bone marrow transplant were treated with infusion of unirradiated donor leukocytes between 0.12 to $1x10^6$ CD3+ T cells/kg body weight. All recipients received cells from HLA-matched unrelated donor and did

not receive GvHD prophylaxis except for T-cell depletion. Complete pathological/clinical response was documented in all treated patients with clinical remissions achieved within 14 to 30 days after infusion. Two patients died but not directly attributed to infusion. Of the 3 who survived and in whom the EBV-associated LPD resolved, 2 had acute Grade II skin GvHD and mild chronic GvHD of skin that responded to topical corticosteroids (Papadopoulos et al., 1994). This showed that transferring unmanipulated lymphocytes from EBV-seropositive donors can restore immune response to EBV and eradicate Post Transplant Lymphoproliferative Disease (PTLD). However there is a significant risk of GvHD due to the high frequency of alloreactive cells (Heslop et al., 1994). Similarly, Hromas and colleagues reported the first successful treatment of adenovirus infection with DLI in a patient who developed severe ADV-associated haemorrhagic cystitis after a T cell-depleted graft, and failed to respond to anti-viral drugs or immunoglobulin. After infusion of $1x10^6$/kg CD3+ cells on day +61 the patient improved over a period of 5 weeks without developing GvHD (Hromas et al., 1994). These pioneering studies supported the rationale for the adoptive transfer of virus-specific T cells (VSTs).

1.3.4 T-cell therapy for EBV

1.3.4.1 EBV infection and PTLD

Epstein-Barr virus (EBV) is an enveloped herpes virus that infects more than 95% of the world's population. It enters its human host via a mucosal route, infects and replicates in oropharyngeal epithelial cells and then infects

lymphocytes in the oropharynx (Bollard et al., 2012). Primary infection in the immunocompetent host results in a mild self-limiting illness, but the virus persists lifelong as a latent infection in B cells and spreads in the community by productive replication in B cells and mucosal epithelium (Fujita et al., 2008). Infected B cells do not produce infectious virus, but instead express viral latency-associated proteins that induce B-cell proliferation and then drive the infected B ell into 'true latency', in which no viral genes are expressed (Bollard et al., 2012). There are four EBV latency states in B cells, three of which are found in Post-transplant lymphoproliferative Disease (PTLD) and each is associated with a different type of virus-gene expression, different levels of immunogenicity, and different types of lymphoma (Bollard et al., 2012; Thorley-Lawson and Gross, 2004). B cells expressing type 3 latency express all the 9 latency-associated EBV proteins, including the immunodominant EBNA3 viral antigens. These T cells express cell adhesion and costimulatory molecules rendering them immunogenic and susceptible to immune-mediated killing by EBV-specific cytotoxic T lymphocytes (CTLs) (Bollard, 2013; Rickinson and Moss, 1997). After HSCT, the lack of functional CTLs can mean the type 3 latency EBV-infected B cells can proliferate unchecked and cause a lymphoproliferative disorder that can progress to lymphoma with increased levels of EBV DNA detected by PCR (Bollard, 2013; Bollard et al., 2004a). PTLD developing after HSCT usually results from donor B cells and appears within the first 6-12 months of the post-transplant period, before immune-reconstitution of EBV-specific CTL takes place (Bollard et al., 2012). EBV-PTLD represents a heterogeneous group of EBV-related diseases characterized by: (i) populations

of monoclonal or oligoclonal cell proliferation defined by cellular markers; (ii) evidence of EBV infection by detecting specific DNA, RNA, or proteins; and (iii) disruption of the underlying cellular architecture by a lymphoproliferative process (Bordon et al., 2012). The overall frequency of PTLD post HSCT is approximately 1%, but varies from 0.45% after HSCT from matched family donors to 11.7-29% after HSCT from T-cell-depleted unrelated donors (Patriarca et al., 2013). Risk factors for the development of PTLD include reduced intensity conditioning in combination with *in vivo* or *ex vivo* T-cell depletion [including use of anti-thymocyte globulin (ATG)], mismatches in HLA and serological EBV status between donor and recipient, degree and duration of immunosuppressive treatment after the transplant (Bollard et al., 2012; Uhlin et al., 2014); and low CD4+ lymphocyte count at day +30 after transplantation (Hiwarkar et al., 2013; Patriarca et al., 2013). After HSCT, PTLD may present with a clinical picture similar to infectious mononucleosis with B symptoms (fevers, sweats, anorexia) and tonsilar enlargement and cervical lymphadenopathy. In highly immunosuppressed patients, the clinical presentation may also be more fulminant with diffuse multiorgan involvement and a clinical picture that is similar to sepsis or GvHD (Bollard et al., 2012).

Initial treatment for PTLD comprises reduction of immunosuppression and rituximab, with or without chemotherapy (Heslop, 2009; Messahel et al., 2006). Treatment by reduction of immunosuppression may be limited by the onset of graft rejection and development of GvHD. The introduction of the monoclonal anti-CD20 antibody rituximab has decreased the PTLD mortality rate

substantially. It has proven effective both as pre-emptive therapy in patients with high EBV viral loads and as treatment for patients with established PTLD, with response rates of 55-100% in both scenarios. However PTLD often recurs because cellular immunity against EBV has not been restored (Bollard et al., 2012; Messahel et al., 2006; Uhlin et al., 2014). Uhlin and colleagues (Uhlin et al., 2014) described clinical outcome of cohort of 1021 patients who underwent HSCT at Karolinska University Hospital in Stockholm between 1996 and 2011 in regards to EBV-related PTLD, with the aim to report on long-term follow-up of patients after the initial viral clearance. The overall frequency of PTLD in the cohort was 4% and only 28% of patients with PTLD were alive 1 year after diagnosis. Thirty-five patients (88%) were treated with rituximab according to standard protocol. The authors conclude that even if rituximab initially resolves the PTLD in most cases, a considerable proportion of patients will eventually succumb from other causes.

1.3.4.2 EBV CTLs

For this reason, alternative strategies to restore EBV-specific immunity have been explored, namely by infusing T cells that target EBV antigens. Because many PTLDs express type III latency, it provides a good model for testing T-cell-based therapies designed to target EBV antigens that are presented on the cell surface by major histocompatibility molecules (Bollard et al., 2012). Also the risk of PTLD correlates inversely with anti-EBV T cell activity in patients, especially in children who have not previously encountered the virus (Abu-Elmagd et al., 2009).

As mentioned previously, unmanipulated donor lymphocyte infusions after bone marrow transplantation have been shown to be effective against PTLD (Papadopoulos et al., 1994). More recently, Doubrovina and colleagues (Doubrovina et al., 2012) reported on a single-center experience with a cohort of 49 patients who were treated with unmanipulated Donor Lymphocyte Infusions (DLIs), EBV-CTL, or both for biopsy proven EBV-LPD emerging after HSCT. 73% of the patient series treated with DLIs and 68% of those treated with EBV-CTLs achieved a sustained CR. However infusing unmanipulated DLIs is associated with an increased incidence of severe or fatal GvHD, due to the presence of alloreactive T cells in the infused cell product (Bollard et al., 2012). Therefore similar to the development of ADV and CMV CTLs, different strategies have been employed to enhance effectiveness of CTLs including collecting donor lymphocytes responding to EBV by apheresis and selecting by cytokine secretion and infusion within days of diagnosis into patients (Moosmann et al., 2010; Uhlin et al., 2012),and developing bank of EBV-specific CTLs from third party donors for recipients with seronegative donors or naïve donors such as in cord blood transplants (Barker et al., 2010; Doubrovina et al., 2012; Vickers et al., 2014).

Heslop and colleagues (Heslop et al., 2010) completed studies in 114 patients who had received infusions of EBV-specific CTLs to prevent or treat EBV+ LPD post HSCT at 3 different centers. Donor T-cell lines directed against EBV antigens were selectively expanded using EBV-LCLs (lymphoblastoid cell lines) generated according to Good Manufacturing Practice. None of the 101 patients

who received CTL infusions as prophylaxis (resulting in up to a 4-log expansion of infused CTLs) developed EBV+ LPD. Thirteen patients were treated with CTLs for biopsy-proven or probable LPD, 11 of whom achieved sustained complete remissions without recurrence. One patient died very early after treatment of progressive disease and a second patient did not respond because the tumor had a deletion of the two epitopes in the EBNA3B recognized by the infused line ((Bollard et al., 2012). Importantly, none of the patients developed *de novo* GvHD after CTL infusion. However this method of production is lengthy and costly due to time needed to generate donor-derived LCLs and the repeated restimulation of donor T cells. The complete production period requires 2 to 3 months of processing time. This precludes the preparation and use of such T cells after PTLD has become manifest. Therefore, more rapid methods to prepare EBV-specific T cells are required to make EBV-specific T-cell therapy more widely applicable.

Moosmann and colleagues (Moosmann et al., 2010) described a method for rapid isolation of clinical-grade EBV-specific T cells from donor blood cells in less than 36 hours by stimulation with a pool of 23 class I and II EBV peptide epitopes derived from 11 EBV antigens, followed by isolation of responding cells by antibody-mediated surface IFN-γ capture and immunomagnetic separation. Six patients with PTLD – three of whom had early stage disease – who received an average dose of $4x10^6$ selected T cells had complete responses and rapid reconstitution of EBV-specific immunity; whereas three patients with more advanced disease did not respond to the treatment. However a note of caution

is that all patients had received additional treatment in addition to EBV-specific CTLs (reduction in immunosuppression, Rituximab and Cidofovir). Similarly Icheva and colleagues (Icheva et al., 2013) described a rapid protocol (30 hours) for isolation of polyclonal EBV nuclear antigen 1 (EBNA-1) –specific T cells by using an IFN-γ capture technique. EBNA-1 protein is involved in the replication of viral episomes, is crucial for the persistence of EBV infection and is the only viral protein required for replication of EBV in its latent form. It is universally expressed in EBV+ PTLD and contains immunodominant T-cell epitopes that induce CD4+ and CD8+ T-cell responses in healthy population (Jones et al., 2010). In the study recombinant EBNA-1 protein was used as stimulant for the earlier enrolled patients and changed to the GMP-grade EBNA-1 Peptivator (overlapping peptides) for the latter patients once became commercially available. Enrichment of cytokine-secreting cells was performed using the cytokine secretion system and the CliniMACS device for immunomagnetic separation (Miltenyi Biotec). Cells were infused directly or cryopreserved without further in vitro expansion. Ten patients with chemorefractory EBV viraemia or EBV+ PTLD were treated with infusion of EBNA-1 specific T cells at mean dose of 5,794 CD3+ cells per kilogram of body weight. One patient developed transient grade 1 to 2 acute skin GvHD 15 days after first donor lymphocyte infusion which responded well to treatment and resolved within 3 to 4 weeks. Eight of 10 patients showed in vivo expansion of EBNA-1-specific T cells detected by intracellular cytokine staining by flow cytometry after stimulation with EBNA-1 antigen. This was associated with a clinical and virological response in 7 of the 8 responders (decrease of viral load

more than 1 log and resolution of PTLD). One patient showed T-cell expansion 6 days after transfer but succumbed as a result of haemophagocytic lymphohistiocytosis and multiorgan failure 11 days after transfer. Overall 2 of 10 patients had died of EBV-related disease, and another patient had ongoing viraemia without clinical symptoms at last follow-up.

Another alternative to increase availability of EBV-CTLs to a larger number of patients (for example, those with EBV-seronegative donors) is the creation of a bank of characterized HLA-typed EBV-specific T cell lines. Haque and colleagues (Haque et al., 2007) first described this approach of generating EBV-specific CTLs from EBV-seropositive blood donor to treat patients with EBV PTLD on the basis of the best HLA match and specific in vitro cytotoxicity. The group reported data from a phase 2 multicenter trial enrolling 33 patients with EBV-positive PTLD from 19 transplant centers who had not responded to conventional therapy and were given EBV-specific CTLs generated from unrelated third-party blood donors. No adverse events were observed from CTL infusions, and response rate (complete or partial) was observed at 64% at 5 weeks and 52% at 6 months. Sixteen patients had no response at 6 months, 5 died before completing treatment. The best results were observed in patients receiving CTLs that were best-HLA matched with the recipient. More recently, Vickers and colleagues (Vickers et al., 2014) also described the establishment of a GMP-compliant allogeneic EBV-specific cytotoxic cell bank from peripheral blood mononuclear cell (PBMC) donations supplied by New Zealand Blood Service (in order to minimize risk of Creutzfeldt-Jacob disease transmission).

Donors are HLA-typed at HLA Class I and Class II (DRB1, DQB1) loci. EBV CTLs are generated by stimulating mononuclear cells with purifed EBV and LCLs and expanded over approximately 2 months. Cells are then cryopreserved in 10% DMSO in bags of $50x10^6$ or $150x10^6$ cells using a controlled rate freezer, before being placed in nitrogen vapour-phase storage at <-150°C. These cells are supplied as an Advanced Therapy Medicinal Product (ATMP) under MHRA 'Specials' Licence. CTLs are infused at doses of $1\text{-}2x10^6$/kg body weight as a course of four infusions given at weekly intervals. Over a 2 year period, CTLs were issued and infused into 11 patients; all had PTLD except for 1 patient with leiomyosarcoma. Overall outcome was good with 8/10 achieving complete remission and only two dying of refractory PTLD. The minimum number of antigens shared was 3/10 and the maximum 9/10. Poor HLA class II matching appears to have contributed to non-clinical response in 2 patients. Overall CTLs were well-tolerated with only one case of likely skin GvHD observed in the 2 days after one of the infusions (Vickers et al., 2014).

1.3.4.3 Solid organ transplant

In the setting of solid organ transplantation, the use of EBV CTLs had been less effective due to the requirement for long-term immune suppression. In order to increase their persistence in vivo, EBV-CTLs have been genetically engineered to render them resistant to calcineurin (CN) inhibitor FK506 through retroviral transfer of a calcineurin A mutant (CNA12) (Ricciardelli et al., 2014). Xenogeneic mice bearing human B-cell lymphoma were injected with autologous CTLs transduced with either CNA12 or eGFP in the

presence/absence of FK506. Adoptive transfer of autologous CNA12-CTLs induced dramatic lymphoma regression despite the presence of FK506, compared to eGFP-CTLs. They also persisted longer, homed to the tumor, and expanded more than eGFP-CTLs in mice treated with FK506; with longer survival in mice receiving CNA12-CTLs and treated with FK506. This novel approach proves to be promising but application in the clinical setting is awaited.

1.3.5 T-cell therapy for CMV

Human cytomegalovirus (CMV) is a member of the β-herpesvirus family and is latent in approximately 70% of the adult population (Ciaurriz et al., 2015). In immunocompetent individuals, life-long asymptomatic latency is established following primary infection. However historically before preemptive treatment was in place, untreated CMV infection and disease in the immunocompromised host had been associated with significant morbidity in the early days after HSCT and led to mortality in nearly 25% of seropositive patients (Boeckh et al., 2003). In patients following allo-HSCT, several risk factors predispose to increased incidence of CMV infection. Positive CMV serostatus of the recipient is a poor prognostic factor, especially in recipients of T cell-depleted marrow or stem cells (Ljungman et al., 2003a). Other risk factors include mismatched donors, immunosuppressive regimens, and use of nonmyeloablative conditioning regimens with addition of alemtuzumab treatment. The most frequently used drugs for prophylactic or preemptive treatment for CMV are ganciclovir, valganciclovir and foscarnet. Although these drugs have been successful in

reducing mortality associated with CMV infection (Bollard et al., 2004b), they have significant side effects with neutropaenia and nephrotoxicity and have been found to impair and delay the development of CMV-specific CTLs (Ciaurriz et al., 2015).

1.3.5.1 CMV-specific T cell clones

As mentioned earlier transfer of unselected donor lymphocytes for treatment of EBV LPD resulted in GvHD in recipients (Papadopoulos et al., 1994). Subsequent approach evaluated use of T-cell clones with specificity for the antigens of the pathogen being treated. Walter and colleagues isolated clones of CMV-specific CD8+ cytotoxic T lymphocytes from blood of sibling donors donating bone marrow by stimulating with autologous fibroblasts pulsed with CMV. Escalating doses of CMV clones (from $3.3x10^6$/kg to 1 $x10^9$/kg) were infused prophylactically into 14 recipients beginning 30 to 40 days after marrow transplantation. The predominant specificity of the clones is directed against CMV structural proteins, such as the matrix proteins pp65 and pp150, which are presented for recognition by cytotoxic T lymphocytes before new virions are formed in infected cells. None of the 14 recipients experienced toxic effects related to the infusion, with 11 patients showing significantly increased cytotoxic activity against CMV in *in vitro* measurements. Two patients had analysis of rearranged T-cell receptor genes in T cells showing persistence of transferred clones for at least 12 weeks. However, anti-CMV activity declined in those deficient in CD4+ CMV-specific T cells, suggesting that helper T cell is needed for long-term persistence. None of the 14 patients developed CMV viraemia or

disease (Walter et al., 1995). Although this study demonstrated proof of principle in terms of measures of immune reconstitution, the patient group was at relatively low risk for CMV-related disease. All patients had undergone T-cell-replete HLA-matched sibling transplants and majority were CMV seronegative. Also the expansion of T-cell clones to achieve adequate dose levels was extremely time-consuming and was therefore limiting in terms of costs and logistics (Peggs, 2009).

1.3.5.2 CMV-specific T cell lines

In another prophylactic study, Peggs and colleagues (Peggs et al., 2003) produced and characterized polyclonal CMV-specific CD4+ and CD8+ T cells by stimulating reactive T cells 2 or 3 times with dendritic cells (DCs) pulsed with CMV antigens derived from a CMV-infected human lung fibroblast cell line to stimulate reactive T cells. The use of the entire CMV antigen therefore enables a broad CTL response to be generated, as opposed to a solely pp65-specific response, for example (Bollard et al., 2004b). Patients were monitored for CMV reactivation and 16 patients were infused with CMV-specific CTL ($1x10^5$ CTL/kg) at the of first PCR reactivity (at a median of 36 days post-transplant). There were no infusion-related toxicities and study showed small doses of CTLs were able to reconstitute immunity with considerable *in vivo* expansion of CMV-specific CTLs, and with no requirement for further antiviral drugs in eight cases (Peggs et al., 2003).

Similarly Einsele and colleagues investigated the therapeutic application of adoptively transferring CMV-specific CTLs to patients with persisting or recurring CMV infection despite the prolonged use of antiviral medications. Polyclonal CMV-specific CTLs were generated by pulsing donor-derived PBMCs with CMV lysate and expanded by using autologous irradiated feeder cells, CMV lysate, and interleukin 2. Eight patients received $1x10^7$ T cells/m^2 after a CD34-selected HSCT from a family donor. Infusions of CTLs were effective in reducing viral load in 7 evaluable patients and 5 patients had sustained antiviral effect (Einsele et al., 2002). This production method avoids the use of DCs and relies on monocytes infected with the CMV lysate for antigen presentation. However the use of infectious CMV in the form of CMV lysate means this method is not suitable for clinical production (Bollard et al., 2004b).

To avoid the use of CMV lysate, monocyte-derived DCs can be pulsed with the immunodominant CD8+ HLA-A2 restricted epitope NLVPMVATV (NLV) derived from pp65. Using this method Micklethwaite and colleagues (Micklethwaite et al., 2007) treated 9 patients who had mostly undergone non-myeloablative HSCT without *in vitro* or *in vivo* T cell depletion with DC co-cultured NLV-specific CTLs. Six of the nine patients showed evidence of an increase in the frequency of specific T cells post infusion, but response did not persist beyond several days to weeks. Three patients developed GvHD within 14 days of T-cell therapy and one patient died of GvHD. However all patients had GvHD prior to cell infusion therefore it could not be concluded the CMV-specific T cells were causative of GvHD but may have caused exacerbation

(Micklethwaite et al., 2007). It was unclear why expansion of NLV-specific T cells were only modest and did not persist; but both exhaustion of antigen-specific T cells during *in vitro* expansion and lack of antigen-specific CD4+ T cell help could have contributed (Peggs, 2009).

1.3.5.3 Direct selection method

Many seropositive individuals have high frequencies of CMV-reactive T cells; making direct selection of CMV-specific T cells from donor samples an attractive option for CMV immunotherapy. This method eliminates the need for *ex vivo* manipulation and offers potential for more rapid production allowing therapeutic rather than prophylactic use of isolated CTL. Two strategies of direct selection have been investigated for production of CMV-specific CTLs. One is based on isolating a pure population of CMV peptide-specific CD8+ T cells directly from donor peripheral blood using staining with specific tetramers followed by selection with magnetic beads (Cobbold et al., 2005; Cwynarski et al., 2001). This method was explored and applied clinically in 2005 where HLA-peptide tetramers containing peptides derived from pp65 or CMV-IE-1 were used to purify CMV-specific CD8+ T cells followed by selection with magnetic beads. CMV-specific CD8+ T cells were infused directly into 9 HSCT recipients within 4h of selection. The median cell dose was $8.6x10^3$ /kg with high purity (median 95.6%). Seven of the patients received the CTLs following initial CMV virus reactivation while 2 patients were treated for persistent viraemia. CMV-specific CD8+ T cells were detectable in all patients within 10 days of infusion and 2

patients had long-term detectable CMV-specific response. Only 2 out of 7 patients treated pre-emptively required antiviral therapy; and 1 patient whose CMV reactivation was refractory to antiviral drugs achieved control within 8 days of T cell infusion. All patients had a reduction in the level of CMV viraemia and there was no significant increase in GvHD rates (Cobbold et al., 2005).

A variation on the HLA-multimer technology is the use of Streptamers® which allows reversible binding to the TCR and thus may have less effect on the T-cell phenotype. Labeling of Strept-Tactin, which functions as “backbone” for the streptamer complex, with fluorochromes or magnetic microparticles allows specific visualization and/or enrichment of antigen-specific CTLs in combination with FACS or MACS. The multimeric peptide MHC class I complexes, which use the binding of Strep-tag II to Strept-Tactin to form complexes, can be easily disassembled by the addition of d-biotin. Because of their low affinity, peptide-MHC class I monomers dissociate from their cognate TCR spontaneously within seconds, without altering T-cell function or activating T cells through cross-linking TCRs, therefore leaving ‘untouched’ T cells (Odendahl et al., 2014).

Another method of rapid, direct selection is based on induction of IFN-γ secretion in response to viral proteins or peptides and selection of responding cells. Using this method, Feuchtinger’s group (Feuchtinger et al., 2010) infused polyclonal CMV-specific T cells generated by stimulation with pp65 followed by isolation of IFN-γ producing cells into 18 patients after allo-SCT with CMV disease or antiviral refractory viraemia. The mean infused cell dose of $21x10^3$/kg resulted in clearance or reduction in viral load in 83%. In-vivo expansion of

CMV-specific T cells was demonstrated in 12 of 16 evaluable patients, without causing induction of GvHD.

Recently, a large Phase II study (Australian Clinical Trial Registry #ACTRN12605000213640 and #ACTRN12607000224426) prophylactically treated 50 allogeneic HSCT recipients with donor-derived CMV-specific CTLs and compared outcomes with a contemporaneous control group of transplant recipients (Blyth et al., 2013). CMV CTLs were initially generated by pulsing dendritic cells with an HLA-A*0201-restricted immunodominant peptide NLV from the CMV pp65 antigen. This method was restricted to donors who were HLA A0201-positive and produced CTLs with restricted specificity that may have been predisposed to escape mutants (Heslop, 2013). The investigators subsequently modified their manufacturing methods to use dendritic cells genetically modified by an adenoviral vector encoding the full pp65 antigen (ad5f35pp65; obtained from the Center for Cell and Gene Therapy, Houston, TX). This method allowed a broader immune response to be produced but is lengthy and requires live virus. Irradiated peptide pulsed or transfected mo-DC were used to stimulate donor T cells (obtained either from peripheral blood or stem cell harvest product) and cells were expanded in culture over 21 days. A single dose of 2×10^7 CMV CTLs/m^2 was infused into 50 eligible patients on or after day 28 post-transplant. Nine patients received a lower cell dose at median of 1.2×10^7 cells/m^2 due to insufficient cell expansion. All patients had undergone conditioned allogeneic HSCT for haematological malignancies, 26 of whom had

T-cell depletion. Twenty-six patients (52%) who received CTLs reactivated CMV (5 post CTL infusion). Nine patients required treatment with Ganciclovir or Foscarnet (1 post CTL infusion). There was 1 case of fatal CMV disease, attributable to high levels of ATG at time of T cell infusion. Although the investigators did not observe a significant difference in CMV reactivation rate between CTL recipients and controls, there was a difference in the requirement for CMV-directed antiviral therapy (17% vs 36%; *P* = .01). Notably there was no increase in acute or chronic GvHD in the treatment group.

In the UK a randomized controlled trial to evaluate safety of CMV-CTLs has recently been completed (CMV-IMPACT:NCT 01077908). The study enrolled CMV-seropositive patients > 16 years old, undergoing T-cell depleted (alemtuzumab) HSCT from a matched sibling CMV-seropositive donor, in 14 UK transplant centers. CMV-specific cells were manufactured by direct selection using either a *Strep*tamer™ (if donor expressed a suitable HLA allele) or gamma-capture technique (CliniMACS® *Cytokine Capture System,* IFN-gamma). Clinical outcome from this study is awaited.

1.3.5.4 CMV-specific TCR-transgenic T cells for immunotherapy

Some patients undergoing allo HSCT have CMV-negative donors and are at highest risk of reactivation of CMV. Adoptive therapy is difficult as naïve CMV-specific T cells are difficult to isolate from CMV-seronegative donors. One way to overcome this is by utilizing retroviral transduction to introduce a TCR with specificity for CMV into polyclonal T cells, thereby redirecting their specificity.

The generated CMV-specific T cell clones are of several HLA restrictions and can recognise the endogenously processed antigen pp65. In a pre-clinical study the genes of four TCRs were cloned and inserted into retroviral vectors to transduce primary T cells from CMV-negative donors. The transgenic T cells expanded *in vivo* in response to endogenously processed antigen and demonstrated increased antigen-specific cytotoxicity and cytokine secretion. These CMV-TCR-transgenic T cells are expected to be effective in controlling acute CMV disease and establishing antiviral memory (Nicholson and Peggs, 2015; Schub et al., 2009). The feasibility of generating donor-derived CMV TCR transduced T cells for treatment of CMV infection post HSCT is currently being investigated in a UK Phase I/II proof of concept pilot study (CMV TCR001; EudraCT 2008-006649-18).

1.3.6 T-cell therapy for ADV

Recovery from ADV infection requires cellular immune reconstitution after allogeneic HSCT. Adoptive immunotherapy using both unmanipulated T cells and virus-specific T cells has been evaluated as approaches to reconstitute antiviral immunity.

As mentioned earlier, Hromas and colleagues (Hromas et al., 1994) reported on the first successful treatment of a patient with severe ADV-associated haemorrhagic cystitis with DLI. However much like the development of CMV and

EBV-specific immunotherapy, strategies have evolved to produce more antigen-specific CTLs in the treatment of ADV infection post-transplant to avoid GvHD.

1.3.6.1 Generation of T cells against ADV

In order to increase antiviral efficacy and to reduce the risk of alloreactivity, techniques were developed to isolate only ADV specific T cells to be given to patients. In 1996, Smith and colleagues (Smith et al., 1996) used donor peripheral blood dendritic cells as antigen-presenting cells to manufacture cytotoxic T cells (CTLs) that recognise ADV. Dendritic cells (DCs) from donors were infected with either wild-type adenovirus serotype 5 (Ad) or Ad5 strain *dl312*, an Ad5 mutant with the E1A region deleted resulting in a virus defective in early and late viral gene transcription. The adenovirus-specific T cells were subsequently expanded using virion-pulsed irradiated DCs. The majority of the CTLs were CD4+ T cells and were directed against the input virion proteins. The group also demonstrated cross-reactivity of the adenovirus-specific CTLs, as CTLs generated against a subgroup C adenovirus recognised and killed cells infected with a subgroup B adenovirus (Smith et al., 1996). Although the ADV-specific CTLs reported by Smith et al were able to recognise ADV from different serologic groups, these CTLS were unable to kill target cells in a standard 4-6-hour assay (requiring 18 hours to kill) and could not be adequately expanded into CTL lines (Leen et al., 2004a)

In 2004 Leen and her group developed a protocol to reactivate ADV-specific memory T cells from donors' PBMCs using clinical grade ADV vector. PBMCs from 6 healthy ADV-seropositive volunteers were stimulated twice with autologous DCs transduced with Ad5f35 (replication-defective ADV vector). Both CD4+ and CD8+ ADV-specific T cells were expanded with autologous Ad5f35GFP-transduced lymphoblastoid cell lines (LCLs) and showed ADV specific killing (Leen et al., 2004a). Because the generation of DCs to act as stimulator cells requires a large volume of blood, a second protocol using Ad5f35GFP-transduced PBMCs as both stimulators and responders were used. Expansion was again carried out with Ad5f35GFP-transduced LCLs, the resultant expanded T cells had specific reactivity against both EBV and ADV. These CTL lines generated using Ad5f35 vector were able to recognise and kill autologous cells infected with wild-type adenovirus isolates from different serotypes and groups including Ad2, Ad4, Ad7 and Ad11 (Leen et al., 2004a).

In 2006 Leen et al reported on the prophylactic clinical use of tri-specific (EBV, CMV, ADV) CTLs on 11 adult and paediatric patients post haematopoietic stem cell transplant. Donor PBMCs transduced with a recombinant adenoviral vector encoding the CMV antigen pp65 (Ad5f35pp65) were used to reactivate CMV and ADV-specific T cells. Subsequent stimulation with EBV transformed LCLs transduced with the same vector reactivated EBV-specific T cells whilst maintaining the expansion of activated ADV and CMV-specific T cells (Figure 3c). Fourteen out of 15 CTL lines responded to all viruses (one individual responded to CMV and EBV but not ADV); and 15/15 donor CTL lines showed

cytolytic activity against all three viruses (Fujita et al., 2008; Leen et al., 2006). Eleven patients received from 5 x 10^6 to 1 x 10^8 cells/m^2 at 35 to 150 days after HSCT. CMV and EBV-specific CTLs consistently expanded in all individuals treated within 4 weeks of administration; whereas ADV-specific CTLs expanded only in those with active or recent infection. All patients with pre-infusion viral infection/reactivation had reduction in viral titer and resolution of disease symptoms, coinciding with expansion of virus-specific T-cells (Leen et al., 2006).

In order to increase the frequency of adenovirus-specific T cells within their CTL lines, Leen and her group removed competition from the immunodominant CMV antigen and manufactured bivirus-specific CTL lines directed only to EBV and adenovirus (Leen et al., 2009). 20 CTL lines were made of which 13 were administered to paediatric stem cell transplant recipients: 7 unrelated and 6 haploidentical transplants. The frequency of adenovirus hexon-specific T cells in the bivirus CTL was significantly higher than in the trivirus CTL study (median ADV cells 308 SFC/10^5 CTL [range 46-350] compared to 86 SFC/10^5 CTL [46-350] in bivirus product) (Leen et al., 2009; Leen et al., 2006). Each patient received from 5x10^6 to 1.35x10^8 cells/m^2 at 40 to 150 days after HSCT. There were no toxicities related to CTL therapy and no subject developed de novo GVHD after cell infusion. None of the 13 patients developed EBV-associated lymphoproliferative disease, and 2 of the subjects had resolution of their adenoviral disease (Leen et al., 2009).

Once it has been established that ADV-specific T cells can be expanded *in vitro* and that they are effective and protective *in vivo,* the next challenge was to overcome logistics of manufacturing these products. The protocol described above of activating donor PBMCs with autologous monocytes transduced with the Ad5f3pp65 vector followed on days 9, 16 and 23 by restimulation with Ad5f35pp65-transduced EBV-LCL takes in total 10-14 weeks. This implies that products have to be manufactured in advance if they were to be made immediately available for acutely ill patients; and comes with it cost implications. Different cell selection and culture practices were therefore explored to develop more rapid and cost-effective strategies for production of CTLs.

1.3.6.2 Cytokine based selection of antigen-specific T cells from donor peripheral blood mononuclear cells

In 2004 Feuchtinger's group in Germany (Feuchtinger et al., 2004) described a clinical-grade strategy to isolate and expand donor-derived human ADV-specific T lymphocytes using the Miltenyi Biotec (Bergisch Gladbach, Germany) interferon-γ (IFN-γ) secretion assay. PBMCs were isolated from suitable donors, 0.1-2x10^9 cells were stimulated with type C adenoviral antigen (BioWhittaker, Verviers, Belgium) for 16 hours. T cells with antigen-specific secretion of IFN-γ were detected on the following day and these cytokine-secreting cells were magnetically enriched using ClinicMACS device (Miltenyi Biotech). A mean number of 3.4x10^6 cells were obtained with a mean purity of 85% ADV-specific T cells. These isolated cells were then expanded *ex vivo* in a median of 18 days

(range 7-29 days) to greater than 10^8 total cells using IL-2 and autologous feeder cell stimulation. The generated T cells showed ADV-specific IFN-γ release and specific killing of ADV-infected B-LCL. Alloreactive proliferation of the generated lines in mixed lymphocyte cultures was significantly reduced when compared to unmanipulated PBMCs (Lang et al., 2004).

The above method of generating ADV-specific T cells was adopted clinically in 2006 by Feuchtinger's group for nine children with systemic ADV infection after HSCT for mainly leukaemia or lymphoma. These children underwent myeloablative conditioning regimen with T-cell depletion for HSCT with either matched or mismatched unrelated, or haploidentical donors. All eligible patients had ADV viraemia not controlled by antivirals and lacked ADV-specific T cells. T-cell transfer was performed if a sufficient ADV-specific T-cell response was detectable in the donor (>0.01% of T cells). The percentage of ADV specific T cells rose from 1.1% to 45.7% after stimulation with ADV antigen. A mean of $14x10^3$/kg (range 1.2-50 x 10^3/kg) T cells were infused at a median of +77 days post-transplant (range +40-+378). T-cell infusion was well tolerated in all nine patients, except for one case with aggravation of pre-existing skin GVHD that was seen at days 10-14. Five out of six evaluable patients had significant decrease of viral DNA in peripheral blood and stool with an *in vivo* expansion of specific T cells. Those without a specific T cell response post adoptive T-cell transfer had either increasing or unchanged viral DNA-load in peripheral blood. Three patients in whom follow-up were possible had sustained ADV-specific T-cell response detected 4-6 months after T-cell transfer. 3 patients died from

adenovirus-associated, pre-existing multi-organ failure; 1 patient died from relapsed NHL. Three out of 4 patients who died did not have specific T-cell response post-immunotherapy. Efficacy was independent of T-cell dose transferred, suggesting efficient *in vivo* expansion (Feuchtinger et al., 2006).

Chatziandreou and colleagues also reported on the successful isolation of ADV-specific T cells using a similar protocol. Again using the Miltenyi IFN-γ secretion and capture assay with adenovirus lysate derived from ADV SpC-infected human embryonic lung fibroblasts, ADV-specific T cells were isolated, expanded and restimulated over 2 weeks. The numbers of eluted virus-specific cells from six ADV-positive donors ranged from 1 to 7 x 10^5 cells, majority being CD4+ cells. After a 2–week culture period, a 1.5-2 log expansion was seen with cell numbers averaging at 1 x 10^7 cells. This would enable infusions of up to 10^5 ADV-specific cells/kg for most adults and larger amounts of paediatric patients. This approach offers the advantage of a short 14-day culture period, which allows for generation of cells in response to first detection of virus during routine screening. Therefore it is less labour intensive and has a more favourable cost:benefit profile (Chatziandreou et al., 2007). Using a similar IFN-γ capture protocol, five patients have been treated at Great Ormond Street Hospital with ADV-specific T cells either from the original donor (n=3) or third-party haploidentical parents (n=2) (Qasim et al., 2013). All 5 children had undergone either *in vivo* or *ex vivo* T cell depletion as part of their conditioning regimen and had peak ADV loads in blood ranging from 5.6 x 10^4/mL to 22 x10^6/mL before cell infusion. IFN-γ secreting ADV-specific T cells in the donations were

enriched to between 19-64% after 24 hours and infused directly without ex vivo expansion (Figure 1.2a), with 4 children receiving 10^4 T cells/kg and 1 child receiving 10^5 T cells/kg at an average of 80 days after the original stem cell graft. Three patients cleared ADV in blood after a single infusion of 10^4/kg and had demonstrable ADV-specific T cells in circulation detected by IFN-γ secretion assay. No acute, infusion-related toxicities were observed. Three patients died, one due to bystander GVHD after cell infusion even though viraemia had resolved (Qasim et al., 2011) the other two failed to clear virus and died at days 175 and 56, respectively (Qasim et al., 2013).

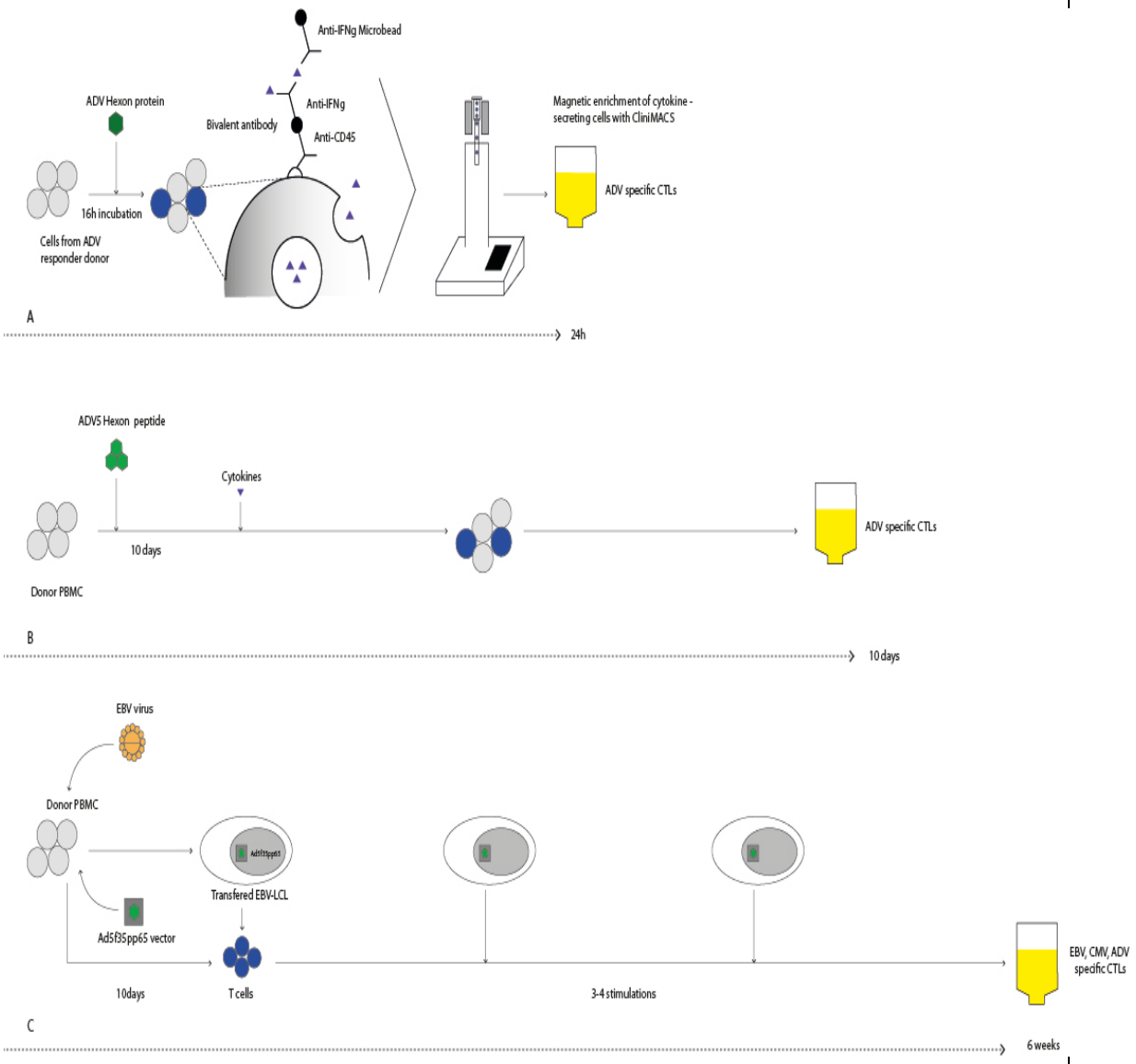
1.3.6.3 Peptide expanded T cells

More recently in order to generate CTLs from a greater majority of healthy donors in a short period of time, Comoli et al used a pool of five 30-mer peptides derived from HAdV5 hexon protein, to generate 21 T-cell lines with limited alloreactivity starting from median of $20x10^6$ donor PMBC and expanded to 75 x 10^6 cells at end of 26 days. This would have been sufficient for infusion aimed at 0.5 x 10^6 cells/kg (Comoli et al., 2008). In 2010 Aissi-Rothe et al used clinical-grade PepTivator-ADV5 Hexon (Miltenyi Biotec, Germany) and 6-hr incubation time to generate IFN-γ secreting ADV-specific T cells which were expanded over a median of 2-week period with IL2 and irradiated autologous feeder cells (Figure 1.2b). Up to $85x10^6$ ADV T cells were generated with a mean of 1.7 log expansion and a reduction of 1.3 log in alloreactivity (Aissi-Rothe et al., 2010).

In order to broaden the antiviral coverage Papadopoulou and colleagues were able to rapidly manufacture single preparation of multivirus VSTs (mVSTs) by direct stimulation of PBMCs with overlapping peptide libraries that incorporate EBV, CMV, ADV, BK virus (BKV) and human herpesvirus 6 (HHV6) antigens. Fourty-eight clinical-grade mVSTs lines were manufactured by exposing PBMCs ($3x10^7$ cells/sample) to overlapping peptide libraries spanning immunogenic ADV (Hexon, Penton), CMV (pp65, IE1), EBV (LMP2, EBNA1, BZLF1), BK (Large T, VP1) and HHV-6 (U11, U14, U90) antigens. VSTs were then expanded over 9-11 days with IL4 and IL7 in a G-Rex device. A mean of 40.1 x 10^7 polyclonal CD4+ and CD8+ cells were produced. T cell subsets expressed central $CD45RO^+CD62L^+$ and effector memory markers $CD45RO^+/CD62L^-$. Fourteen out of the 48 lines (29%) generated had activity against all five stimulating viruses (pentavalent) as assessed by IFN-γ ELIspot assay, 19% were tetravalent, 25% were trivalent and 22% were bivalent. Only one mVST line was found to be monovalent and one failed to recognize any of the targeted viruses. None of the CMV-seronegative donors produced lines containing CMV-directed T cells. These mVSTs were infused prophylactically (n=3) or as treatment for active infection/reactivation (n=8) for up to four viruses at doses of 0.5 to 2 $x10^7$ cells/m^2 between days 38 and 139 post transplant. One patient developed de novo GvHD of the skin (stage II) 4 weeks after receiving cells and resolved with topical steroids. In all patients with viral reactivation expansion of the mVSTs and clinical responses were observed and those patients treated prophylactically remained virus-infection free for >3 months

(Maecker-Kolhoff and Eiz-Vesper, 2015; Papadopoulou et al., 2014); Papadopoulou et al., 2013). This represents the first successful attempt using adoptive T cell transfer for the treatment of BKV and HHV6 in HSCT recipients. BKV is a ubiquitous polyomavirus that establishes latent, asymptomatic infection in >90% of general population. It can cause severe and intractable disease in HSCT recipients with 60 to 80% having urinary shedding of BKV and 5-15% developing BKV-associated haemorrhagic cystitis (Tomblyn et al., 2009). HHV6 is a member of the β-herpesvirus family and persists lifelong in a latent form after primary infection which occurs in >90% of individuals by age of 2 years. HHV6 reactivation and viraemia occur in 40 to 60% of HSCT recipients and has been found to be an independent predictor of acute and chronic GvHD and lower survival rates, mainly because of non-relapse mortality (de Pagter et al., 2008). In this study, among the 7 patients who reactivated BKV, 5 had complete and 1 had partial responses; including 3 who had severe haemorrhagic cystitis pre-infusion and achieving marked symptomatic and virological responses within 2 to 4 weeks of receiving cells. And in two patients who reactivated HHV6, VSTs produced clinical benefit and viral clearance coincided with an increase in the frequency of T cells targeting HHV6 antigens (Papadopoulou et al., 2014).

Figure 1.3 Protocols for generating virus-specific T cells



A – Donor identified as ADV responder by IFN-γ secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood mononuclear cells (PBMC) isolated and incubated overnight with ADV Hexon protein. Responding cells captured with IFN-γ reagent, anti-IFN-γ microbead and magnetically enriched with CliniMACS (Miltenyi Biotec). (Qasim et al., 2013)

B – Donor PBMC incubated over 10 days with ADV5 hexon peptide and cytokines. Expanded cells isolated and infused into patients after QA/QC testing. (Aissi-Rothe et al., 2010)

C – EBV-transformed B cell lines (EBV-LCLs) generated from donor PBMCs by infecting with EBV virus. Donor PBMCs are transfected with Ad5f35pp65 vector (replication-competent adenovirus-negative) and later restimulated several times by EBV-LCLs that have been transduced with the same vector. (Leen et al., 2006)

1.3.6.4 Stimulation with viral DNA plasmids

In 2011 the Baylor group took an alternative approach to rapidly select virus-specific T cells. Instead of using adenovectors to stimulate T cells, dendritic cells nucleofected with DNA plasmids encoding LMP2, EBNA1 and BZLF1 (EBV), Hexon and Penton (ADV), and pp65 and IE1 (CMV) were used as antigen-presenting cells. Secondly, EBV-LCLs were removed and replaced by gas permeable culture device (G-Rex) that promotes expansion and survival of large cell numbers after a single stimulation. Activated T-cells were cultured in the presence of IL-4 (1,000 u/mL) and IL-7 (10ng/mL). This approach reduced the time of manufacturing from 10 weeks to 10 days, as well as the cost of production by >90% (Gerdemann U, 2011). Using this method, 22 trivirus and 14 bivirus CTL lines were produced with a 1.5 log expansion from $15x10^6$ starting PBMCs. 10 patients with viral reactivation (either single or dual) were

treated between day 27 and 52 months post HSCT, with each patient receiving 0.5 to $2x10^7$ cells/m^2. Complete virological responses associated with increased frequency of virus specific T cells was seen in 80%. One patient developed stage 2 skin GVHD post infusion but no other toxicities were observed (Gerdemann et al., 2013a). Similarly this approach was used to develop a single preparation of polyclonal (CD4+ and CD8+) CTLs that is specific for 7 viruses (EBV, CMV, Adenovirus, BK, human herpes virus-6, respiratory syncytial virus, and Influenza) (Gerdemann et al., 2012).

1.3.6.5 Isolation protocols using T-cell activation markers

Apart from using IFN-γ production as a way to capture antigen-specific T cells, alternative isolation strategies based on other T-cell activation markers have been investigated. Khanna et al. generated antigen-specific T cells lines for ADV, EBV, CMV, A fumigatus, and C albicans based on magnetic cell separation of CD154+ T cells after 16 hours of stimulation with antigens, followed by expansion in presence of IL2, IL7 and IL15 over 14 days. Purity of the product was between 8 to 15%, with a higher frequency of virus-specific T cells compared to fungus-specific T cells (Khanna et al., 2011). Leibold et al compared the specificity, expansion/differentiation potential, and Th1 response against CMV and ADV after isolation of antigen specific T cells based on IFNγ release or expression of activation markers (CD137 ND CD154). Isolation of T cells based on expression markers is feasible and less time consuming, but it

resulted in smaller proportion of Th1 cells compared to IFNγ capture which may correspond to less effector function in vivo (Leibold et al., 2012).

Because CD4+ T cells are critical in human ADV infection, Haveman and colleagues explored the possibility to selectively expand and isolate ADV-specific CD4+ T cells. PBMCs were stimulated with 15-mer pan-DR binding CD4+ T cell epitopes of ADV serotype 5 peptides using artificial APCs, composed of liposomes harbouring ADV peptide/HLA class-II complexes (Haveman et al., 2013). The resultant T-cell lines after 7-day culture period produced mainly pro-inflammatory cytokines (TNFα, IFNγ, MDC, RANTES and MIP-1α), expressed perforin and granzyme B, had specific ADV-killing and were not alloreactive (Haveman et al., 2013).

1.3.6.6 Selection of Adenovirus-specific T cells by HLA-peptide multimer

As described in the previous section on generation of CMV-CTLs, peptide-major histocompatibility complex (pMHC) multimers (tetramers or pentamers) containing immunodominant CMV $CD8^+$ epitopes have been used to demonstrate the safety and efficacy of direct adoptive transfer without ex vivo expansion. Similarly Chakupurakal and colleagues (Chakupurakal et al., 2013) (Gunther et al., 2015)developed pMHC tetramers containing 8 ADV-specific class I epitopes within the hexon and major capsid protein, and used these to determine the properties of ADV-specific $CD8^+$ T cells directly ex vivo, and to

identify the optimal ADV peptides for future development in $CD8^+$ T-cell immunotherapy.

Table 1.4 8 ADV-specific class I epitopes used to identify optimal tetramer for enriching ADV-specific CD8+ T cells (Chakupurakal et al., 2013)

Class I HLA Restriction	Sequence	Abbreviation
A*01	TDLGQNLLY	TDL
A*02	GLRYRSMLLGNGRY	GLR
A*02	TFYNHTFKKV	TFY
A*02	LLYANSAHAAL	LLY
A*02	YVLFEFFDVV	YVL
A*24	TYFSLNNKF	TYF
B*07	KPYSGTAYNSL	KPY
B*07/35	MPNRPNYIAF	MPN

Of the 8 tetramers tested, HLA-A*01 TDL tetramer appears to be most appropriate for use in enriching ADV-specific $CD8^+$ T cells. Enriched TDL-specific T cells from donors recognized Ad5 and peptide-loaded antigen presenting cells and secreted IFN-γ in response (as measured by ELISA). They also showed proliferative potential in CFSE assay. Immunophenotyping of fresh PMBCs by flow cytometry in 8 HLA A*01 donors revealed that TDL-specific T cells displayed a minimally differentiated central memory phenotype: $CD45RA^{high}$, $CD45RO^{high}$, $CCR7^{high}$, $CD62L^{low}$, $CD27^{high}$, $CD28^{high}$, $CD57^{low}$, and $LFA\text{-}1^{high}$. TDL-specific T cells were also shown to lyse target cells infected with heat-inactivated wild-type viruses responsible for the majority of infections in HSCT recipients, including ADV3,5,11,12,19a, and Ad40 (species B1, C, B2, A, D and F, respectively). Gunther and colleagues (Gunther et al., 2015) adoptively

transferred TDLGQNLLY-HLA-A*01 streptamer selected donor T cells to a HLA-A*01-positive HSCT recipient who had become infected with adenovirus 2 and not able to clear infection with pharmacological treatment. The frequency of specific T cells 5 weeks after adoptive T-cell transfer was 2.33% of all $CD3^+$ T cells compared to 0.03% in healthy HLA-A*01 donor. There was also concurrent decrease of viral load from 10^5 virus copies/mL before transfer to undetectable levels after infusion. The group also identified a novel immunodominant HLA-B*07:02-restricted peptide epitope VPATGRTLVL from protein 13.6 K and demonstrated the high proliferative, cytotoxic, and IFN-γ-producing capacity of peptide-specific T-cells. The results suggest that the peptide epitope VPATGRTLVL may be suitable for paediatric adoptive transfer therapy as this peptide is conserved in species C adenoviruses which cause the majority of adenoviral infections in children. Taken together it would appear that ADV epitope-specific $CD8^+$ T cells enriched by tetramer selection may be therapeutic in HSCT recipients after adoptive transfer; as these cells appear to be minimally differentiated with a high proliferative potential and could therefore expand in the presence of virus and maintain effector functions. The phenotype of these T cells should also allow them to persist in the recipient for a longer duration in comparison with differentiated T cells.

1.3.7 Third-party Virus-specific T cells (VSTs)

For patients receiving an allogeneic cord blood transplant or a transplant from a virus-seronegative donor, an alternative option for VSTs is with partially HLA-

mismatched third-party T-cell donors. Recently an allogeneic cell registry *alloCELL* was established in 2013 at Hannover Medical School to gain more insight into virus specific memory T cell repertories and to identify the most efficient target antigens for adoptive immunotherapy. The T cell frequencies against ADV, CMV, EBV, HHV6 peptides and peptide pools available in good manufacturing practice (GMP) quality were determined in 204 healthy donors via IFN-γ enzyme-linked immunospot (ELISpot) assay and by flow cytometric analysis using pMHC multimers. It has now also been extended to provide data on the assessment of specific individual memory T cell repertoire in response to polyoma (BK) virus as well as melanoma (Melan-1/Mart-1) and tumor antigens (WT-1). The registry now contains record of memory T-cell repertoire for more than 550 donors, as well as the HLA type (class I and II). The enrichment of clinical-grade antigen-specific T cells from donors is performed under GMP conditions using the IFN-γ CliniMACS cytokine capture system and overlapping GMP-grade peptide pools spanning the entire sequence of an immunogenic epitope (Sukdolak et al., 2013; Tischer et al., 2014) (www.alloCELL.org).

In a recent multicenter study, banked third-party virus-specific T cells (VSTs) were administered to 50 patients with severe, refractory CMV, ADV or EBV infections (Leen et al., 2013). Thirty-two virus-specific lines were generated from individuals with common HLA polymorphisms immune to EBV, CMV or ADV; of which 18 lines were administered to 50 post-HSCT patients with severe, refractory illness due to infection with one of these viruses. The virus specific T cells were generated by transduction of PBMC with clinical grade Ad5f35pp65

vector followed by stimulation with EBV-transformed LCL that had been transduced with the same chimeric vector. The VSTs were then cryopreserved until required. Patients were excluded from the study if they had received T-cell serotherapy within 28 days of proposed administration date. The cumulative rates of complete or partial responses at 6 weeks post infusion were 74% for the entire group. No immediate infusion-related adverse events were noted, 2 patients developed de novo GVHD (grade 1). Six out of eight patients who did not have line available and continued with standard therapy died of their viral disease. The lineage of the VSTs is largely central or effector memory-derived T cells. This approach of using 'off-the-shelf' third-party VSTs appear to remove some of the barriers to the wider application of cell therapy in viral reactivations post-transplant. It avoids the lengthy time and cost of producing individual lines, and does not appear to cause GVHD from alloreactivity of the third-party cells (Leen et al., 2013).

1.3.8 Virus-specific T cells (VSTs) from umbilical cord blood

Increasingly umbilical cord blood (UCB) has emerged as an important alternative source of stem cells for patients undergoing allogeneic stem cell transplant. UCB transplants (UCBT) allow more rapid procurement of the graft, requirement for less-stringent HLA matching, higher likelihood of finding a match for ethnic minorities, and decreased incidence of GvHD. However a major drawback is delayed engraftment and immune recovery and subsequent

increased susceptibility to viral infections post-transplant (Walker et al., 2007). Unlike adoptive transfer of VSTs from an adult stem cell donor, there are considerable barriers to successful use of adoptive immunotherapy with VSTs post UCBT, including small numbers of T-cells available for manipulation and their naïve phenotype (Thompson et al., 2015). Cord blood (CB) T cells also have lower cytotoxic activity and higher activation-induced cell death than do peripheral blood-derived T cells. Development of CB CTLs requires priming of naïve T cells rather than simple expansion of preexisting memory T cells. To overcome these obstacles Hanley et al described a method to generate single cultures of CTLs from CB that are specific for multiple viruses. EBV-infected B cells are transduced with a clinical-grade Ad5f35pp65 adenoviral vector and expanded in the presence of cytokines IL-7, Il-12 and IL-15 over a 2 week period. These cytokines decrease antigen concentration threshold, direct T-cells toward a central memory phenotype and influence TH1 and T-cytotoxic type 1 cell differentiation. In combination these cytokines augment the generation of antigen-specific CTLs from naïve T cells. T-cell lines were prepared from 9 different CB units and a median of $85.5x10^6$T cells were harvested which would have been sufficient for T-cell adoptive transfer at $1x10^7/m^2$. The generated VSTs showed specific IFNγ response in response to EBV, CMVpp65- and ADV-hexon-expressing target cells by ELISpot assays (Hanley et al., 2009). Also by use of the G-rex culture device, expansion of VSTs to clinically relevant numbers ($>6x10^7$) was possible using only 20% fraction of thawed UCB units (Hanley et al., 2012). To date, 9 patients have been treated at the Center for Cell and Gene Therapy at Baylor College of Medicine in Texas with UCB-

derived mVSTs. Six were treated prophylactically and 3 preemptively on evidence of viral reactivation/infection (one with CMV and ADV; two with EBV reactivation). Viral infections were resolved in all three patients with detection of transferred virus-specific T cells in peripheral blood by means of IFNγ ELISpot and/or deep T-cell receptor sequencing. All infusions were well tolerated and not associated with GvHD (Hanley et al., 2015).

1.3.9 Virus specific T cells (VSTs) from seronegative donors

About 20% of the adult population is seronegative to ADV (Garnett et al., 2002) which renders the approaches described above of manufacturing ADV-specific T cells by expansion of memory T cells impossible. Similar to the use of TCR-redirected CMV-specific T cells to treat CMV-infections, Dorrie and colleagues described a method of expanding ADV-specific T cells by stimulation with the HLA-A*0101-restricted, immunodominant, and cross reactive epitope LTDLGQNLLY (LTD) from the hexon protein of ADV-species C. ADV-specific TCR α/β chain sequences are then cloned and transfected via mRNA electroporation into CD8+ α/β and γ/δ T cells. Both TCR-transfected α/β and γ/δ T cells secreted cytokines after antigen-specific stimulation, and efficiently lysed adenovirus-infected target cells (Dorrie et al., 2014). However γ/δ T cells, when compared to α/β counterparts, offer the advantage of being involved in viral defense without the potential to induce GVHD in patients (Oevermann et al., 2012). This has not been trialed in clinical studies but could potentially be an alternative means of ADV immunotherapy to using third-party ADV specific T-

cells for patients whose original HSCT donors have no detectable ADV-specific T-cells.

1.4 AIMS of THESIS

Adenovirus infection is an important cause of morbidity and mortality in HSCT recipients, especially in the paediatric group. Current antiviral treatment can be unsatisfactory in their side-effect profile and does not always facilitate viral clearance. Reconstitution of viral immunity in the post-transplant setting is essential for viral clearance and prevention of severe disease. The ASPIRE (Adenovirus Specific Paediatric Immune REconstitution) study is a Phase I/II clinical trial aimed to investigate the safety of Adenovirus- specific T cells given to high-risk paediatric patients post allogeneic transplant to treat reactivation/infection of adenovirus. The trial product, Cytovir ADV, is an Advanced Therapy Medicinal Product (ATMP) comprising ADV specific T cells derived from donor peripheral blood mononuclear cells (PBMCs) that have been expanded *ex vivo.*

The aim of this thesis is to:

1. Evaluate the safety and efficacy of Cytovir ADV by collecting clinical data on patients who are registered on the study. Data collected include stem cell transplant details, days of viral reactivation and viral load, adverse events after cell infusion, and immune reconstitution as evaluated by IFN-γ cytokine capture

assay. This group is compared with a contemporaneous group of patients who developed adenoviraemia but were not registered on the study.

2. Generate ADV-specific T cells from PBMCs collected from healthy adult volunteers using the same method as in the ASPIRE study and characterise the expanded T-cell population in regards to their phenotype as well as antigen-specific function, ability to cause bystander activation and defining the cytokine profile in supernatant collected after T-cell expansion.

The data collected helps to define a group of paediatric patients who will benefit from adoptive T-cell therapy for treatment of ADV infection/reactivation post-HSCT. The optimal T-cell population to be infused will also be discussed.

Table 1.5 Clinical trials using virus-specific cytotoxic T cells in the HSCT setting

Ref (Centre)	Virus-specificity	Expansion protocol	Antigen Used	Infused number & type of cells	Patients treated	Clinical results
(Leen et al., 2006) (Texas)	EBV, CMV, ADV	Donor PBMCs infected with vector; restimulated repetitively with irradiated EBV-LCLs transduced with same vector over 10-12 weeks.	Clinical grade Ad5f35pp65 vector.	Median 5 x 10^7 polyclonal cells/m^2 infused at 35-150d after HSCT (median 62d)	11 infused (children and adults; 10 prophylactically, 1 treated for ADV infection)	3/3 cleared CMV and 3/3 cleared EBV infection/PTLD without antivirals; 3 patients with infection and 1 with disease cleared ADV post-CTL. No GVHD
(Feuchtinger et al., 2004) (Tuebingen)	ADV	IFN-γ selection after 16h stimulation. Cytokine secreting cells magnetically enriched .	Adenoviral antigen type C (nonclinical grade)	1.2-50x10^3/kg ADV-reactive polyclonal T cells infused	9 children with ADV infection.	5 out of 6 with ADV responded. 1 died at 30 days from ADV infection. 5 deaths (3 due to ADV infection)
(Leen et al., 2009) (Texas)	EBV + ADV	PBMCs infected with vector. Responder cells restimulated weekly with irradiated autologous LCL transduced with same vector IL-2 was added twice weekly from day 14. CTLs cryopreserved after 3 or 4 simulations.	$Ad5f35^{null}$ vector MOI 200vp/cell	20 CTL lines with EBV and ADV specificity produced, 13 lines infused. Dose of 5x10^6 to 1.35 x 10^8 cells/m^2 at 40 to 150 days after HSCT (median 77 days).	13 children [(M)MUD or haplo] 2 with active ADV disease; 11 prophylactic	No toxicities or GVHD, monitored for 3 months. Only detected increases in ADV-sp T cells in peripheral blood in those with active ADV infection (2 out of 13).

(Qasim et al., 2013) (London)	ADV	PBMCs stimulated for 16hrs with ADV-hexon antigen. Cytokine secreting cells selected using anti-IFN-g microbeads and Miltenyi Mini-MACS column within 24h	Commercially available purified ADV-hexon antigen (The Binding site, UK)	3 received γ-captured cells from original stem cell donor Cells (10^4 – 10^5 /kg) received on average 80 d after original graft (range 34-122)	5 patients treated (3 with original donor; 2 third-party haploidentical donor) -	Blood viraemia resolved in 3. IFN-γ secreting ADV specific T cells present in 4 patients. 3 died – 1 of bystander GVHD after clearing virus
(Leen et al., 2013) (Texas)	ADV, CMV, EBV	Banked 3rd-party PMBCs transduced with vector and stimulated with EBV-LCL transduced with same vector	Ad5f35pp65 vector	32 virus-specific lines from individuals with common HLA polymorphisms immune to EBV, CMV or ADV. Each patient received up to $2x10^7$ cells/m^2	18 lines administered to 50 patients with severe viral illness with one of the viruses	Cumulative rates of complete or partial responses at 6 weeks was 74% for whole group. 2 de novo GVHD (grade 1).
(Gerdemann et al., 2013c) (Texas)	ADV, CMV, EBV	Donor PBMCs stimulated with nucleofacted DCs and cultured over 2-3 weeks with IL4 and IL7.	DCs nucleofacted with range of EBV, CMV, ADV viral antigens.	22 trivirus and 14 bivirus CTL lines Each patient received 0.5 -2 $x10^7$ cells/m^2	10 patients with viral reactivation treated between day 27 and month 52 post HSCT	Viral clearance and increased frequency of VSTs in 80%. 1 stage 2 skin GVHD

CHAPTER 2

ASPIRE – <u>A</u>denovirus **<u>S</u>**pecific **<u>P</u>**aediatric **<u>I</u>**mmune **<u>R</u>**econstitution

2.1 STUDY SYNOPSIS

Protocol Title	A Phase I/II study to investigate the safety of Adenovirus specific T cells given to high-risk paediatric patients post allogeneic haematopoietic stem cell transplant (allo-HSCT) to treat reactivation of adenovirus.
Protocol Number	CM-2011-02
Eudra CT Number	2011-001788-36
ISRCTN number	ISTCTN22322271
Investigational Product	ADV specific T cells derived from donor peripheral blood mononuclear cells (PBMCs) expanded *ex vivo* (Cytovir ADV)
Indication	Treatment of ADV infection post allogeneic haematopoietic stem cell transplant
Objectives	**Primary Objective:** • To evaluate the safety of ADV specific T cells **Secondary Objectives:** • To evaluate the pharmacokinetics (PK) of ADV specific T cells with respect to absolute T cell counts • Time to absence of ADV viraemia • Use of antiviral and other anti-infective drugs • Time in-hospital stay **Exploratory Objective:** • To evaluate persistence and expansion of adoptively transferred ADV specific T cells

Study Design	This is an open-label safety study assessing the effect of pre-emptive administration of ADV-specific T cells (Cytovir ADV). A maximum of 15 patients will receive these cells.
Planned Number of Subjects	It is estimated that donor cell therapy will be prepared for 50 patients with the aim of registering and treating a maximum of 15 patients who have reactivated ADV.
Subject Population	Paediatric recipients of an allo-HSCT using T cell depletion from a matched unrelated, mis-matched unrelated or family donor, or haploidentical donor. Mismatched is defined as mismatches at ≥ 1/10 alleles.
Treatment Regimen:	Patients will commence the study and be consented to receive Cytovir ADV on reactivation of ADV (defined as two consecutive positive PCRs > 1000 copies/ml) post-transplant, in addition to antiviral drugs that would be administered as part of standard of care. Patients will only be treated if they are considered stable as deemed by the attending physician. The primary dose will be $1x10^4$/kg total CD3+ T cells. Patients will be monitored at these 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 will be assessed to receive a secondary maximum target dose of $1x10^5$/kg total CD3+ T cells and will be monitored for a further 180 days.

Endpoints:	**Primary Endpoint:** • Toxicity: incidence and severity of GvHD, cytopaenias, grade 3-4 AEs
	Secondary Endpoints: • Number of reported serious adverse events (SAEs) (Suspected, Unexpected Serious Adverse Reactions [SUSARs] and Suspected, Expected Serious Adverse Reactions [SESARs]) • Absolute (by PCR) ADV viraemia at each time point • Number of treatment days with antiviral drugs • Number of treatment days with other anti-infective drugs • Number of in-hospital days during study period **Exploratory Endpoint:** • Number of detectable ADV specific T cells *in vivo* assessed at each time point
Statistical Methods:	This is primarily a safety study so analysis will be of numbers of adverse events related to treatment. No control group is included in this Phase I/IIa study but data on allo-HSCT outcomes in 273 recipients performed at GOSH/ ICH has been collated as part of the preparation for this study. This large database is available as an historical comparison.

Independent Review of Data	An Independent Data and Safety Monitoring Committee will review data as they emerge from the study. The IDSMC will have the ability to halt the trial if pre-agreed stopping criteria are reached. These criteria will be documented in a Charter signed by all Committee members.

2.2 INTRODUCTION

Cytovir™ ADV has been developed by Cell Medica Ltd. as an Advanced Therapy Medicinal Product (ATMP) comprising Adenovirus (ADV)-specific T cells derived from peripheral blood mononuclear cells, expanded *ex vivo* - for the treatment of ADV infection in immunocompromised transplant recipients. The purpose is to accelerate reconstitution of the immune system with respect to its ability to control ADV infection or reactivation; and ultimately to provide patient with protective immunity to control *de novo* ADV infection or reactivation.

Clinical experience is drawn from previous trial conducted at Great Ormond Street Hospital where 5 children were treated with ADV-specific T cells derived from the original donor (n=3) or third-party haploidentical parents (n=2) (Qasim et al., 2013). Enrichment of IFN-γ secreting cells was performed using the *Cytokine Secretion System™* and the *CliniMACS™* device for immunomagnetic separation (both Miltenyi Biotec, Bergisch Gladbach, Germany) and ADV-specific T cells were infused directly after 24 hours without *ex vivo* expansion. Three patients cleared ADV in blood after a single infusion of 10^4 /kg and had demonstrable ADV-specific T cells in circulation detected by IFN-γ secretion

assay. However there was time delay in treatment with recalling of original donors and children received cell therapy at an average of 80 days after original stem cell graft; whilst median days to viral reactivation is 15 days (Hiwarkar et al., 2012). Hence the need for quicker access to immunotherapy was identified. In the current study, Cytovir ADV will be manufactured upfront at time of HSCT rather than at time of viral reactivation, to ensure timely administration at time of need.

Cytovir^{TM} ADV contains a population of naturally occurring ADV-specific T cells which are recovered from the peripheral blood of a donor who is fully or partially HLA matched with the recipient. Cells are expanded *ex vivo* by cultivation with synthetic ADV peptide antigens, cytokines and human serum. The expanded ADV specific T cells are harvested, washed and cryopreserved. At the time of administration Cytovir^{TM} ADV is thawed and infused intravenously. The therapeutic mode of action is adoptive immune reconstitution following the transplantation of virus specific T cells. The T cells will be derived from the same donor who provides the graft for the recipient's allo-HSCT procedure.

2.3 PRODUCT CHARACTERISTICS AND ADMINISTRATION

The raw material for Cytovir ADV is peripheral blood mononuclear cells derived from whole blood or mobilised apheresis obtained from HLA matched donors. To manufacture Cytovir ADV, Peripheral Blood Mononuclear Cells (PBMCs) are isolated by density gradient centrifugation with ficoll-paque. ADV-specific T cells are selectively expanded with synthetic ADV peptides and specific cytokines (IL-4 and IL-7) that promote rapid proliferation of the stimulated T cell population.ADV specific T cells are selectively expanded for ten days. At the end of the process the cells are harvested, washed, counted and re-suspended at the appropriate concentration in cryopreservation medium containing Human Serum Albumin (HSA) and Dimethyl Sulfoxide (DMSO) prior to controlled rate freezing for storage in liquid nitrogen vapour.

Following Qualified Person (QP) release (purity, enumeration of ADV specific T cell content) the patient-specific dose of ADV specific T cells can be made available for infusion. Appropriate aerobic and anaerobic cultures will be performed and confirmation of negative results subsequently passed to the Principal Investigator (PI). Cells that are unused will be destroyed as clinical waste following study closure or with appropriate consent, may be used anonymously for research or training purposes

The frozen cells are supplied to the hospital in a sterile, IV bag. Prior to patient administration the frozen cells are thawed and infused within 30 minutes of thawing. Cytovir™ ADV is administered as a single IV infusion following allo-HSCT on PCR evidence of ADV reactivation (two consecutive positive PCRs).

Each infusion is patient specific (HLA matched) and will contain ADV specific T cells typically comprised of CD4+ and CD8+ cells with minor cell populations including monocytes, B lymphocytes and NK cells. The first dose given will be at $1x10^4$ /kg body weight total CD3+ T cells. A second dose of $1x10^5$ /kg can be given at least 28 days after the first dose if patient experiences persistent significant adenoviraemia requiring treatment.

2.3.1 Release criteria

TEST	**TEST LIMITS**
Appearance	Clear and colourless solution
Purity	> 80% CD3+ T cells
Viability	> 70% CD3+ T cells
Safety - Sterility - Mycoplasma - Endotoxin	 Absence of growth Not Detected < 10.0 (EU of endotoxin per mL of product)
Potency	≥ 10^2 IFNγ+ T cells per kg BW of recipient

2.3.2 Composition of one dose of Cytovir ADV

COMPONENT	QUANTITY PER DOSE
$CD3^+$	≤ $1x10^5$ per kg BW of recipient
ADV specific T cells	≥ $1x10^2$ per kg BW of recipient
Infusion bag with 15-70mL of the ATMP in 4.5% human serum albumin (HAS), 10% dimethyl sulphoxide (DMSO)	One to several depending on final $CD3^+$ numbers and patient body weight

2.4 ADVERSE EVENTS

There is little clinical experience with Cytovir™ ADV. The transfusion could theoretically cause GvHD. Events which are recognised and expected complications of the preceding allogeneic transplantation procedure, conditioning or concurrent medications used in the transplantation procedure or follow-up phase, will be exempt from the requirement to report serious adverse events if they are included in the list below:

- Central line, cannula, or gastrostomy tube complications requiring revision
- Cytopaenias (either autoimmune, drug-induced or due to hypoplastic graft) with resultant need for transfusion, risk of bleeding and infection
- Deranged liver function or Jaundice
- Deranged renal function
- Electrolyte disturbances
- Enteropathy or pneumatosis intestinalis

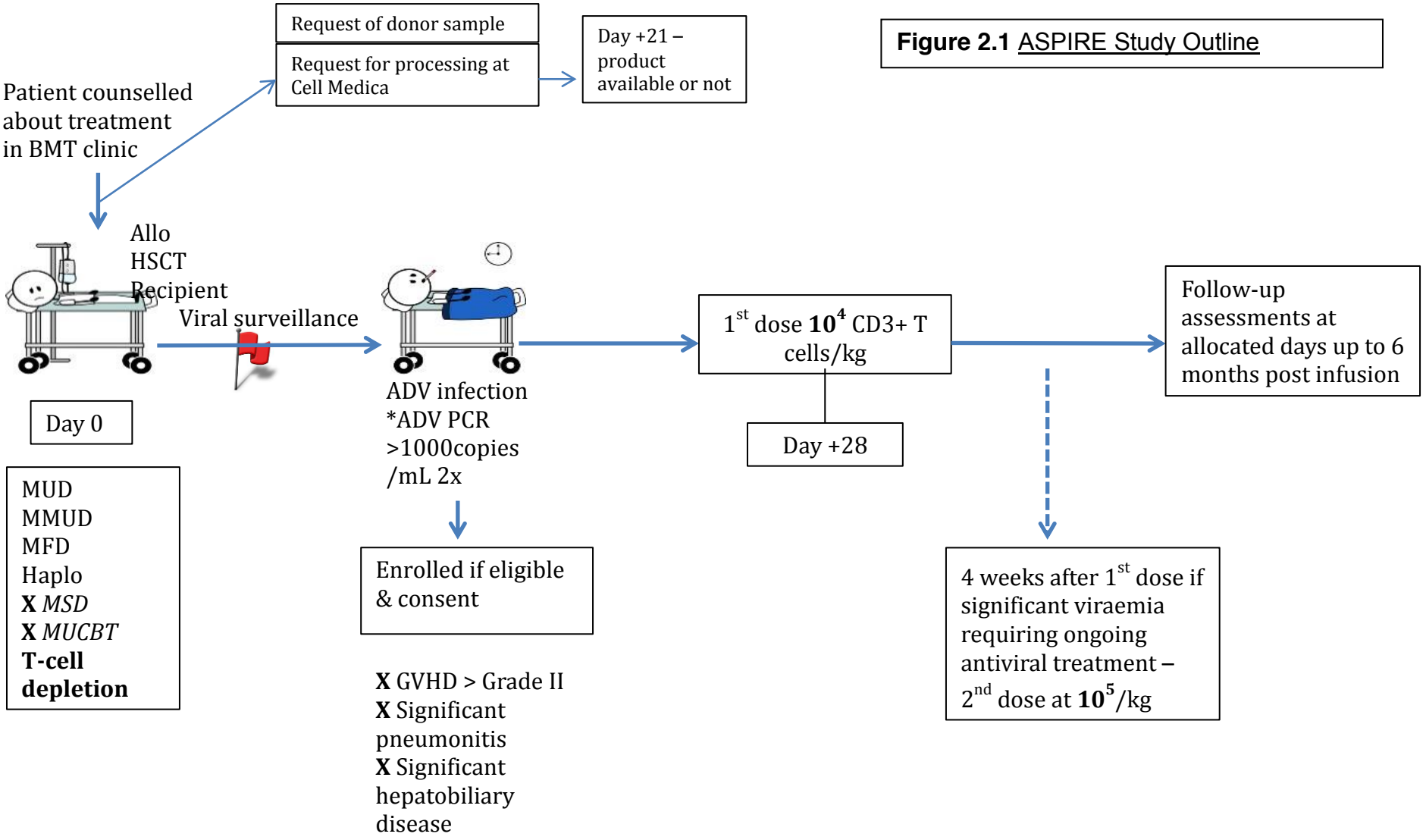
- Haemorrhagic cystitis
- Hypertension or Hypotension
- Infections (bacterial, fungal and viral)
- Mixed chimerism
- Mucositis
- Pancreatitis
- Pericardial or pleural effusion
- Pneumonitis
- Rejection or secondary graft failure
- Relapse of malignancy
- Seizures related to infection, hypertension/electrolyte imbalance/drugs
- Thrombotic thrombocytopenic purpura
- Toxicities related to drugs given independent of the study including derangement of electrolytes, renal and liver function, hypertension
- Veno-occlusive disease of the liver

2.5 STUDY DESIGN

The study will test the hypothesis that human ADV specific T cells can enhance ADV specific immune responses post allo-HSCT, and reduce the requirement for antiviral therapy and hospitalisation without toxicity or increasing GvHD. See Figure 2.1 (Outline of the Study) below. In advance of study participation, the manufacturer will prepare cells for 50 patients of whom a maximum of 15 patients will be enrolled and registered if they reactivate ADV and are eligible to

receive cells: patients are treated when they exhibit ADV viraemia of at least 1000 copies/mL. The use of standard antiviral therapy will not be amended. The dosing will be one single dose of $1x10^4$/kg total CD3+ lymphocytes with the option of a second dose, if available. The second dose will be a maximum of $1x10^5$/kg (minimum $1x10^4$/kg) total CD3+ lymphocytes given if the patient has significant ADV viraemia requiring antiviral therapy at ≥ 4 weeks after the first infusion. Cell administration will not occur until 28 days post-transplant due to continuing *in vivo* persistence of conditioning antibodies such as alemtuzumab or Antithymocyte Globulin (ATG). These could directly impact on the viability of infused T-cells. ADV specific T cells will be administered from days 28 post allo-HSCT and patients will be followed up for 6 months following infusion, with assessments at approximately 14, 30, 60, 90, 120, 150 and 180 days post-infusion.

Figure 2.1 ASPIRE Study Outline



The study opened at Great Ormond Street Hospital in Q1 2013, and two new sites were recently recruited and opened in December 2014 – The Great North Children's Hospital in Newcastle-upon-tyne, and Royal Manchester Children's Hospital, UK. Patients will be enrolled up till June 2015 with the last patient visit conducted in December 2015. These centers are specialist paediatric UK transplant centers experienced in the management of children undergoing allo-HSCT. The study is conducted in compliance with the protocol, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) for Good Clinical Practice (GCP) and all applicable regulatory requirements. All necessary regulatory and ethical approvals have been obtained before patient recruitment began. Potential donors will be flagged by the transplant team at the relevant study site and Bone Marrow Donor registries requested to procure from donors in accordance with standard procedures. These donors may be seen at any of the procurement sites under the jurisdiction of the relevant registry.

2.6 SELECTION CRITERIA

2.6.1 Patient Inclusion Criteria – for the manufacture of Cytovir ADV (Screening Phase)

The patient inclusion criteria include:

1) Age < 16 years of age

2) Laboratory tests to determine negative serology for HIV1+2, HepBcAb & HepBsAg, HepC, HTLV 1&2, Syphilis.

3) Recipient of allo-HSCT with *in vivo* or *ex vivo* T cell depletion from matched unrelated, mis-matched unrelated or family donor, or haploidentical donor

2.6.2 Patient Inclusion Criteria – for study enrolment and cell infusion (Treatment Phase)

A patient will be eligible for enrolment in the study if, in addition to the screening patient inclusion criteria listed above, all of the following criteria apply:

1) For all subjects, a parent or legal guardian must give informed consent. Patients aged 11-15 will be encouraged to provide witnessed written assent.

2) The patient has two consecutive positive ADV PCR results >1000 copies/ml

2.6.3 Patient Exclusion Criteria – for the cell infusion of Cytovir ADV

A patient will NOT be eligible for enrolment in the study if any of the following criteria are met:

1) Significant GvHD ≥ Grade II

2) Significant pneumonitis

3) Significant hepato-biliary disease

2.6.4 Donor Inclusion Criteria

Donors for suitable participants will be selected for the manufacture of Cytovir ADV from patients already scheduled to undergo an allo-HSCT. Eligible unrelated, haploid or mismatched family donors will have already passed a medical for stem cell donation.

The donor inclusion criteria include:

1) The donor will be selected from a registry that has approved the protocol and consent procedure.
2) Donor must have met all regulatory requirements for transplant.
3) Laboratory tests as described in Appendix 6
4) Healthy donor – having passed medical for stem cell donation
5) Negative serology for HIV (type 1 & 2), Hepatitis B and C and Syphilis
6) Written informed consent
7) Age 16 years or older

2.6.5 Donor Exclusion criteria in accordance with registry criteria

1) Pregnant or lactating and to be assessed prior to donation
2) Platelets < 50x109/L

2.7 SUMMARY OF CLINICAL USE OF CYTOVIR ADV TO DATE

Patient Study ID	**Date of dose (Day post HSCT)**	**ADV PCR on administration**	**% of CD3 IFNγ ADV response in donor T cells**	**No. of Cidofovir doses (pre /post Cytovir ADV)**	**Serious Adverse events**
A1/02	1st dose – 10th April 2013 (Day +43) 2nd dose – 8th May 2013 (Day +71)	PCR 1: 3890 PCR 2: 5900	17.79	1/2	None
A1/03	14th May 2013 (Day +97)	PCR 1: 9659 PCR 2: 50,151	21.99	1/2	Severe Grade 4 GvHD skin and gut. Died 23 months post SCT.
A1/04	25th April 2014 (D+28)	PCR 1: 26,280 PCR 2: 201,697	1.36	1/4	Astrovirus encephalitis Died 9 months post SCT.
A1/13	14th November 2013 (D+30)	PCR 1: 241,411 PCR 2: 28, 687	3.98	3/0	Pancreatitis

A1/17	8th January 2014 (D+62)	PCR 1: 919,156 PCR 2: 523,863	11.8	5/0	None
A1/42	15th October 2014 (D+28)	PCR 1: >20 million PCR 2: >20 million	1.49	2/0	None

Table 2.1 Characteristics of enrolled patients before infusion of Cytovir ADV

Patient Study ID	Age	Primary Condition	Graft	Donor type	Conditioning regimen	GvHD prophylaxis	Co-infection at T-cell transfer	Other sites of ADV infection at T-cell Transfer	Immuno-suppression at T-cell Transfer	Antiviral drugs before T-cell Transfer
A1/02	5yrs 8 mth	Relapsed Acute Lymphocytic Leukaemia (ALL)	BM	9/10 (1A) mismatched related (father)	-TBI (200cGyx4 Days -7 to -4) -Etop (60mg/kg Day -3) -ATG (20mg/kg x3 Days -3 to -1)	-CSA-A -MTX	EBV viraemia (>2m copies/mL)	- Stool	- Topical steroids for Grade 1, stage 2 skin GvHD - Rituximab for EBV viraemia	1 dose of CDV
A1/03	9 mth	Haemophagocytic lymphohistiocytosis (HLH) secondary to MUNC 18-2 deficiency	PBSC	9/10 (1DQ) mismatched unrelated	-Flu (30 mg/m^2 x5 Days -6 to -2) -Treo (14g/m^2 x3 Days -6 to -4) -Alem (0.2mg/kg x5 Days -7 to -3)	- CSA-A - MMF	-	- Stool - NPA	- Low dose Prednisolone (0.2mg/kg)	1 dose of CDV
A1/04	18 mth	Cartilage Hair Hypoplasia (*RMRP* mutation) and associated immunodeficiency	PBSC	10/10 matched unrelated	-Flu(30mg/m^2 x5 Days -7 to -3) -Mel (140mg/m^2 Day -2) -Alem (0.2mg/kg x5 Days -8 to -4)	- CSA-A - MMF	Sapovirus in stool	- Stool	-	1 dose of CDV
A1/13	11 yrs	X-linked adrenoleukodystrophy	PBSC	9/10 (1C) mismatched unrelated	-Cyclo (60mg/kg x2 Days -7 to -6) -TBI (3cGy x1 Day -4) -Flu (30mg/m2 x5 Days -8 to -4) -Alem (0.2mg/kg x5 Days -8 to -4)	- CSA-A - MMF	BK virus – haemorrhagic cystitis	- Stool	-	3 doses of CDV

A1/17	5 yrs	Relapsed Acute Myeloid Leukaemia (AML)	PBSC	Haplo with alpha/beta/ CD19 depletion	-Flu (40mg/m^2 x4 Days -6 to -3) -Treo (14 g/m^2 x3 Days -7 to -5) - Thio (5mg/kg x2 Day -4) - ATG (5mg/kg x3 Days -5 to -3)	- CSA-A	-	-	-	5 doses of CDV
A1/42	13 year old	Juvenile Idiopathic Arthritis/Macrophage Activation Syndrome/Haemophagocytic Lymphohistiocytosis	PBSC	10/10 matched unrelated	-Flu (30mg/m^2 x5 Days -7 to -3) -Mel (140mg/m^2 Day -2) -Alem (0.2mg/kg x5 Days -8 to -4)	- CSA-A - MMF - Prednisolone	-	- Stool - Gut biopsy	-	2 doses of CDV

Alem = Alemtuzumab; ATG = Anti-thymocyte globulin (Fresenius); BM = bone marrow; CDV = Cidofovir; CSA-A = Cyclosporine A; Cyclo = Cyclophosphamide; Etop = Etoposide; Flu = Fludarabine; Haplo = haploidentical; Mel = Melphalan; MMF = Mycophenolate Mofetil; MX = Methotrexate; NPA = Nasopharyngeal Aspirate; PBSC = peripheral blood stem cell; TBI = Total Body Irradiation; Treo = Treosulphan

Table 2.2 Cytovir ADV infusion and response

Patient Study ID	Adoptive T-cell transfer (days after SCT)	Blood ADV load (copies/mL) at time of T-cell transfer	% of CD3 IFNγ ADV response	Virologic Response	Immunologic response/in-vivo expansion of transferred ADV T cells	No. of Cidofovir doses post Cytovir ADV	Serious Adverse Events	Clinical Outcome
A1/02	1st dose ($1x10^4$ $CD3^+$/kg) – 10th April 2013 (Day +43) 2nd dose ($1x10^5$ $CD3^+$/kg) – 8th May 2013 (Day +71)	PCR 1: 3890 PCR 2: 5900	17.79	ADV load continued to rise to 250,000copies/mL; Load reduced to 261 copies/mL 12 days after 2nd dose; remained negative at 6months follow-up	Detectable ADV specific responses by Elispot 56 days post 2nd dose (20 spots/10^5 cells), 53 spots/10^5 cells at 91 days.	2	None	Alive
A1/03	14th May 2013 (Day +97)	PCR 1: 9659 PCR 2: 50,151	21.99	ADV load undectable 38 days post infusion.	No IFNg response 85days post infusion	2	Severe Grade 4 GvHD skin & gut	Died 23 months post SCT
A1/04	25th April 2014 (Day +28)	PCR 1: 26,280 PCR 2: 201,697	1.36	ADV load reduced to 820 copies/mL 12 days post infusion.		4	Astrovirus encephalitis	Died 9 months post SCT
A1/13	14th November 2013 (Day +30)	PCR 1: 241,411 PCR 2: 28,687	3.98	ADV load reduced to 2,142 copies/mL 7 days post infusion but rose to 32,324 11 days post. Undetectable since 39 days post.	Detected ADV specific response by IFNγ capture 27 days post infusion. Response sustained at 6 months detected by Elispot (501spots/10^5 cells)	0	Pancreatitis (associated with rise in ADV load) 9 days post infusion.	Alive

A1/17	8th January 2014 (Day +62)	PCR 1: 919,156 PCR 2: 523,863	11.8	ADV load decreased to 29,091 copies/mL 23 days post; undetectable since 113 days post.	Detected ADV specific response by Elispot 126 days post infusion (75.3 spots/10^5 cells)	0	None	Alive
A1/42	15th October 2014 (Day +28)	PCR 1: >20million PCR 2: > 20 million	1.49	ADV load decreased to 26,892 5 days post; remained undetectable since 2 weeks post infusion.	Detected ADV specific response by Elispot and IFNγ capture by 13 days post infusion	0	None	Alive

2.7.1 Detailed description of each treated subject (Fig. 2.2)

A1/02 – a 5 year and 8 month old boy with relapsed Acute Lymphocytic Leukaemia (ALL) underwent a 1A mismatched (9/10) related bone marrow graft from his father on 26.2.2013 with TBI (200cGy x4 Days -7 o -4), Etoposide (60mg/kg Day-3) and ATG-Fresenius (20mg/kg x3 Days -3 to -1).GvHD prophylaxis was undertaken with Cyclosporin and Methotrexate. Full donor chimerism was detected on D+37 post transplant. He developed mild skin GVHD (stage 2, grade 1) that was under control with topical steroids. Adenoviraemia was first detected on D+36 with 3890 copies/mL rising to 15,189 copies/mL one week later. Adenovirus had been detected in the stools since D+11. Cidofovir treatment was initiated and subject received Cytovir ADV on D+43 post HSCT. Subject also experienced EBV reactivation on D+34 with viral load rising to and remaining at greater than 2 million copies/mL despite receiving 4 doses of Rituximab. At the same time subject also developed mild liver derangement with conjugated hyperbilirubinaemia (highest Bilirubin 41 uMol/L, GGT 1090 U/L, ALT 532 U/L). Persistent culture-negative fevers raised the possibility of EBV Lymphoproliferative Disease, and whilst there was no radiological or tissue confirmation of LPD, a trial of methylprednisolone 1mg/kg was initiated on D+58. This brought about control of fever and dose was weaned to 0.5mg/kg on D+70 and stopped in D+74. A GI endoscopy showed no macroscopic evidence of GvHD or LPD.

After the initial dose of Cytovir ADV at $1x10^4$ CD3+/kg, subject continues to have adenoviraemia up to 250,130 copies/mL and was given second higher dose at $1x10^5$ on D+71. ADV viral load reduced to 261 copies/mL 12 days later

and remained undetectable at follow-up 6 months after the second dose. EBV viral load also became undetectable by D+83 (12 days after receiving second dose of Cytovir ADV). Because of persistent cytopaenia (neutrophils and platelets) subject also received a CD34 top-up on Day +84.

ADV specific responses were first detected by Elispot 56 days after 2^{nd} dose (20 spots/10^5 cells) and at 91 days post (53 spots/10^5 cells). On 56^{th} day after 2^{nd} infusion, interferon-γ capture assay also detected ADV specific response in the $CD8^+$ population (0.51% of lymphocytes were $CD8^+$ IFN-γ^+ when stimulated with ADV, compared to 0.26% of unstimulated lymphocytes. An increase >0.05% from unstimulated background level is taken as a positive response).

A1/03 - a 9 month-old girl with haemophagocytic lymphohistiocytosis (HLH) secondary to MUNC 18-2 deficiency underwent a 1DQ mismatch (9/10) unrelated haematopoietic stem cell transplant conditioned with Fludarabine (30mg/m^2 x5 Days -6 to -2), Treosulfan (14mg/m^2 x3 Days -6 to -4) and Alemtuzumab (0.2mg/kg x5 Days -7 to -3); and received GvHD prophylaxis with Cyclosporin and Mycophenolate mofetil. Pre-transplant therapy for HLH included Dexamethasone, Etoposide, Cyclosporin as well as rabbit ATG. Full donor chimerism was first detected on D+13. Subject developed late reactivation of adenovirus in the blood on D+89 with 9659 copies/mL rising to 50,151 copies/mL 3 days later, but without clinical signs of ADV infection; although the virus had been detected in the stools since Day +13 and was also present in NPA. Pre-transplant subject had EBV viraemia and was treated with

three doses of Rituximab. Treatment for ADV viraemia was started with Cidofovir and subject received Cytovir ADV at $1x10^4$ CD3+ /kg on Day +97. ADV viral load became undetectable 38 days after CTL infusion. However subject developed stage 2 skin GvHD 64 days post infusion (D +161 post HSCT). This initially improved with topical steroid treatment but subject presented to the local hospital with profuse diarrhoea 29 days later with an upper and lower GI endoscopy showing histological features of GVHD. Subject was diagnosed with late acute Grade 3 GvHD (stage 3 skin, stage 2 gut). Treatment was started with systemic steroids, infliximab, cyclosporin and gut rest. The event was reported as a serious adverse event (SAE) possibly related to study treatment and subject was taken off study due to the development of >Grade 2 GvHD. At 22 months post-transplant subject continued to have severe skin and gut GvHD. Her gut GvHD further deteriorated to stage 4 and became refractory to steroid treatment. Treatment with basiliximab, infliximab, mycophenolate mofetil, sirolimus and mesenchymal stromal cells failed to bring about improvement in her symptoms of melena and gut failure. Due to the extensive amount of immunosuppression required to treat her GvHD, patient experienced ADV reactivation associated with respiratory failure with imaging showing signs consistent with a pneumonitis. There was also concurrent EBV reactivation with viral load up to 3 million copies/mL in blood, but no clear clinical signs of LPD. Patient was placed on palliative care and sadly passed away 23 months post her stem cell transplant for MUNC 18-2 HLH from acute respiratory failure due to ADV pneumonitis in the context of gut failure and

chronic steroid dependent gut and skin GvHD. IFNγ capture 85 days after CTL infusion failed to show ADV specific response.

A1/04 – please refer to summary of adverse events below.

A1/13 – an 11-year-old boy with X-linked adrenoleukodystrophy underwent a reduced intensity (RIC) Busulfan (AUC=60mg/L/hr), Fludarabine and Alemtuzumab conditioned bone marrow transplant with a low CD34 dose of $1.07x10^6$/kg from a 1C mismatched unrelated donor. Initially subject fully engrafted but developed fevers associated with dropping counts on D+32 with a bone marrow trephine showing aplastic marrow confirming acute graft rejection. Subject proceeded to an immunosuppressive second transplant with peripheral blood stem cells from the same donor, conditioned with cyclophosphamide (60mg/kg x2 Days -7 to -6), TBI (3 gy x1 Day -4), Fludarabine (30mg/m^2 x5 Days -8 to -4) and Alemtuzumab (0.2 mg/kg x5 Days -8 to -4); and Ciclosporin and Mycophenolate Mofetil as GvHD prophylaxis. Full donor chimerism was detected on D+13.

Subject experienced Adenovirus reactivation during his first transplant on D+17 with a peak titer of 324,370 copies/mL. A single dose of Cidofovir was given with good response with undetectable ADV in blood until D-1 of the second transplant. ADV load of 45,210 copies/mL was detected on D+2, peaking at 241,411 copies/mL on D+21. ADV was also detectable in the stool. Cidofovir

treatment was started and although subject did not have clinical signs of ADV infection he developed haemorrhagic cystitis secondary to BK virus. Cytovir ADV at dose of $1x10^4$ CD3+/kg was given on D+30. ADV viral load in blood dropped from 16,257 copies/mL on day of infusion to 2,142 copies/mL one week later. ADV specific T cell response was first detected in the $CD4^+$ population by IFNγ capture 27 days after infusion (0.42% of lymphocytes were $CD4^+$ $IFN\gamma^+$ when stimulated with ADV compared to 0.31% when unstimulated). Response was sustained at 6 months post as detected by Elispot (501 spots/10^5 cells ADV stimulated c.f 274 spots/10^5 cells with negative control).

One serious adverse event (SAE) was reported for A1/13 as 9 days after cell infusion subject developed pancreatitis with raised lipase and amylase. This co-incided with a rise in ADV load to 32,324 copies/mL on D11 peaking to 409,159 copies/mL 4 days later. The pancreatitis was thought to be possibly related to Cytovir ADV but other possible causes included conditioning subject received for the transplant, concurrent adenovirus infection, a known complication of transplant or medication mycophenolate mofetil that was used as GvHD prophylaxis. Subject was started on treatment with octreotide, analgesics, total parenteral nutrition and intravenous antibiotics. He made complete recovery after 39 days and ADV load in blood remained undetectable since. The SAE was subsequently closed and patient remained on study.

A1/17 – is a 5 year old girl with relapsed AML who underwent a haploidentical stem cell transplant with alpha/beta/CD19 depletion conditioned with

Fludarabine (40mg/m^2 x4 Days -6 to -3), Treosulfan (14 g/m^2 x3 Days -7 to -5), Thiotepa (5mg/kg x2 Days -4) and ATG (Fresenius) (5mg/kg x3 Days -5 to -3); with Ciclosporin as GvHD prophylaxis. Full donor chimerism in peripheral blood was detected on D+18.

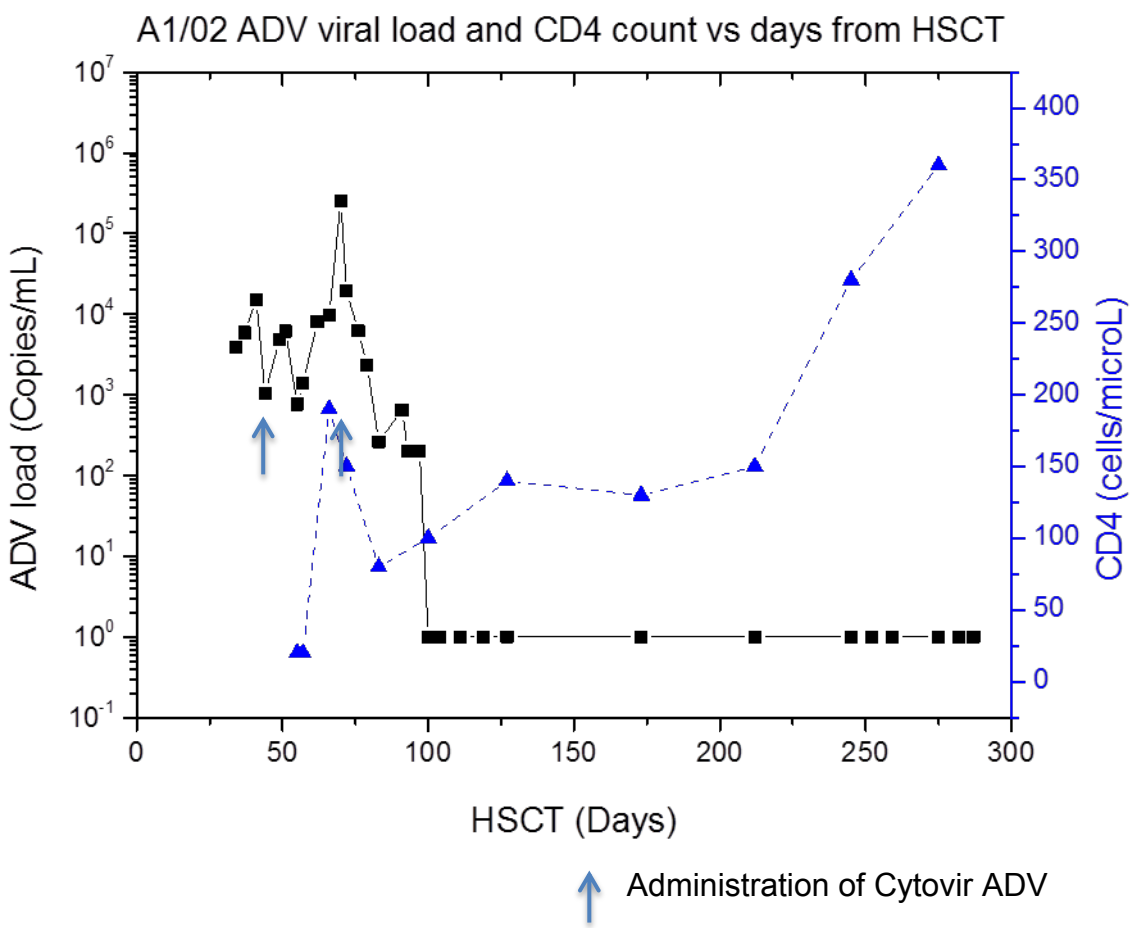
Subject experienced early reactivation of ADV in blood with low levels detected on D+4 at 870 copies/mL. This rose to 17, 814 copies/mL on D+7 and treatment was started with cidofovir. After two doses, ADV level hovered between 6000-9000 copies/mL for two weeks before rising again to 25,765 copies/mL on D+29. Two further doses of cidofovir were given one week apart but despite total of 4 doses of cidofovir ADV load continued to rise and peaked at 919,156 on D+53. Subject remained clinically well with no signs of ADV disease. Subject had good initial T-cell recovery with CD3 count of $0.32x10^6$/mL on D+60 but there was no specific IFN-gamma response to ADV stimulation. Subject received Cytovir ADV at $1x10^4$ CD3/kg on D+62. ADV load reduced from 523,863 copies/mL day before infusion to 49,091 copies/mL 23 days later and became completely undetectable from 113 days onwards. ADV specific T cell response by Elispot was first detected 126 days after infusion (75.3 spots/10^5 cells ADV stimulated c.f to 28.3 spots/10^5 cells unstimulated). Subject did not experience any serious adverse events.

A1/42 – is a 13 year old girl with JIA/MAS/HLH who received a fully matched unrelated stem cell transplant conditioned with Fludarabine (30mg/m^2 x5 Days -7 to -3), Melphalan (140mg/m^2 Day -2) and Alemtuzumab (0.2mg/kg x5 days -8

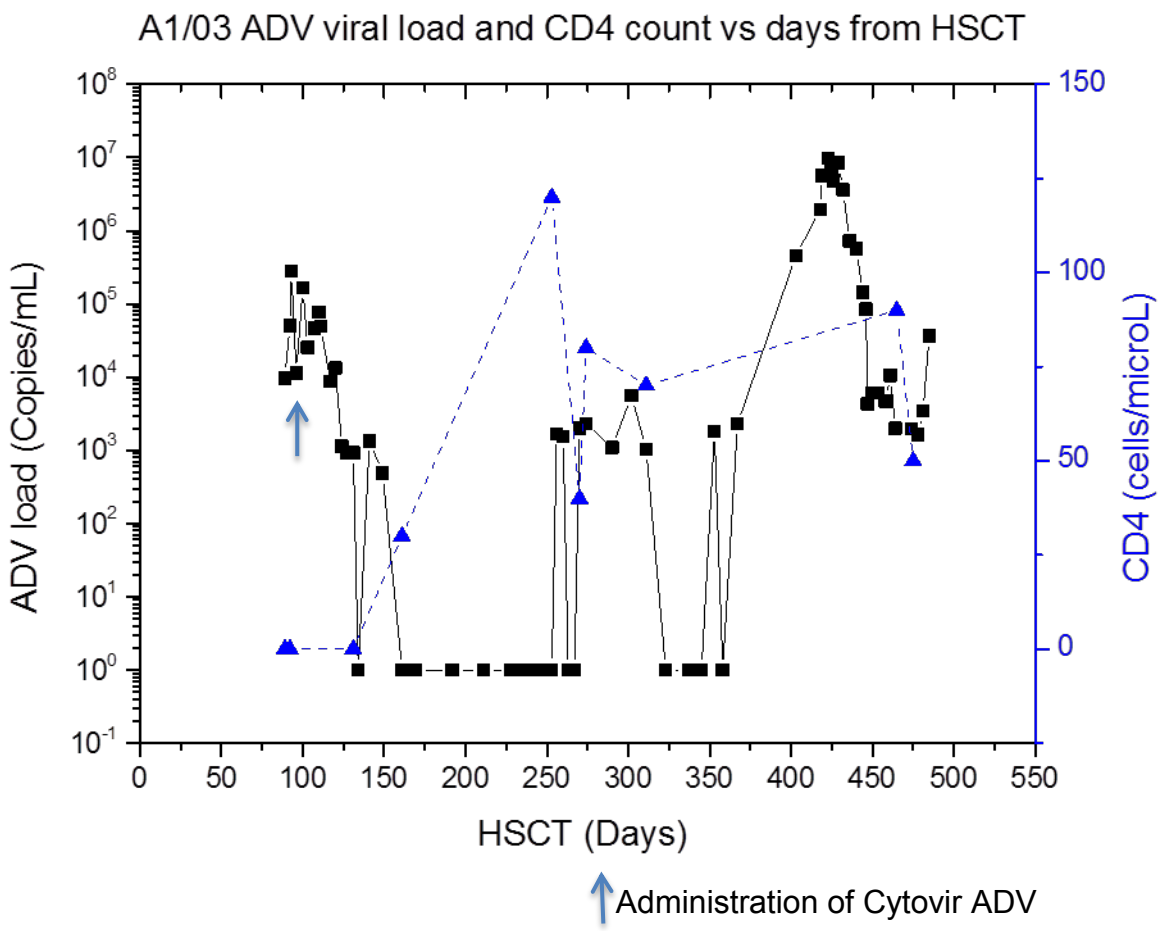
to -4); and ciclosporin, mycophenolate, and prednisolone as GvHD prophylaxis. Full donor chimerism in peripheral blood was detected on D+13. Subject developed early adenoviraemia on D+12 with load of 28, 712 copies/mL. Cidofovir (5mg/kg) was started on D+15 when load had risen to 166, 049 copies/mL; followed by second dose one week later. Clinically subject had evidence of Adenovirus gut disease with diarrhoea and vomiting and isolation of the virus in stool and endoscopic biopsies. She did not have evidence of pneumonitis or hepatitis. Adenovirus 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 at $1x10^4$ CD3/kg on D+28. ADV load reduced to 26,892 after 5 days and became and remained undetectable after 2 weeks. ADV specific T cell response was detected by Elispot and IFNγ capture from 13 days post infusion up until the last monitoring visit, although the degree of positive response decreased as viral load decreased.

Figure 2.2 ADV viral load and CD4 count vs days from HSCT for subjects A1/02, A1/03, A1/04, A1/17, A1/42

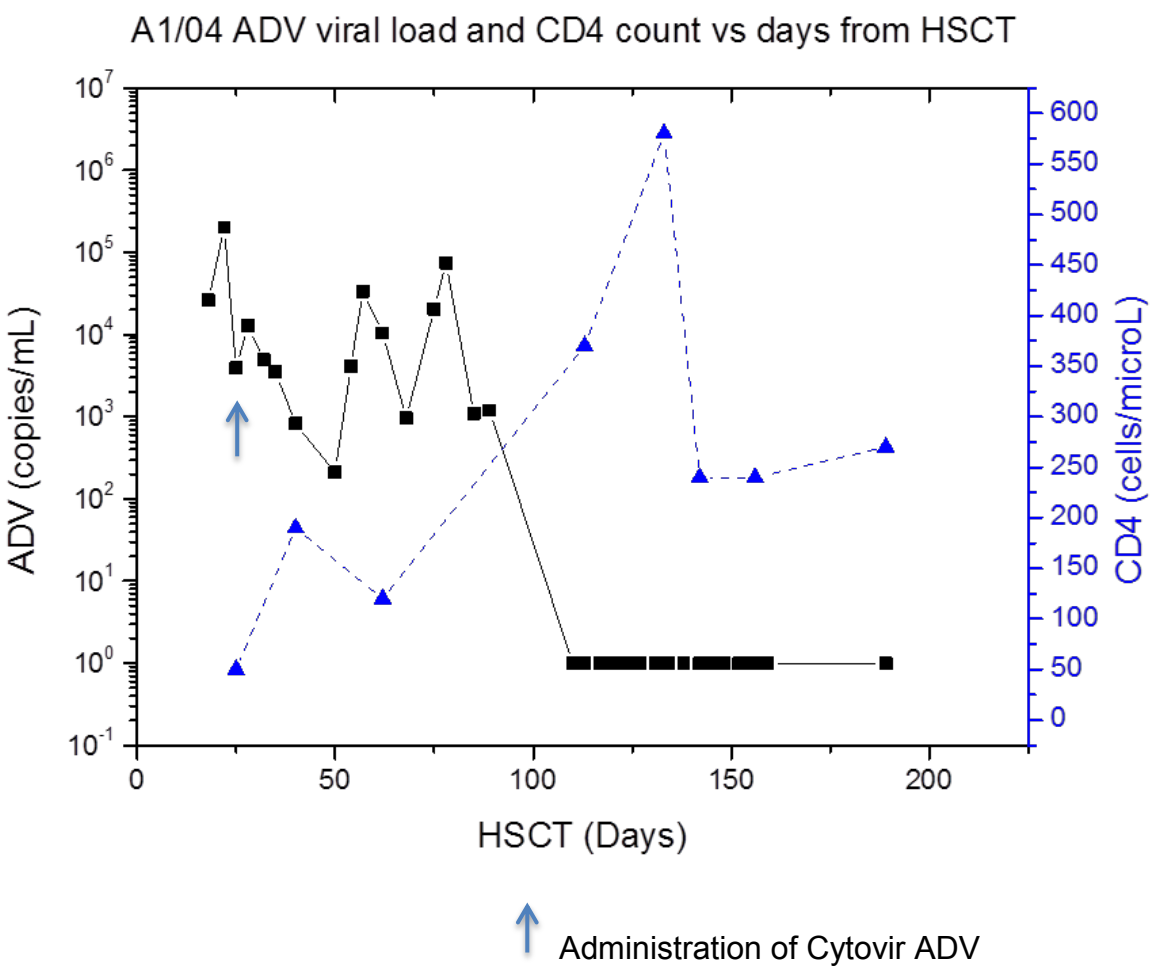
A



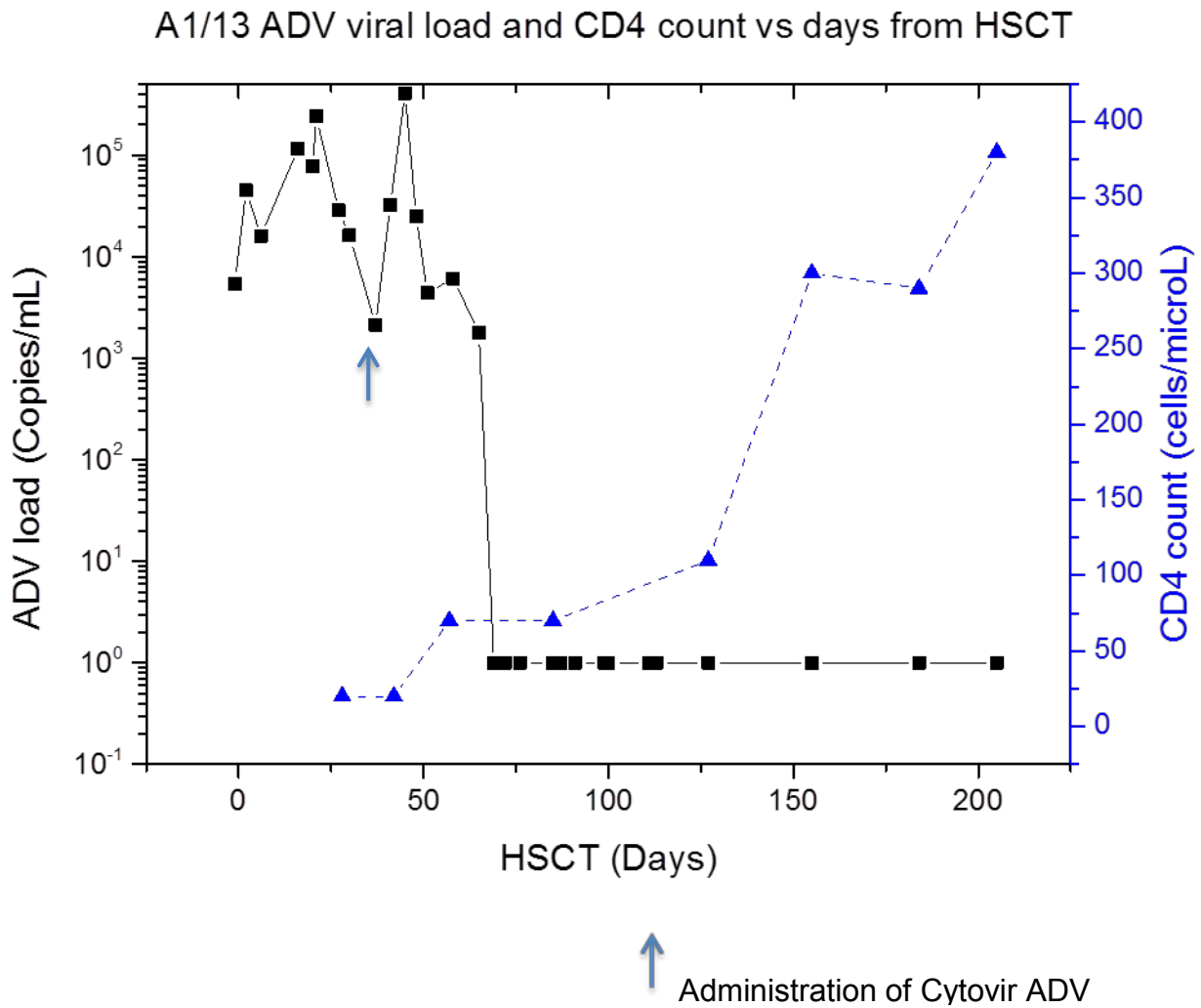
B


A1/03 ADV viral load and CD4 count vs days from HSCT
ADV load (Copies/mL)
CD4 (cells/microL)
HSCT (Days)
Administration of Cytovir ADV

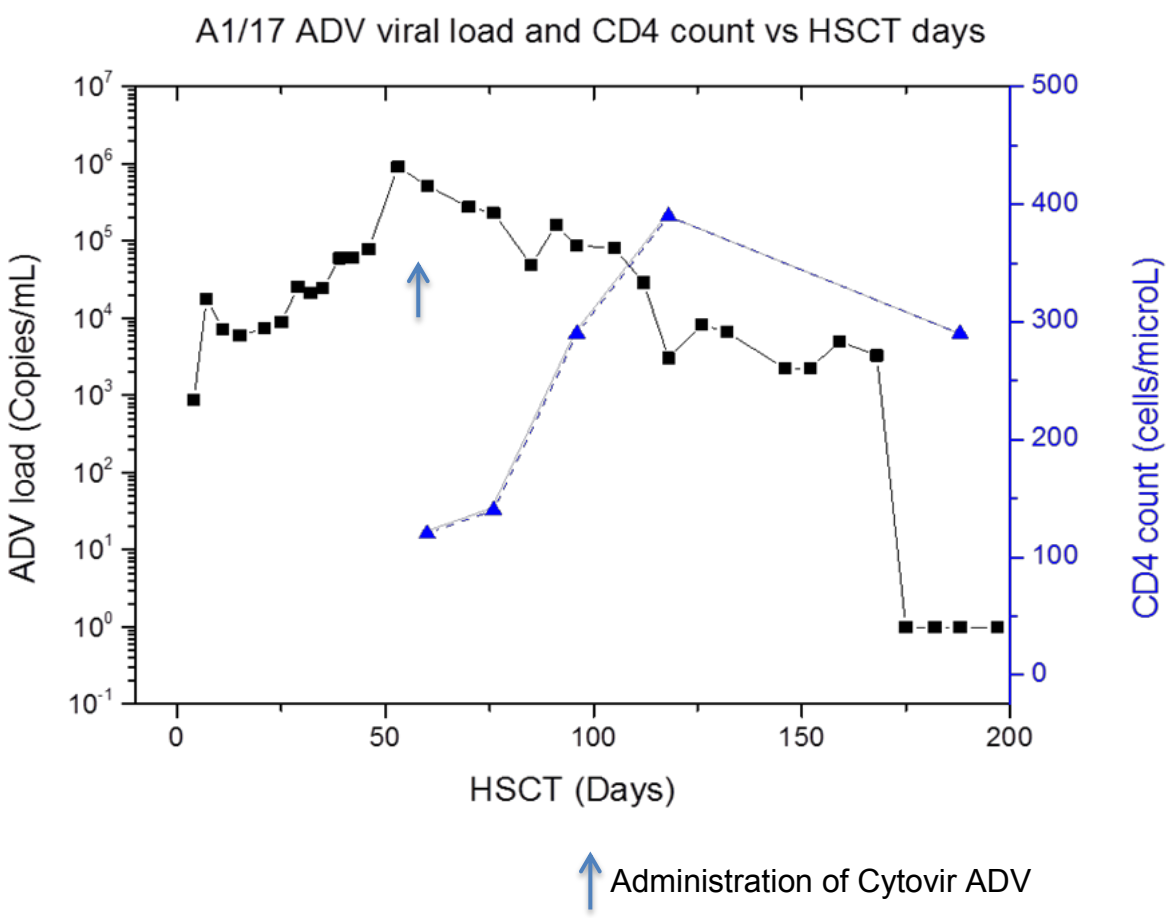
C


A1/04 ADV viral load and CD4 count vs days from HSCT
ADV (copies/mL)
CD4 (cells/microL)
HSCT (Days)
Administration of Cytovir ADV

D


A1/13 ADV viral load and CD4 count vs days from HSCT
ADV load (Copies/mL)
CD4 count (cells/microL)
HSCT (Days)
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
0
50
100
150
200
400
350
300
250
200
150
100
50
0
Administration of Cytovir ADV

E


A1/17 ADV viral load and CD4 count vs HSCT days
ADV load (Copies/mL)
CD4 count (cells/microL)
HSCT (Days)
Administration of Cytovir ADV

F

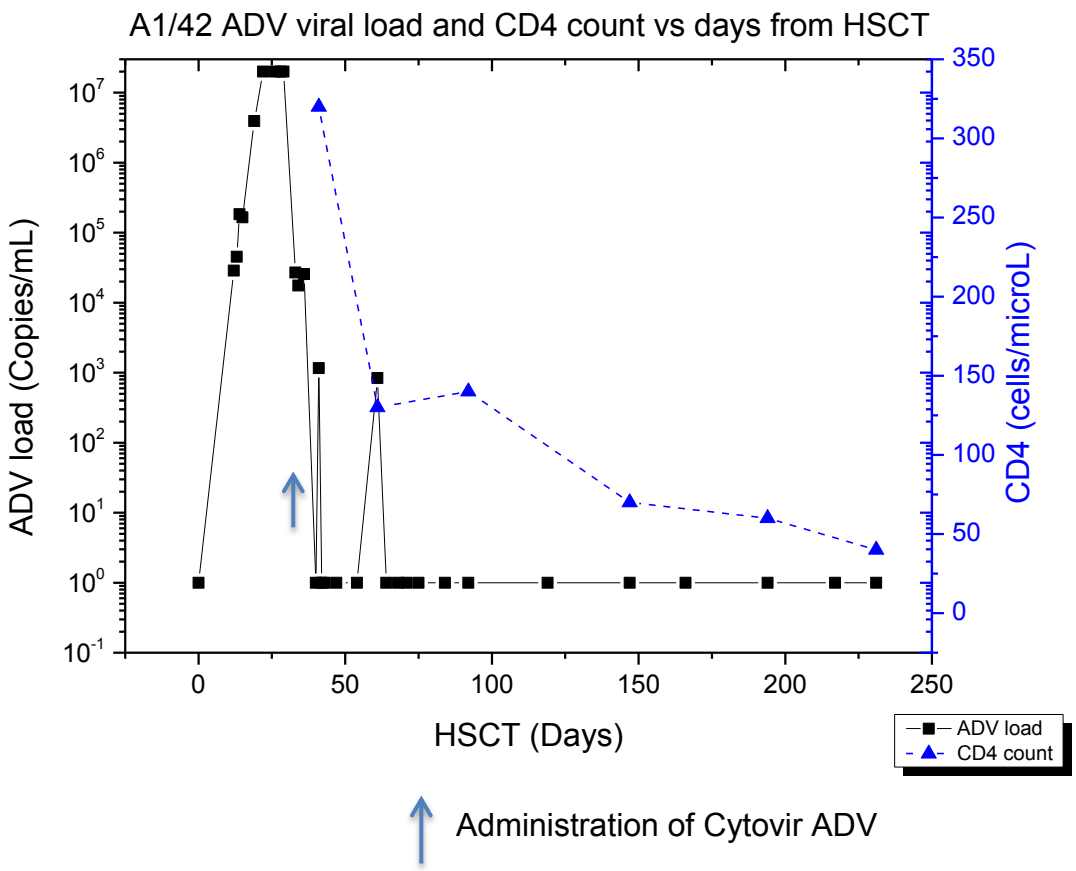
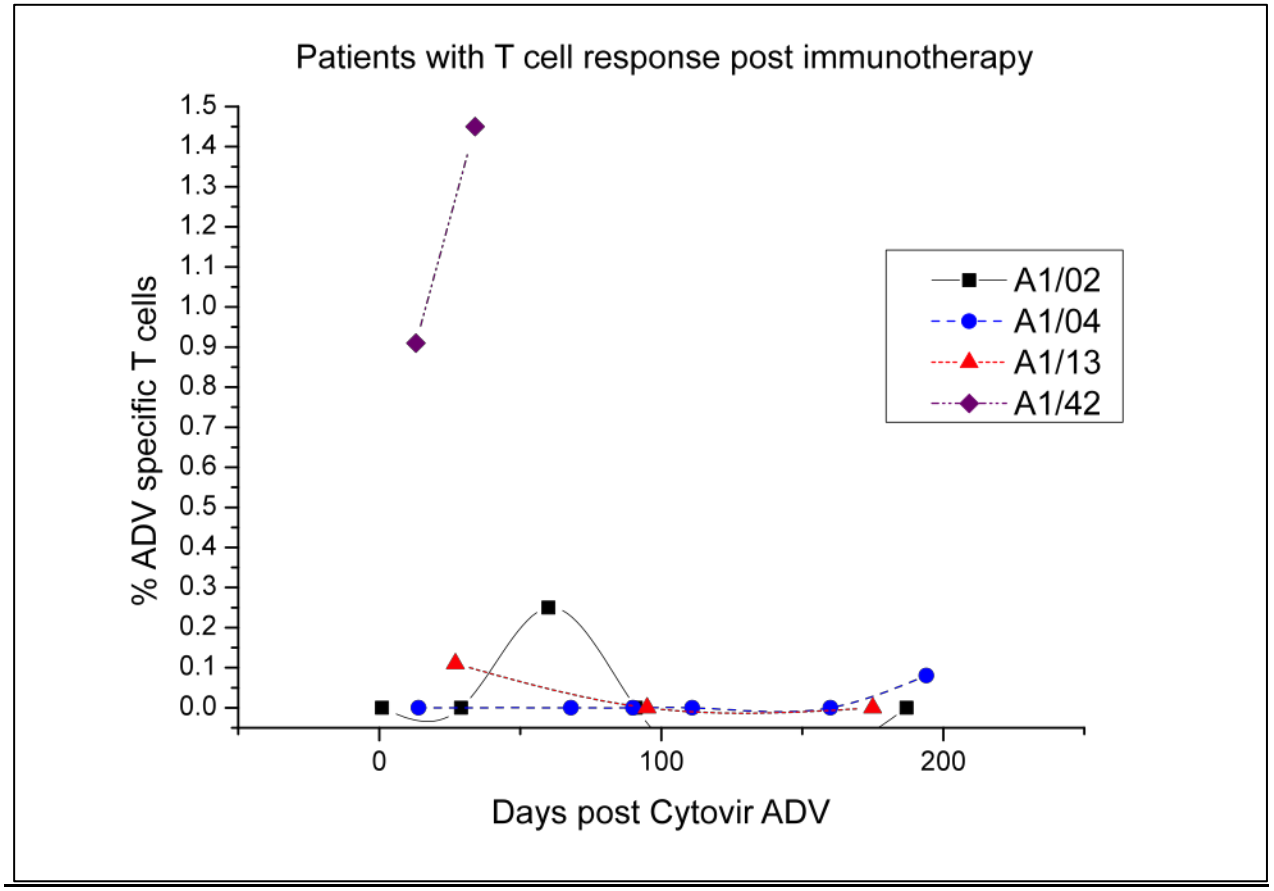

A1/42 ADV viral load and CD4 count vs days from HSCT
ADV load (Copies/mL)
CD4 (cells/microL)
HSCT (Days)
ADV load
CD4 count
Administration of Cytovir ADV

Figure 2.3 ADV specific T-cell response in treated patients (as determined by IFNγ cytokine assay)



2.7.2 Summary of adverse events

To date, three serious adverse events have been reported to the Clinical Administration Office for study CM-2011-02. Of these, only one was initially classified as possibly related to the study treatment and reported to the MHRA and NRES as a SUSAR on 16th May 2013 in line with mandatory requirements. Following additional investigations the event was subsequently downgraded to 'unlikely related'. A summary is provided below for information.

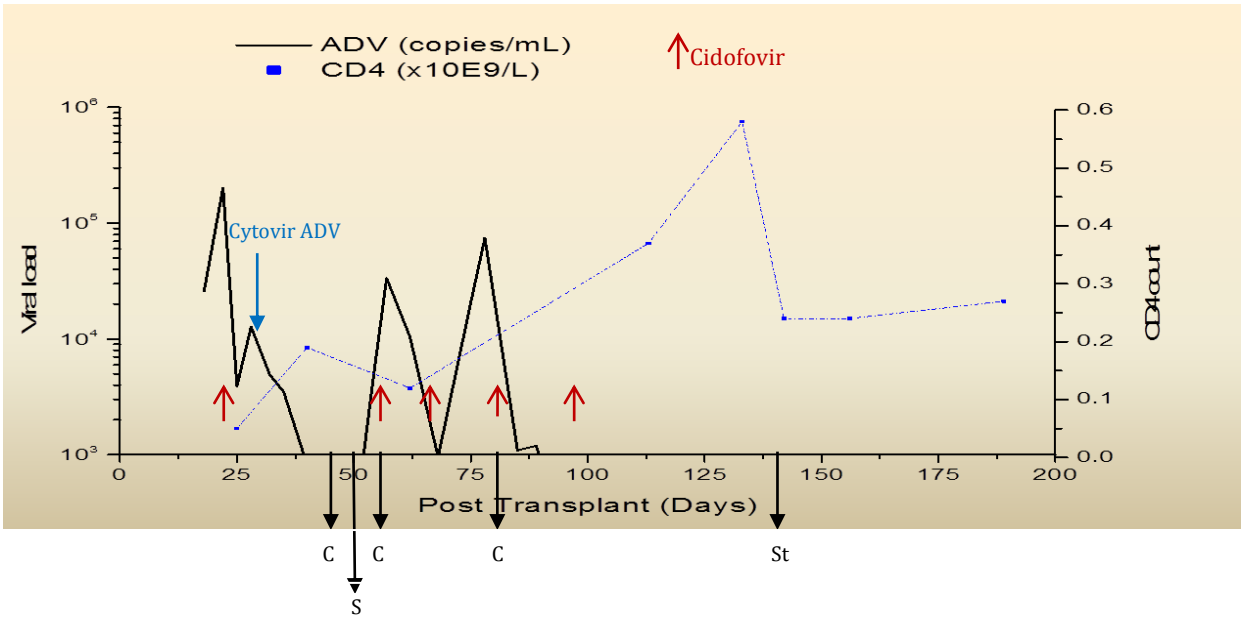
Study subject A1/04, an 18 month old boy with Cartilage Hair Hypoplasia and associated immunodeficiency due to RMRP mutations, underwent a peripheral blood stem cell transplant on 28 March 2013 from a 10/10 HLA matched unrelated donor following conditioning with fludarabine (150mg/m^2 x5 Days -7 to -3), melphalan (140mg/m^2 Day -2) and alemtuzumab (0.2mg/kg x5 Days -8 to -4) for in vivo T cell depletion. Ciclosporin A and Mycophenolate Mofetil were given as GvHD prophylaxis. Transplant was uncomplicated with neutrophil engraftment achieved by day +13 and full donor chimerism detected on day +19. Persistent stool viruses (Sapovirus, Adenovirus) were detected on stool PCR. Adenoviraemia occurred from day 18 onwards with peak load of 201,697 copies/mL on day 21. This required treatment with Cidofovir (5mg/kg 1x dose) and Cytovir ADV (donor-derived Adenovirus-specific CTLs at $1x10^4$/kg total CD3+ cells) on Day +28. Adenovirus load reduced to 820 copies/mL 12 days after infusion. Two weeks later the child became acutely unwell with irritability, dystonia and reduced consciousness. Encephalopathy secondary to drug toxicity, infectious complications or inflammation associated with immune reconstitution were considered in the differential diagnosis. Ciclosporin was

stopped on Day +43 and replaced by methylprednisolone therapy (1mg/kg). MRI of the brain showed progressive changes with loss of volume (Figure 1) and EEG revealed increased amplitudes of on-going slow activity over posterior regions with no focal features or epileptiform abnormalities. PCR of CSF 2 days, 2 weeks and 1 month after the onset of symptoms did not detect adenovirus, herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus, Epstein Barr virus (EBV), BK virus, norovirus, sapovirus, astrovirus (HAstV 1-8), rotavirus, enterovirus, measles, parechovirus, JC virus, HHV6, HHV7 and Toxoplasma. Brain biopsy (Fig 2.6) performed 8 weeks after neurological deterioration was also negative for these pathogens as well as 18s (fungal) and 16s (bacterial) ribosomal PCRs. Histological examination of the brain showed an unusual pattern of pathology with neuronal cell death and microglial activation. Lymphocytes were inconspicuous on microscopy and there were no acute inflammatory cells. Immunohistochemistry for CD68 showed diffuse cortical and subcortical microgliosis. Staining for a range of infectious agents, including prion disease, was negative. And immunostaining for adenovirus showed occasional cells with cytoplasmic staining, which was likely to be non-specific. To further elucidate on the cause for this patient's deterioration, unbiased pyrosequencing using RNA isolated from the brain biopsy was performed by virology laboratory. This identified a novel strain of Astrovirus, HAstV-VA1/HMO-C-UK1(a) and was determined to be cause of child's encephalitis after confirmation by PCR assays (Brown et al., 2015). PCR assays confirmed presence of the novel astrovirus in retrospective samples of CSF, serum and stool. Withdrawal of immunosuppresion was associated with

the development of skin GVHD (grade 2 stage 3) requiring treatment with methylprednisolone (2mg/kg/d). As a result kinetics of T cell reconstitution were reduced with CD3 count of $0.35x10^9$/L 6 months post HSCT, and in the context of ongoing neurological impairment with recurrent respiratory and gastrointestinal complications, the patient died 9 months after transplantation.

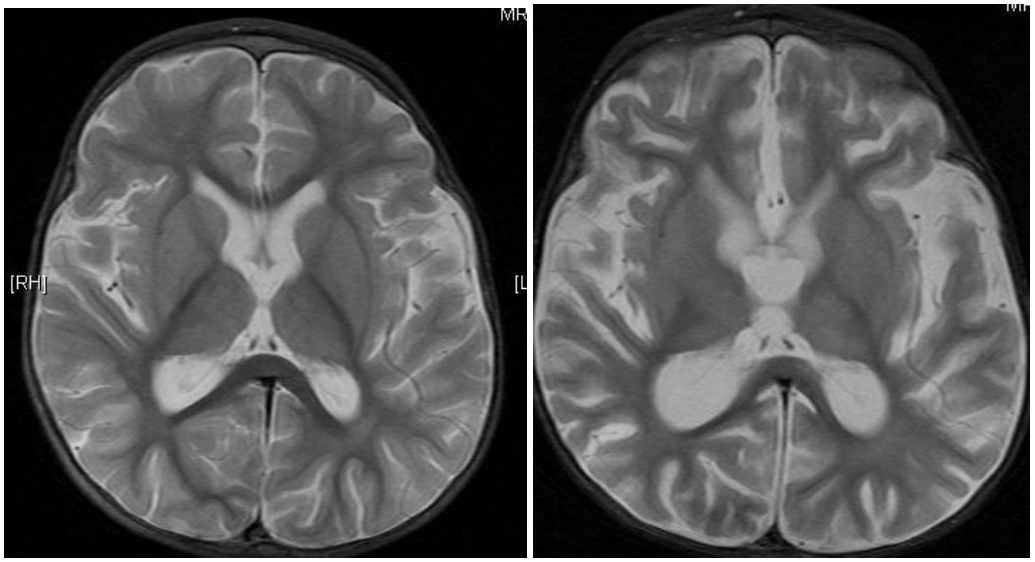
A meeting of the IDSMC concluded the death of patient A1/04 was unlikely to be related to treatment with the study drug and recommendation was given that the study may continue without modification.

Figure 2.4 Clinical summary for A1/04 (ADV viral load and CD4 count vs post-transplant days)



Time points when Astrovirus was identified in retrospective samples. **C** = CSF **S** = serum **St** = stool

Figure 2.5 MRI scans on A1/04 1 month apart showing progressive cerebral atrophy and widening of ventricles and sulci



May 2013 June 2013

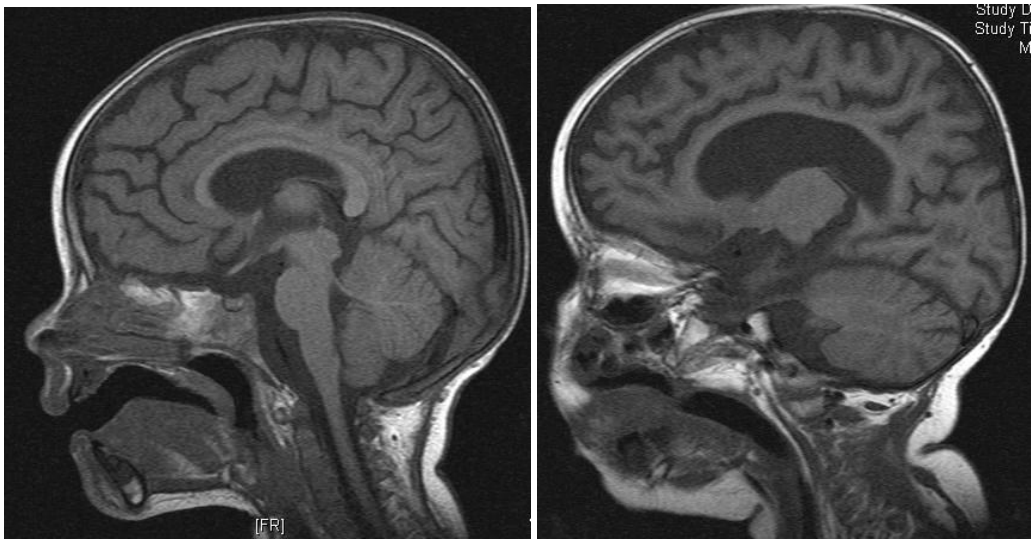
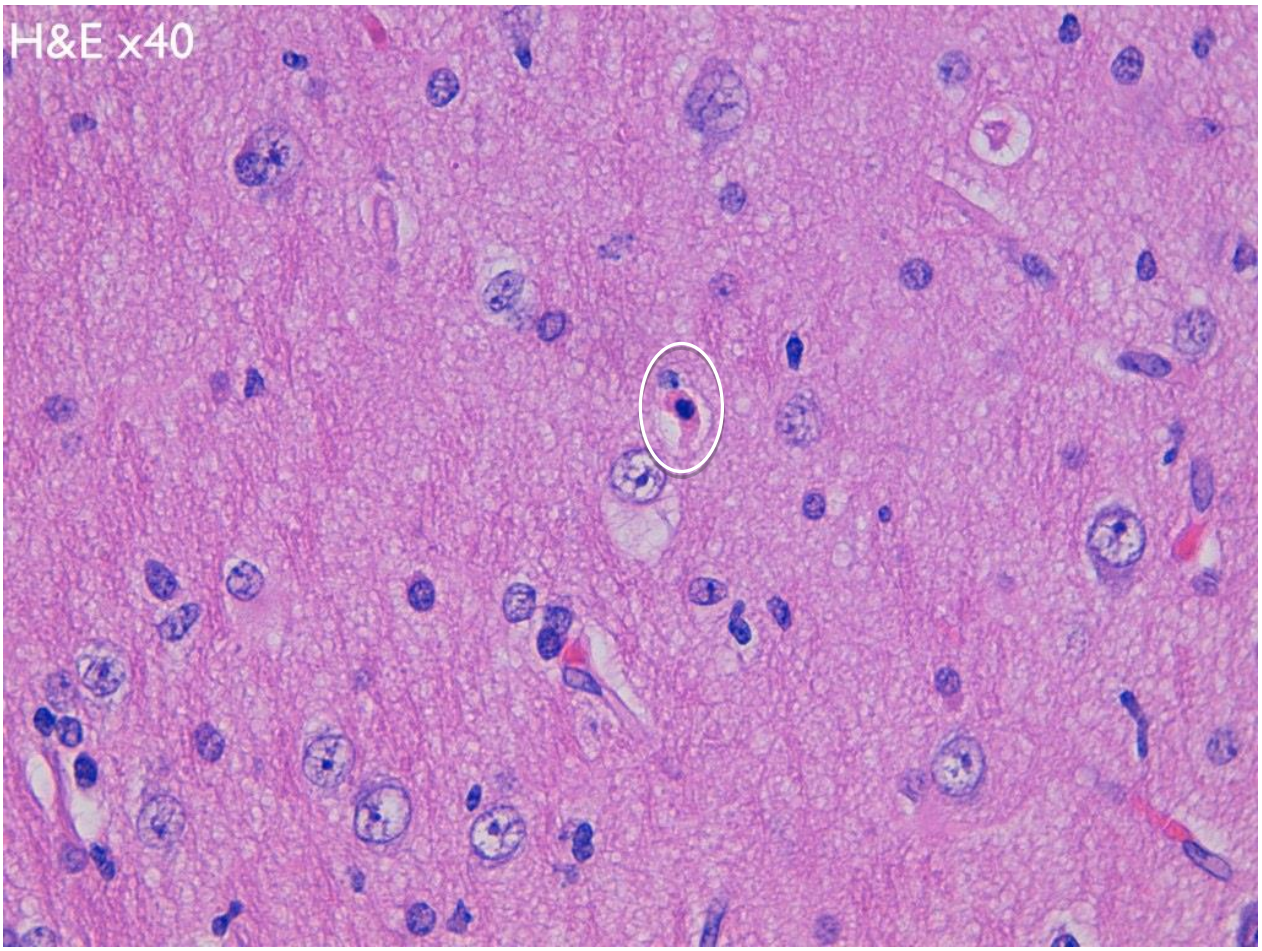
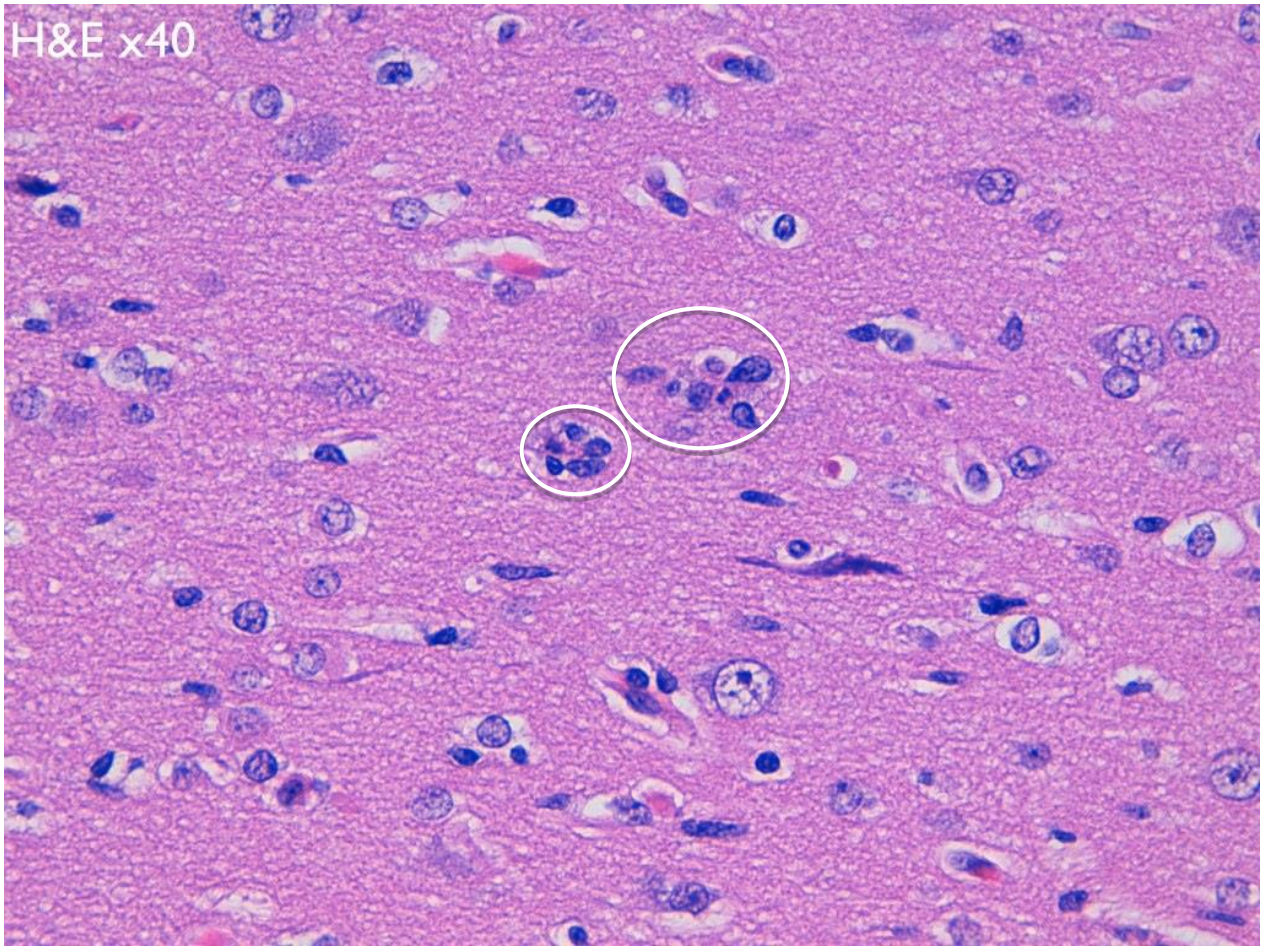


Figure 2.6 Brain biopsy slides on A1/04

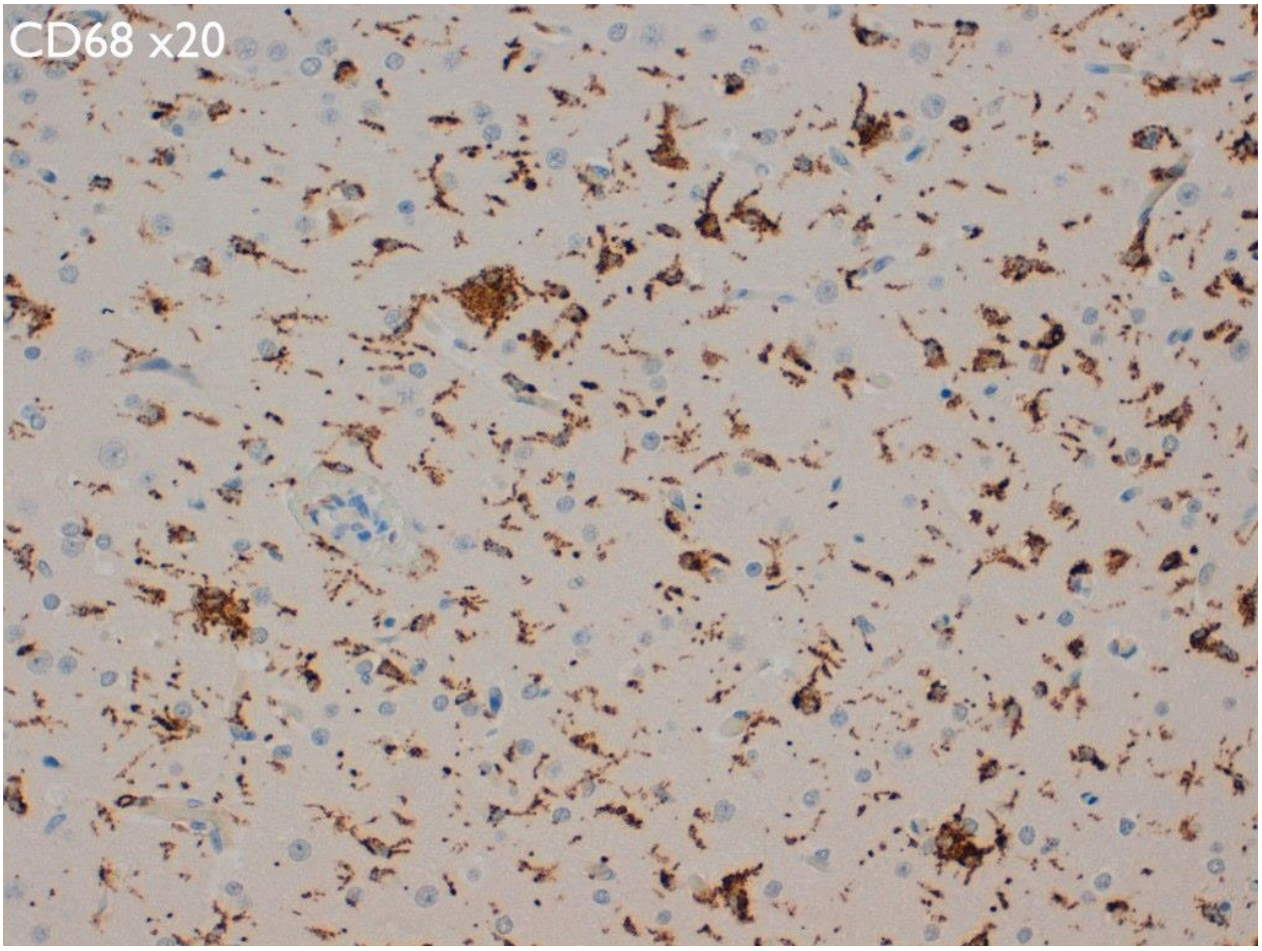
a) Neuronal death



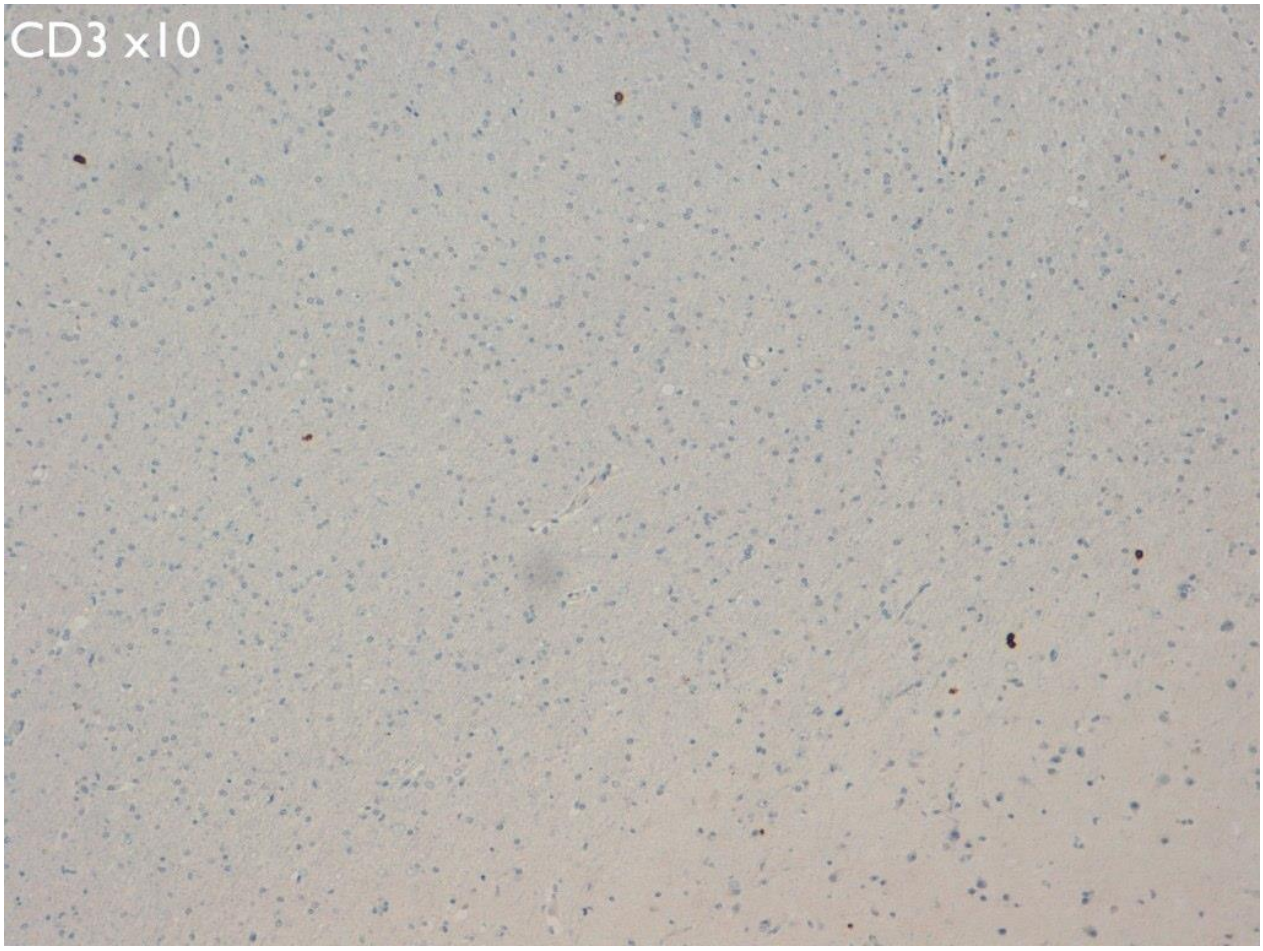
b) Neurophagia



c) Microglia



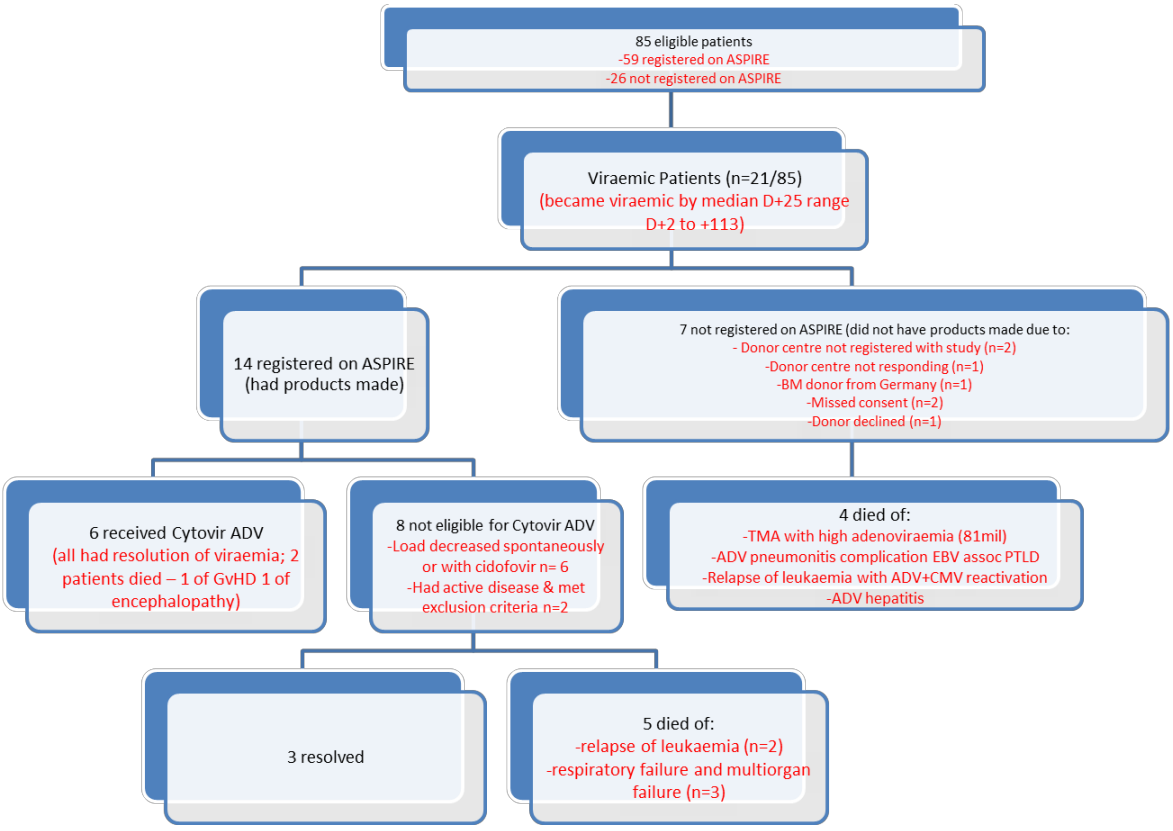
d) No increase in CD3 staining



2.8 PATIENTS WITH ADENOVIRAEMIA DURING TRIAL PERIOD WHO DID NOT RECEIVE CYTOVIR ADV

During the trial period from January 2013 to May 2015 a total of 21 patients became viraemic by median Day +25 (range +2 to +113) (Figure 2.7). Out of these 21 patients, 14 patients were registered on ASPIRE study of which 6 were treated with Cytovir ADV. The remaining 8 patients did not meet criteria to receive treatment with Cytovir ADV because of 1) ADV viral load in blood decreased spontaneously or with Cidofovir treatment (n=6) 2) active ADV infection and met exclusion criteria (n=2). Five of these 8 patients eventually died of multiorgan failure (n=3) or relapse of leukaemia (n=2). Two of the deaths were attributable to ADV infection. Seven patients were not registered on ASPIRE study (and therefore did not have Cytovir ADV products made) due to various reasons: 1) donor center was not registered with the study (n=2) 2) donor center did not respond in time with donor consent (n=1) 3) bone marrow donor from Germany early on in the trial when overseas shipment of peripheral blood had not gained approval (n=1) 4) missed consent (n=2) 5) donor declined (n=1). Out of these 7 patients, 4 died of ADV related diseases.

Fig 2.7 Patients eligible to enter ASPIRE (January 2013 to May 2015)



2.8.1 Patients who were registered on ASPIRE but not eligible for Cytovir ADV on reactivation

Out of the 14 patients who were registered on ASPIRE study and had products made from their original donors, 8 developed adenoviraemia post HSCT but did not receive ADV CTLs either because 1) viral load decreased spontaneously or with treatment with Cidofovir (n= 6) or 2) because exclusion criteria for product administration were met (n=2). The details of each of these patients around their transplant periods are as follows:

A1/11 was a 2-year and 9-month old boy with Idiopathic Severe Aplastic Anaemia who underwent a fully-matched 10/10 family donor peripheral blood stem cell transplant with Fludarabine/Cyclophosphamide/Alemtuzumab conditioning. ADV became detectable in blood on D+31 post transplant at load of 3,001 copies/mL, and again on D+32 at 4,655 copies/mL. ADV viral load in blood became and stayed undetectable from D+35 onwards without antiviral treatment. ADV was also detectable in stool from D+21 and became undetectable from D+87. Patient received treatment with Ganciclovir and Foscarnet for CMV viraemia from D+35 onwards with good response. Patient is currently well 2 years post-transplant.

A1/16 was a 4 year old boy with Autosomal recessive chronic granulomatous disease who had undergone a fully-matched 10/10 unrelated donor bone marrow transplant with reduced intensity Fludarabine/Busulphan/Alemtuzumab conditioning. ADV became detectable in blood on D+41 post transplant at load of 11,635 copies/mL and again on D+46 (38,206 copies/mL) and D+48 (24,322 copies/mL). ADV became undetectable in blood from D+52 onwards. ADV was detectable in stool from D+17 to D+52 and became undetectable from D+87 onwards. Patient did not require antiviral treatment with Cidofovir for adenoviraemia however did receive ganciclovir and foscarnet treatment for CMV viraemia (D+27 to D+40) and one dose of Rituximab for EBV viraemia on D+52. Patient is well 2 years post-transplant.

A1/19 was a 5-year old boy with XIAP deficiency and EBV-driven HLH who initially underwent a fully matched bone marrow transplant with Fludarabine/Cyclophosphamide/Alemtuzumab/Anti-CD45 monoclonal antibody conditioning. Six months later he developed relapse of HLH as well as adenoviraemia with load up to 6 million copies/L. He received a second transplant from the original donor this time with peripheral blood as stem cell source and reduced intensity conditioning regimen with Fludarabine/Cyclophosphamide/Alemtuzumab. Patient was in intensive care at the time and died 6 days after the second transplant with a high adenovirus load in blood of 11 million copies/mL.

A1/23 was an 8-month old boy with RAG1 Severe Combined Immunodeficiency who underwent haploidentical stem cell transplant with Treosulfan/Fludarabine/Thiotepa/ATG conditioning and alpha/beta T cell depletion. ADV was detectable in blood before transplant up to 14,000 copies/mL on D-22. Adenoviraemia peaked post-transplant on D+9 to 236,950 copies/mL but decreased to low level by D+30 (1344 copies/mL). ADV was also detectable in stool and nasopharyngeal aspirate. Given the low level of viral load patient did not meet criteria for ADV CTLs. Respiratory failure on D+35 resulted in admission to intensive care for invasive ventilation and patient passed away on D+51 from pulmonary haemorrhage and multiorgan failure. ADV viral load in blood on D+48 was 3564 copies/mL.

A1/25 was an 18-month old girl with bilineage acute leukaemia who underwent a 9/10 1A mismatched unrelated bone marrow transplant conditioned with treosulfan/fludarabine/thiotepa/ATG. She developed adenoviraemia on D+20 with viral load peaking at 42,500 copies/mL on D+24. Patient was treated with 3 doses of Cidofovir (D+22, D+29, D+41) with eventual clearance of ADV in blood on D+67. As viral load had reduced to 3000 copies/mL by D+31 decision was made to continue with Cidofovir treatment alone. Unfortunately patient experienced a relapse 4 months after transplant and eventually died 8 months post-transplant.

A1/27 was a 7-year old boy with secondary AML/MDS diagnosed after treatment for cerebellar medulloblastoma. He received a fully matched unrelated donor stem cell transplant with reduced intensity Fludarabine/Melphalan/Alemtuzumab conditioning. Patient developed low level adenoviraemia on D+36 at 1,019 copies/mL which peaked to 662,281 copies/mL on D+47. One dose of Cidofovir was administered on Day +57 with resultant decrease in viral load and disappearance from blood by D+84. Given good response to Cidofovir decision was made not to treat with ADV CTLs. Six months after transplant patient suffered relapse and severe gut GvHD and eventually died.

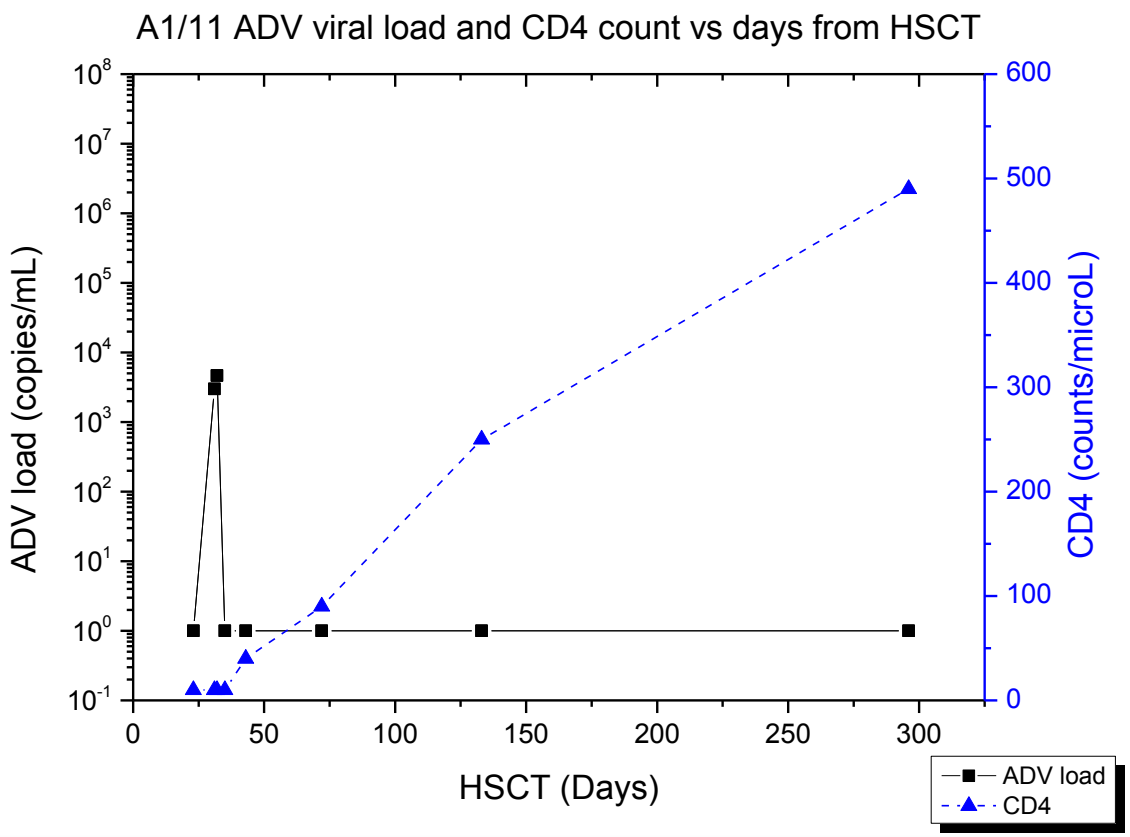
A1/29 was a 3-year old boy with XIAP deficiency and EBV-driven HLH. He underwent a 9/10 1DQ mismatched unrelated donor transplant conditioned with

Fludarabine/Cyclophosphamide/Alemtuzumab/Anti-CD45 monoclonal antibody. Adenovirus was first detected in blood on D+25 at 6,954 copies/mL rising to 55,000 copies/mL two days later. Patient was already unwell in PICU intubated and ventilated and eventually died on D+29. Because of concurrent pneumonitis at time of viral reactivation, patient did not meet eligible criteria to receive CTLs.

A1/51 was a 4.5 year old boy with X-linked Chronic Granulomatous Disease who underwent a 9/10 (1A) mismatched unrelated bone marrow transplant with reduced intensity Busulfan/Fludarabine/Alemtuzumab conditioning. ADV reactivation occurred in blood on D+34 starting with level of 1,910 copies/mL and peaking to 55,525 copies/mL on D+45. Treatment with Cidofovir was commenced on D+40 and total of 3 doses were given with good response. ADV viral load reduced to 3,633 on D+62 and became undetectable from D+68. Stool samples were positive for ADV from D+21 to D+111 and became undetectable from D+140. ADV was also detected in nasopharyngeal aspirates from D+32 to D+52 without causing respiratory symptoms. Patient is well 8 months post-transplant.

Figure 2.8 Graphs of ADV viral load and CD4 count (where available) of patients who were registered on study but not eligible for Cytovir ADV

A



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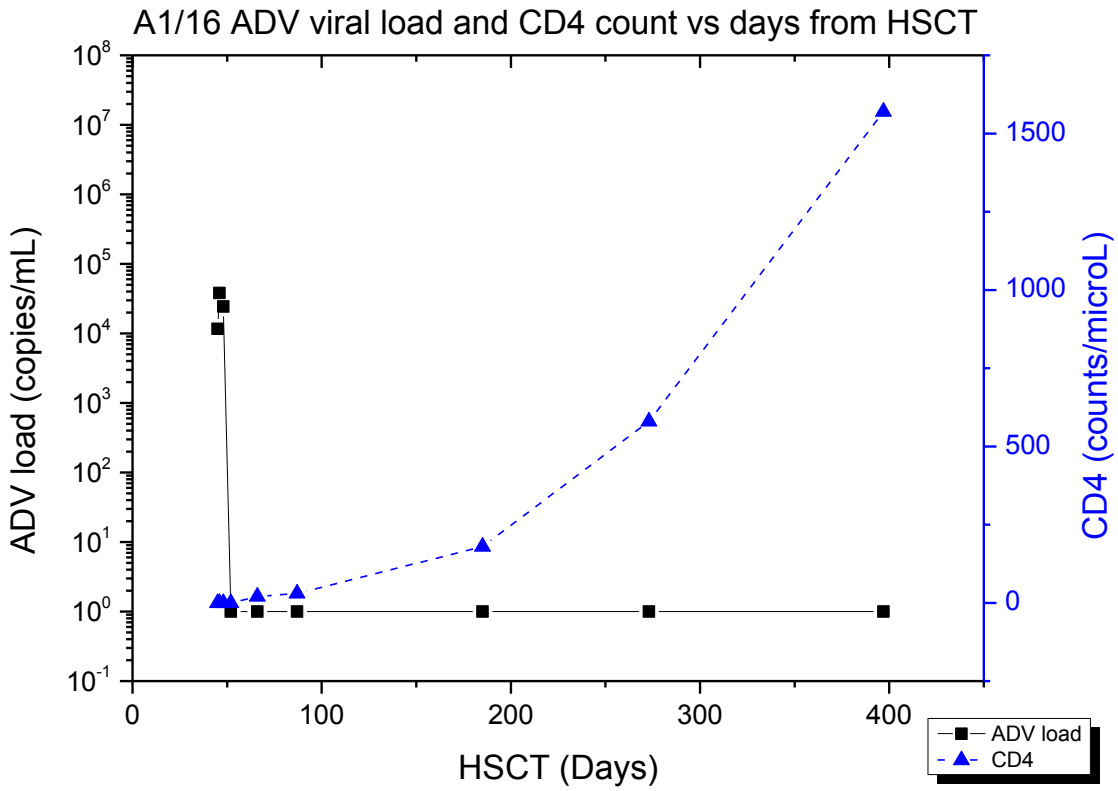
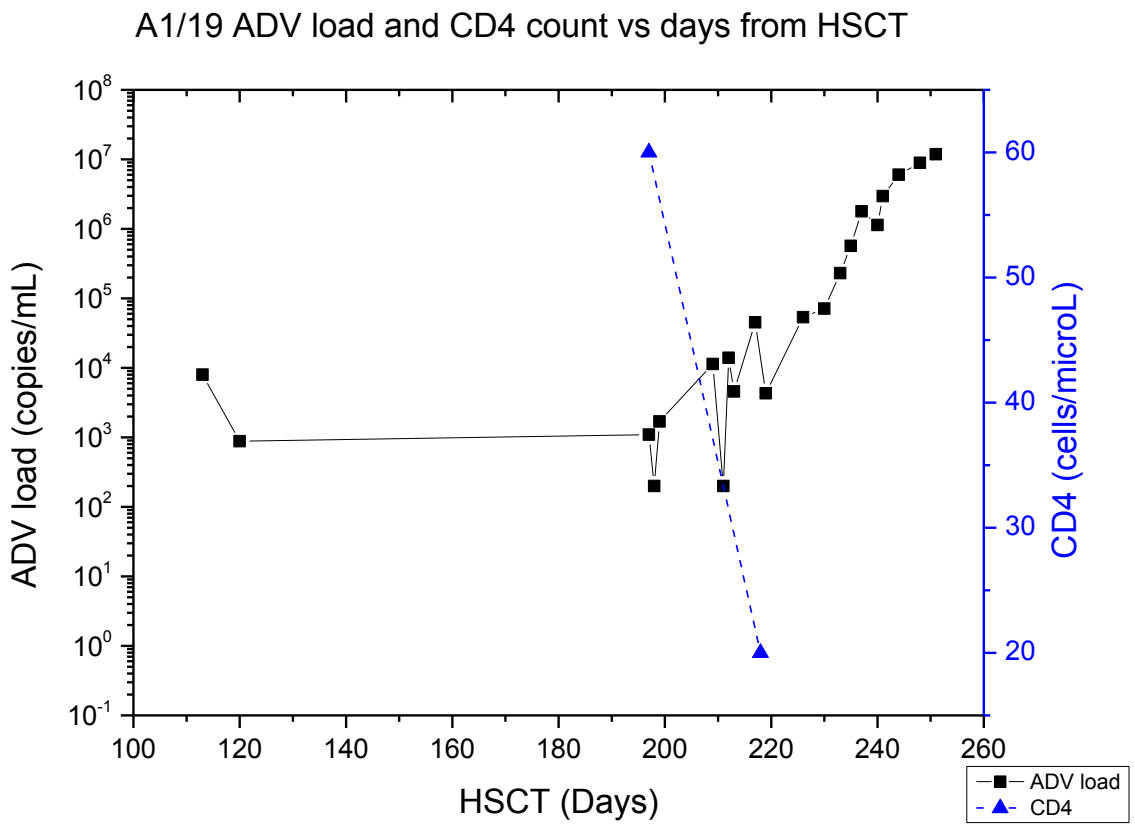
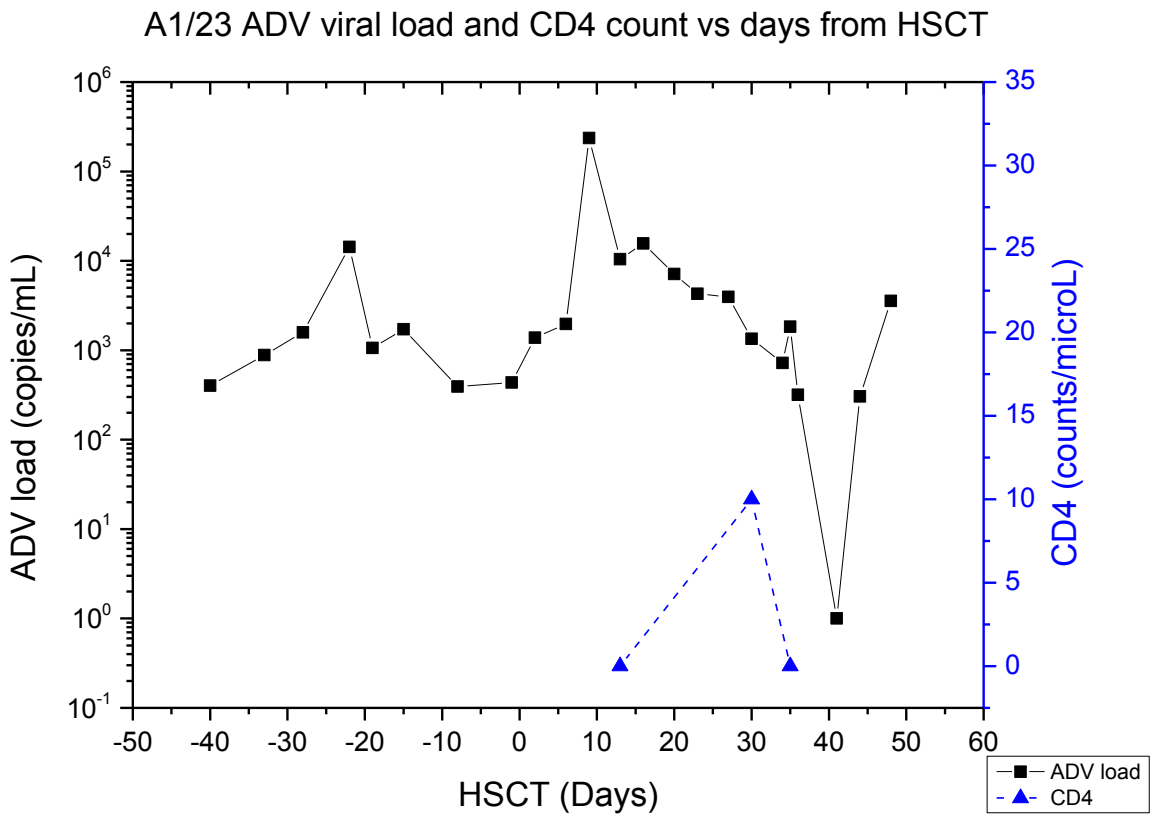

A1/16 ADV viral load and CD4 count vs days from HSCT
ADV load (copies/mL)
CD4 (counts/microL)
HSCT (Days)
ADV load
CD4

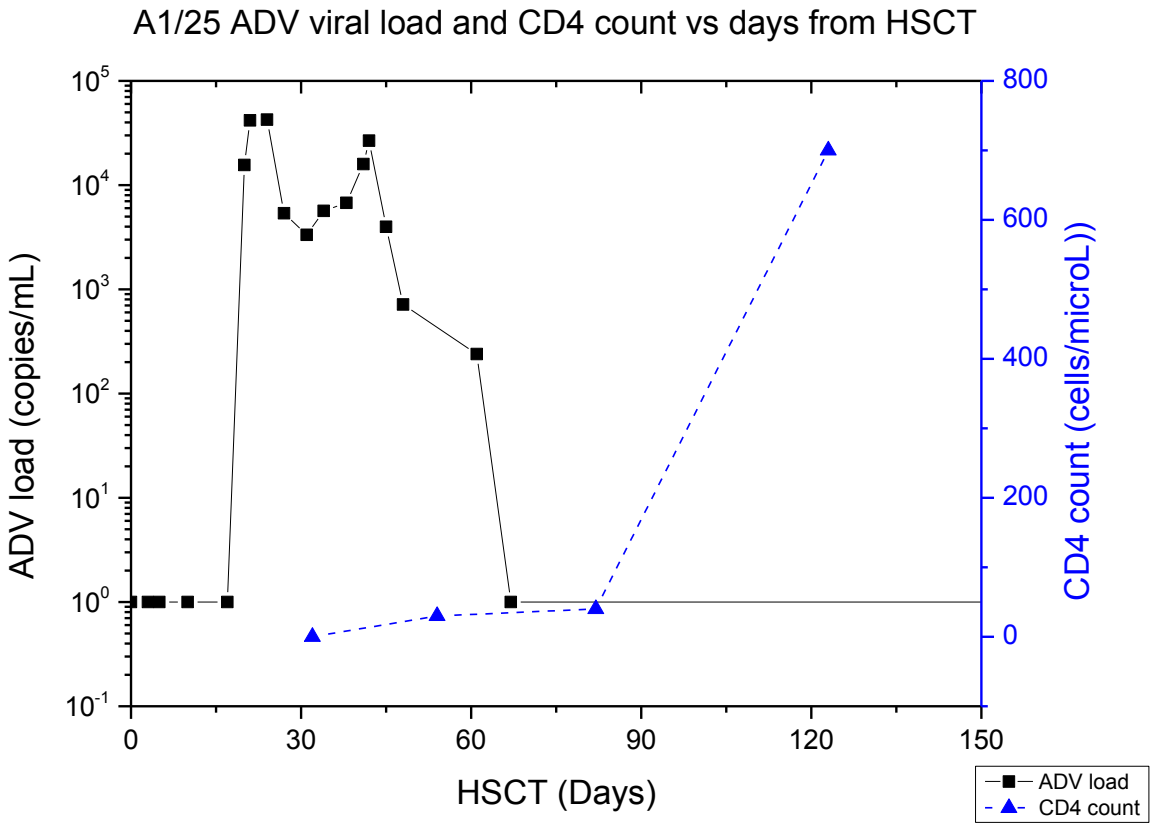
C


A1/19 ADV load and CD4 count vs days from HSCT
ADV load (copies/mL)
CD4 (cells/microL)
HSCT (Days)
ADV load
CD4

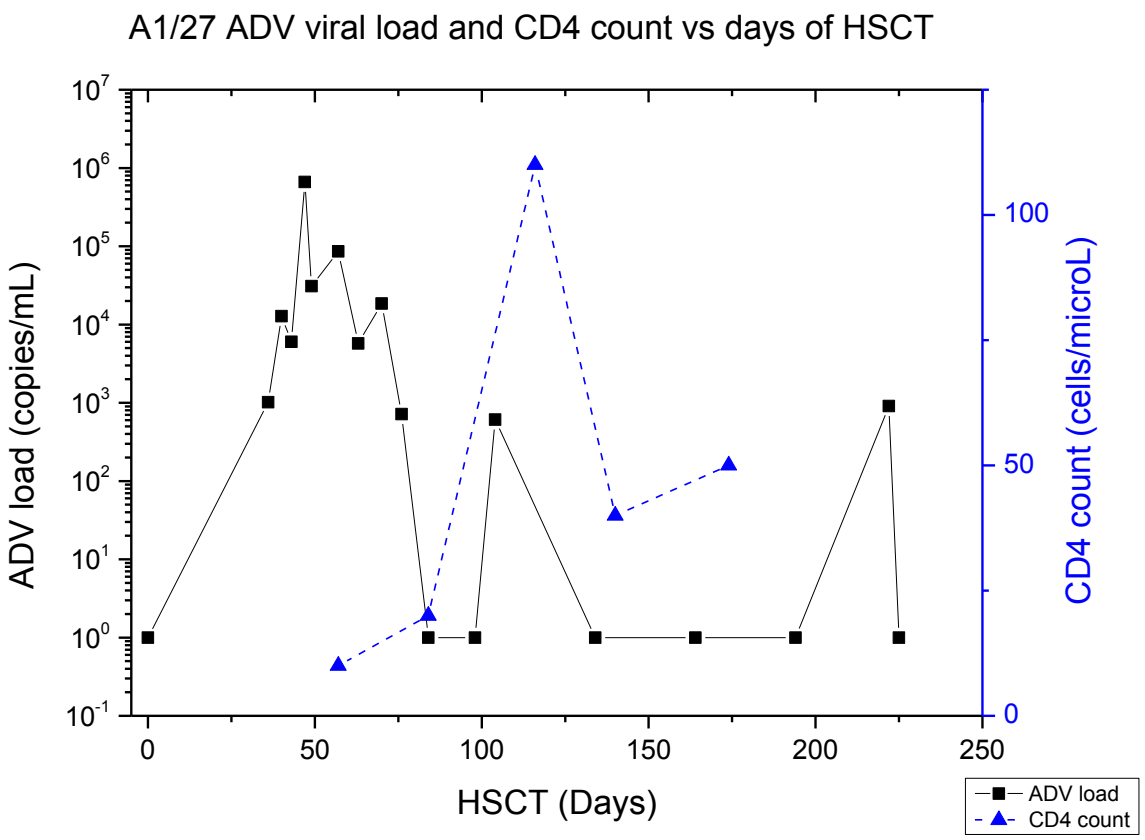
D


A1/23 ADV viral load and CD4 count vs days from HSCT
ADV load (copies/mL)
CD4 (counts/microL)
HSCT (Days)
ADV load
CD4

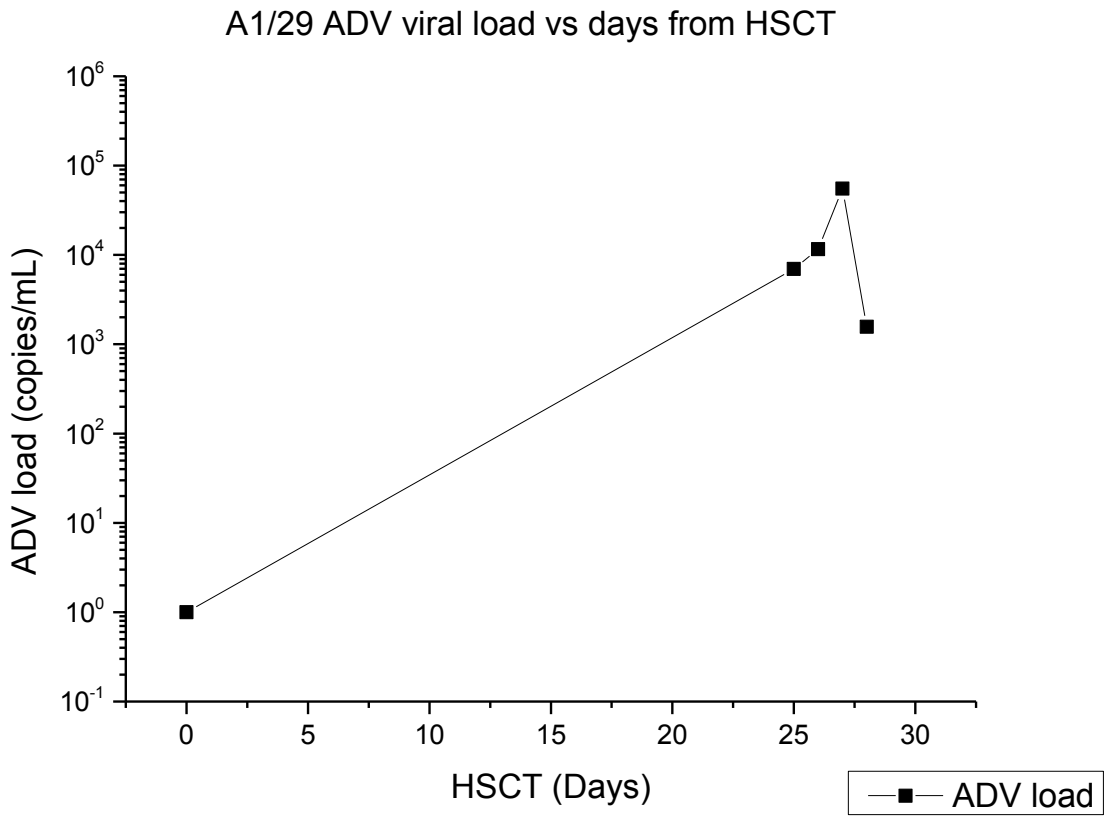
E


A1/25 ADV viral load and CD4 count vs days from HSCT
ADV load (copies/mL)
CD4 count (cells/microL))
HSCT (Days)
ADV load
CD4 count

F


A1/27 ADV viral load and CD4 count vs days of HSCT
ADV load (copies/mL)
CD4 count (cells/microL)
HSCT (Days)
ADV load
CD4 count

G


A1/29 ADV viral load vs days from HSCT
ADV load (copies/mL)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
0
5
10
15
20
25
30
HSCT (Days)
ADV load

H

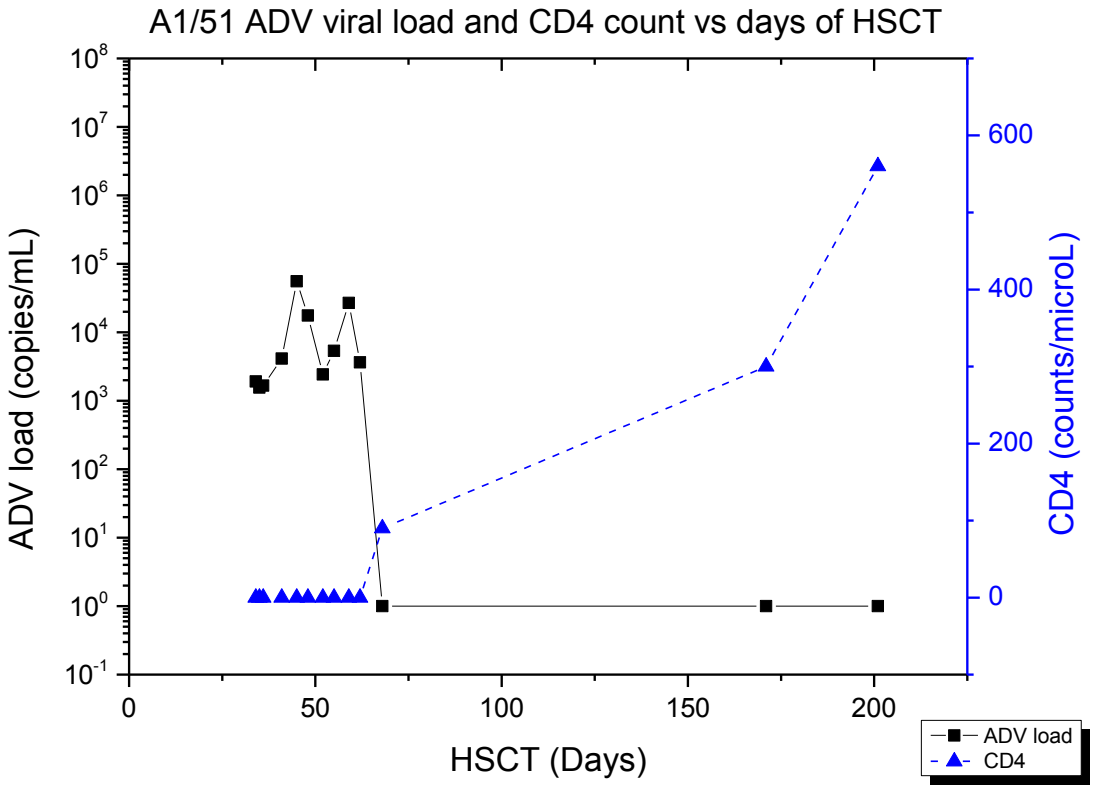

A1/51 ADV viral load and CD4 count vs days of HSCT
ADV load (copies/mL)
CD4 (counts/microL)
HSCT (Days)
ADV load
CD4

Table 2.3 Patient Characteristics (those with viraemia who received Cytovir ADV vs those with viraemia who did not receive Cytovir ADV)

	Received Cytovir ADV (n)	**Did not receive Cytovir ADV (n)**
Age [Years]		
<1	0	1
1-3	2	2
3-5	0	3
5-10	2	2
10-15	2	0
Diagnosis		
ALL	1	1
Familial HLH	2	0
Immunodeficiency	1	3
AML	1	1
ALD	1	0
XIAP deficiency	0	2
SAA	0	1
Donor Graft Type		
MUD	2	3
MMUD	2	3
Haplo	1	1
MMFD	1	0
MFD		1
Donor Source		
BM	1	3
PBSC	5	5
T cell depletion		
In vivo depletion (ATG)	2	2
Alemtuzumab	4	6
Graft manipulation	1	1

Antiviral Drugs		
Cidofovir before ACT	6	5
Cidofovir after ACT	3	NA
Time of 1st positive ADV PCR in blood [days post HSCT] (median, range)	12 (2-89)	3 (2-36)
Peak ADV load	>20 million	11 million
Site of local infection		
Stool	5	5
Nasopharyngeal Aspirate	2	3
Urine	1	0
Outcome		
Alive	4/6	3/8

Abbreviations: ALL = Acute Lymphoblastic Leukaemia; HLH = Haemophagocytic Lymphohistiocytosis; AML = Acute Myeloid Leukaemia; ALD = Adrenal Leukodystrophy; XIAP = X-linked inhibitor of Apotosis Protein; SAA = Severe Aplastic Anaemia; MUD = Matched unrelated donor; MMUD = Mismatched unrelated donor; Haplo = haploidentical; MMFD = Mismatched family donor; BM = bone marrow; PBSC = peripheral blood stem cell; ACT = advanced cell therapy

2.8.2 Patients who were eligible to enter ASPIRE but not registered and developed ADV reactivation/infection during transplant

During the trial period 26 patients (Figure 2.7) were eligible to enter the study but for a number of reasons were not registered. These reasons included 1) missed consent 2) overseas bone marrow donor (the approval to process bone marrow harvest as starting material came later for the German registry. Approval not gained for NMDP registry) 3) A German donor center not

registered with the study 4) non-response from registry 4) lab closure 5) donor declined consent. Out of these 26 eligible patients, 7 patients developed adenoviraemia post-transplant; 4 patients died, with ADV a contributing cause in all cases. The clinical details of each of these patients are as follows:

Patient KD – KD was an 11 year old boy with Chediak-Higashi Syndrome and Haemophagocytic Lymphohistiocytosis who underwent a 9/10 (1C) mismatched unrelated donor stem cell transplant with CD34 selection and CD3 add-back. Conditioning with carried out with Fludarabine (30mg/m^2 x5 Days -7 to -3), Melphalan (140mg/m^2 Day -2) and Alemtuzumab (0.2mg/kg x5 Days -8 to -4); with Ciclosporin and Mycophenolate Mofetil (MMF) as GvHD prophylaxis. He was not registered on the ASPIRE study because the NMDP donor center was not registered with the study. He developed adenoviraemia on D+75 at 1,210 copies/mL which quickly rose to 216,020 copies/mL on D+82. Treatment with Cidofovir was started and adenoviral load in the blood became non-detectable from D+140. This also co-incided with immune reconstitution with CD4 count rising to 0.13x10^9/L on D+140 and steadily rising since.

Patient RL – RL was a 9 month old boy who presented at the age of 4 months old with M5 Acute Myeloid Leukaemia with a high risk MLL rearrangement t(10:11). He received chemotherapy and underwent a fully-matched 10/10 unrelated donor bone marrow transplant for high risk AML in second morphological remission. Conditioning was carried out with Busulfan (targeted

AUC <70mg/Lhr), Cyclophosphamide (60mg/kg x2 Days -3,-2), melphalan (140mg/m^2 Day -1), and Alemtuzumab (0.2mg/kg x5 Days -7 to -3). Cyclosporin A was used as GvHD prophylaxis. He was not registered on the ASPIRE study as the donor was from a German registry and donating bone marrow, and the required approvals were not yet in place to receive and process peripheral blood sample necessary for manufacturing of CTLs (Cytovir ADV) from overseas donor. RL developed adenoviraemia early in the post-transplant period with viral load of 5,000 copies/mL on D+15 and quickly progressed to >2 million copies/mL on D+22. The viral load remained persistently high. And although he achieved 100% donor chimerism on D+28, he had no donor T cell recovery with lymphocyte count steadfastly remaining zero. RL also developed high level and persistent CMV viraemia despite treatments with foscarnet, ganciclovir, and cidofovir for his adenoviraemia. On D+43 RL developed oxygen dependence requiring up to 2L via nasal cannulae along with interstitial changes on CXR suggestive of viral pneumonitis. Adenovirus was detected in nasopharyngeal aspirates from D+28 onwards. Adenoviral load in blood reached a peak of 15 million copies/mL on D+89. In addition, a bone marrow investigation on D+90 revealed frank relapse with 70% blasts. He also developed hepatitis with raised liver enzymes and bilirubin, thought most likely secondary to adenoviral and/or CMV infection. Child was eventually taken to a hospice for end of life care and died shortly after.

Patient AW – AW was an 8 year old girl who received a mismatched (9/10 1DQ) unrelated donor peripheral blood stem cell transplant for relapsed Acute

Lymphoid Leukaemia. Conditioning was carried out with Total body irradiation (200 cGYx2# Days -8 to -6), Etoposide (60mg/kg D-3) and Anti-thymocyte Globulin (ATG; 20mg/kg Days -2,-1). GvHD prophylaxis was undertaken with ciclosporin and methotrexate. AW was not registered on the ASPIRE study as opportunity to take consent from the donor was missed. Full donor chimerism was achieved on D+25. AW developed adenoviraemia on D+50 initially at low level of 571 copies/mL but rising to 823,541 copies/mL on D+77. Treatment with Cidofovir was started. Adenoviral load peaked at 13 million copies/mL on D+85. Cidofovir treatment was continued until D+105 and adenovirus became undetectable in blood from D+133 onwards. This co-incided with reconstitution of CD4 count from zero to $0.15x10^9$/L on D+133. AW has remained virus free since.

Patient EH – EH was a 7 month-old boy who received a 12/12 fully matched unrelated donor peripheral blood stem cell transplant for hereditary multiple intestinal atresia. Conditioning was carried out with Treosulfan (12 g/m^2 x3 Days -6 to -4), Fludarabine (30 mg/m^2 x5 Days -6 to -2), and Alemtuzumab (0.2 mg/kg x5 Days -8 to -4); and patient received Ciclosporin and Mycophenolate Mofetil as GvHD prophylaxis. EH was not registered on the ASPIRE study as opportunity to take consent from the donor was missed. He achieved full donor chimerism on D+18. Significant adenoviraemia was first detected on D+13 at 1,542 copies/mL, with a maximum load of 7,213 copies/mL on D+20. He was not started on treatment with Cidofovir and had no signs of ADV infection. Adenovirus became non-detectable in blood from D+75 onwards with steady

increase in CD4 count from D+75 at $0.80x10^9$/L and increasingly steadily to $0.85x10^9$/L on D+354. Adenovirus was also detected in nasopharyngeal aspirates between D+4 to D+52; and detected at low levels in stool between D+3 and D+59.

Patient BJ – BJ was a 2year 11month old boy with X-linked CGD who received a fully matched unrelated donor bone marrow transplant with reduced intensity conditioning. Conditioning regimen consisted of Busulphan (targeting cumulative AUC 45-65mg/L/hr), Fludarabine ($30mg/m^2$ x6 Days -8 to -3), and Alemtuzumab (0.5 mg/kg total split over days -8 to -6). Patient received Ciclosporin and Mycophenolate Mofetil as GvHD prophylaxis. BJ was not registered on the ASPIRE study as the German donor center was not registered with the study at the time. He achieved full donor chimerism in peripheral blood on D+18 post transplant. Adenovirus was detected in the blood on D+56 at 49,419 copies/mL and remained persistent with a peak level of 81 million copies/mL on D+270, despite treatment with Cidofovir (total of 13 doses). BJ also developed other complications including gut GvHD requiring heavy immunosuppressants including steroids, infliximab, basiliximab and tacrolimus. He also developed TMA and failed treatment with Eculizumab. He was later found to be heterozygous for the *CFH* c.3356A>G p.(Asp1119Gly) pathogenic mutation which most likely explained his post BMT thrombotic microangiopathy. He was also heterozygous for the *C5* c.2654G>A polymorphism which is associated with a poor response to eculizumab. The highest CD4 count recorded during the post-transplant period was $0.12x10^9$/L on D+209. After

much discussion with other specialist teams and centers, and with his parents, symptom care took over active management and child passed away D+275 post transplant.

Patient CD – CD was a 6 year old boy with relapsed Acute Lymphoid Leukaemia who received a fully matched unrelated donor peripheral blood stem cell transplant. Conditioning was undertaken with Total Body Irradiation (200 cGy x 2# - Days -9 to -7), Etoposide (60mg/kg Day -4) and Anti-thymocyte globulin (20mg/kg x3 – Days -4 to -2). Patient received Ciclosporin and Mycophenolate Mofetil as GvHD prophylaxis. CD was not registered on the ASPIRE study due to lack of response from donor center regarding donor consent for the study. He achieved full donor chimerism in peripheral blood on D+24 post transplant. Adenovirus was first detected in the blood on D+13 at 46,273 copies/mL. The viral load rose to 329,632 copies/mL on D+17 and treatment with Cidofovir was started. Eventually viral load decreased to <200 on D+194. Adenovirus was also detected in the stools from D+11. CD also developed EBV viraemia on D+39 starting at 60,872 copies/mL and quickly rising to 256,895 copies/mL 3 days later. Treatment with Rituximab was started. Despite being treated with a total of 7 doses of Rituximab, his EBV viral load remains elevated with image findings consistent with Lymphoproliferative Disease. CD's post-transplant period was also complicated by the development of acute grade 4 gut and grade 3 skin GvHD requiring treatment with high dose steroids, infliximab, basilixumab, and mesenchymal stem cells. He was discharged home on D+186 post-transplant. He continued to be on

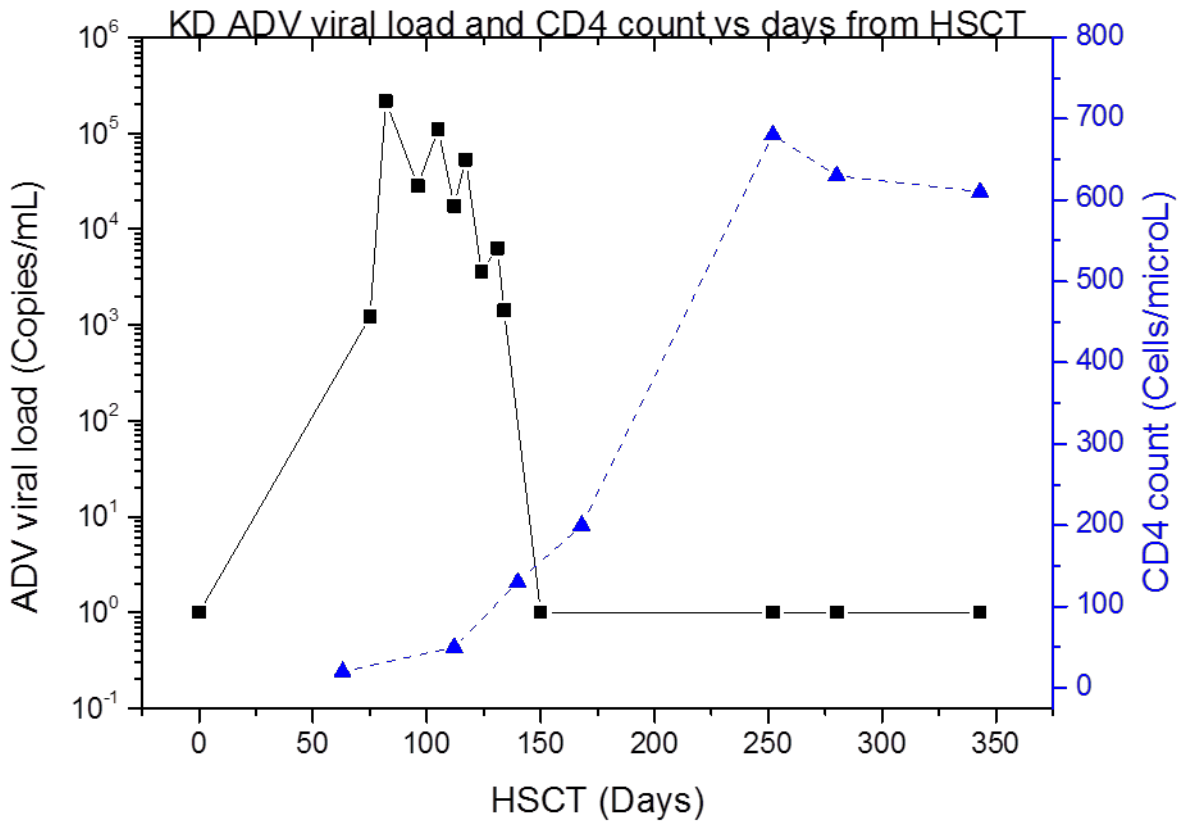
immunosuppression for control of his GvHD. He was readmitted on D+552 with non-specific symptoms of lethargy and fatigue, and finding of palpable abdominal mass on examination. This was later confirmed to be monomorphic Post Transplant Lymphoproliferative Disease on biopsy. He was started on treatment with methylprednisolone 60mg/m^2 according to COP protocol, cyclophosphamide 300 mg/m^2, Vincristine 1mg/m^2 and Rituximab 375 mg/m^2. Two days after the start of his chemotherapy he developed respiratory distress and required admission to PICU for intubation and ventilation. Adenovirus was detected in the blood on D+556 at 5,414 copies/mL quickly rising to 18,194 on D+559 with peak of 1,134,740 copies/mL on D+570. A bronchoalveolar lavage detected adenovirus with CT of 14. Treatment with Cidofovir was started but ADV load continued to rise. Because of the heavy immunosuppression he required immune reconstitution remained poor with only CD4 count of 0.04 x 10^6/L by D+570. Sadly CD did not show any sign of clinical improvement despite maximal therapy and died D+575 post-transplant of ADV pneumonitis complicating EBV associated PTLD.

Patient NR – NR was 7 year old girl with RAG 1 and 2 deficient SCID who received a 9/10 (1C) mismatched unrelated donor peripheral blood stem cell transplant. Reduced intensity conditioning was carried out with Fludarabine (30mg/m^2 x5 Days -7 to -3), Melphalan (140mg/m^2 Day -2) and Alemtuzumab (0.2mg/kg x5 Days -8 to -4). NR received Ciclosporin and Mycophenolate mofetil as GvHD prophylaxis. NR was not registered on the ASPIRE study as donor declined consent. NR developed 100% donor chimerism on D+21.

Adenoviraemia was detected early post-transplant on D+5 at 6,716 copies/mL. The viral load quickly rose to 10,682,000 copies/mL on D+12; peaking and persisting at >20 million copies/mL (max detectable level by PCR in our lab). Taking into account a high risk of GvHD but acknowledging the severity of ADV reactivation, immunosuppression was weaned from day +13 onward. Treatment with weekly cidofovir was started on Day+11. NR also developed deranged liver function from D+21 with rising ALT, gammaGT and bilirubin most likely reflecting an ongoing ADV related hepatitis. NR also developed gastro-enteric bleeding likely reflecting ADV enteritis. NR further deteriorated on D+28 post-transplant with deranged clotting, rising ALT and ascites. She developed signs of confusion, rising ammonia and sustained a sudden onset of seizures followed shortly after by hypoxic related cardiac arrest. CT scan of the head revealed an intracranial right parietal haematoma. Unfortunately NR's condition did not stabilize on PICU and sadly passed away on day +29 after another severe hypotensive episode which caused irreversible cardio-vascular arrest. The cause of death was identified as liver failure as a consequence of Adenovirus hepatitis; in the context of severe immunosuppression 29 days after a mismatched unrelated stem transplant for RAG I/II deficient severe combined immunodeficiency.

Figure 2.9 Graphs of ADV viral load and CD4 count (where available) of patients who were eligible to enter study but did not get registered

A



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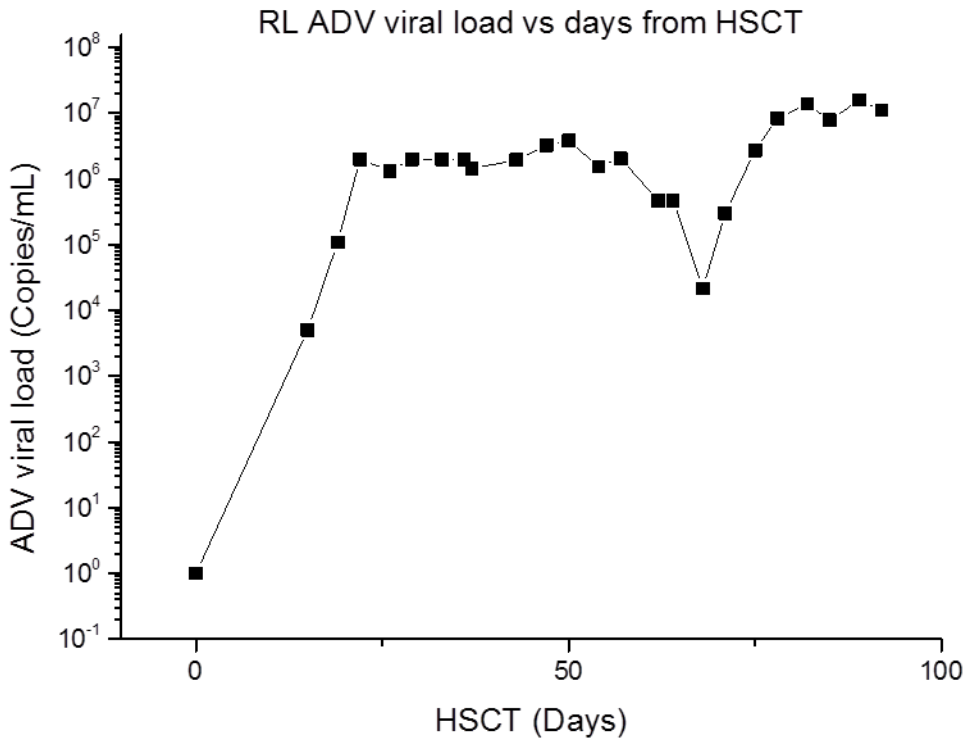
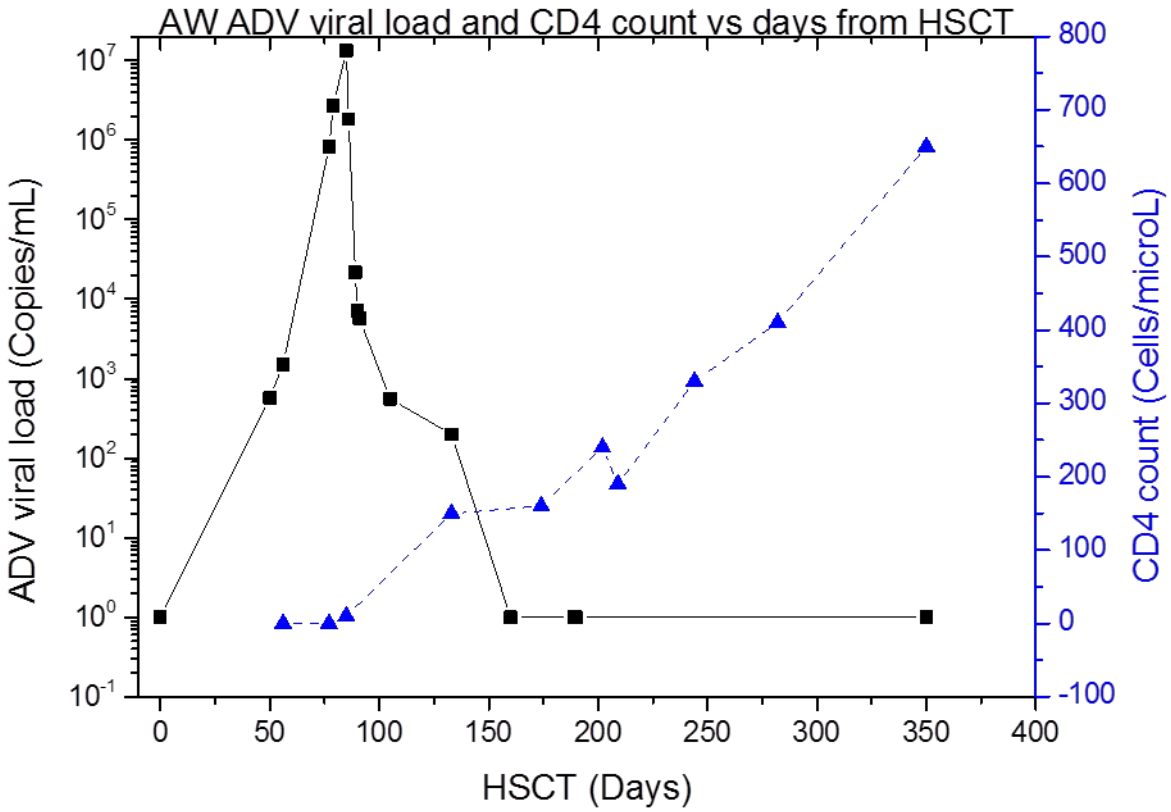
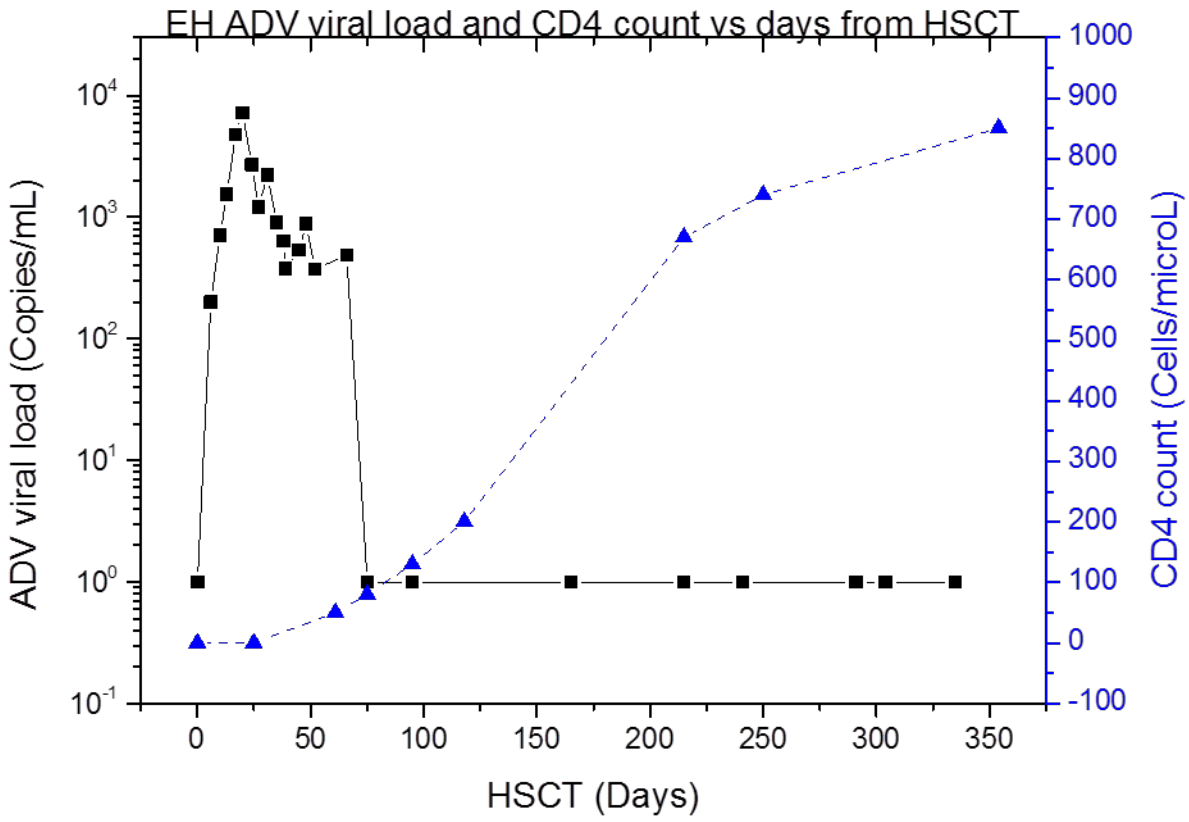

RL ADV viral load vs days from HSCT
ADV viral load (Copies/mL)
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
0
50
100
HSCT (Days)

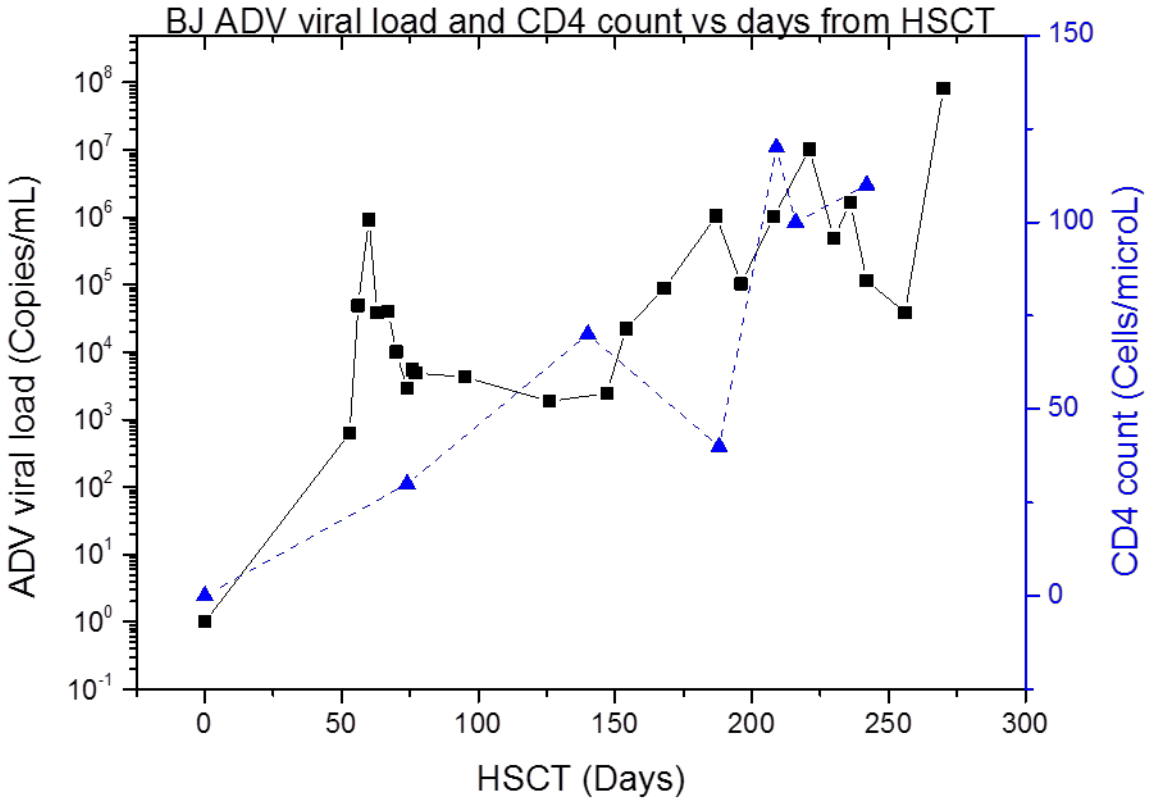
C


AW ADV viral load and CD4 count vs days from HSCT
ADV viral load (Copies/mL)
CD4 count (Cells/microL)
HSCT (Days)
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
800
700
600
500
400
300
200
100
0
-100
0
50
100
150
200
250
300
350
400

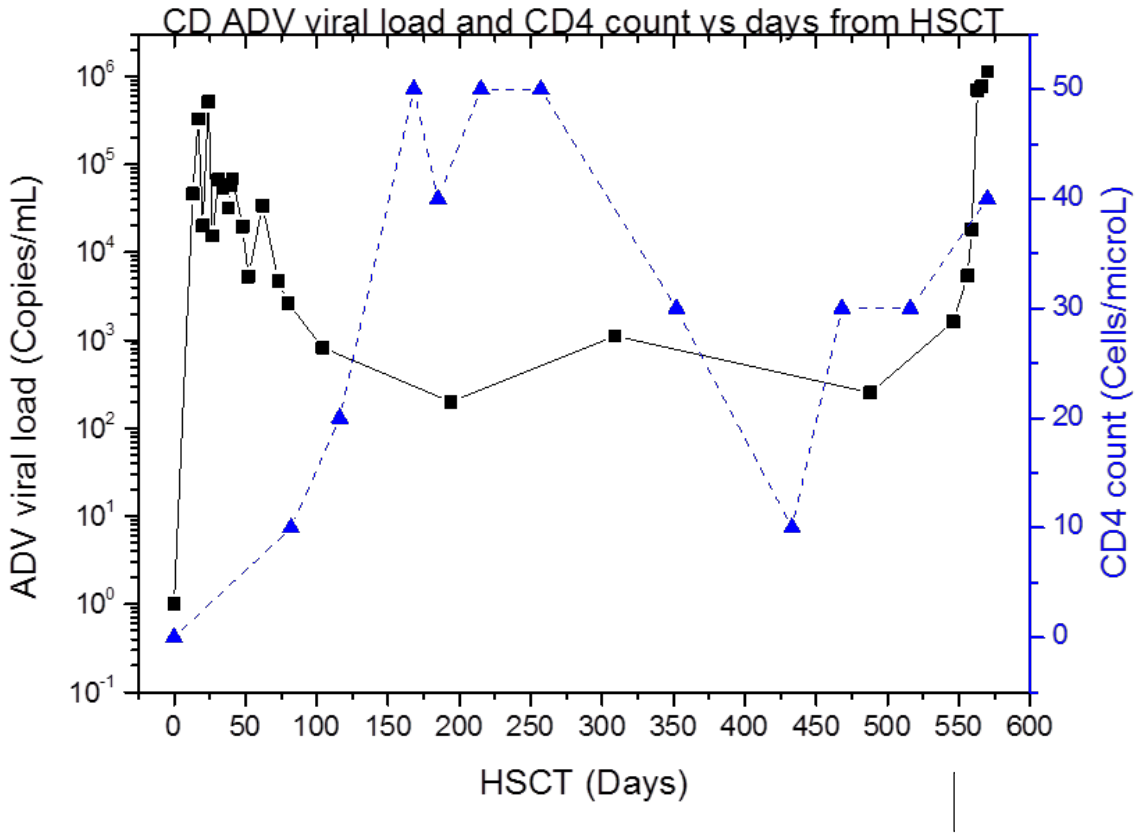
D


EH ADV viral load and CD4 count vs days from HSCT
ADV viral load (Copies/mL)
CD4 count (Cells/microL)
HSCT (Days)
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
1000
900
800
700
600
500
400
300
200
100
0
-100
0
50
100
150
200
250
300
350

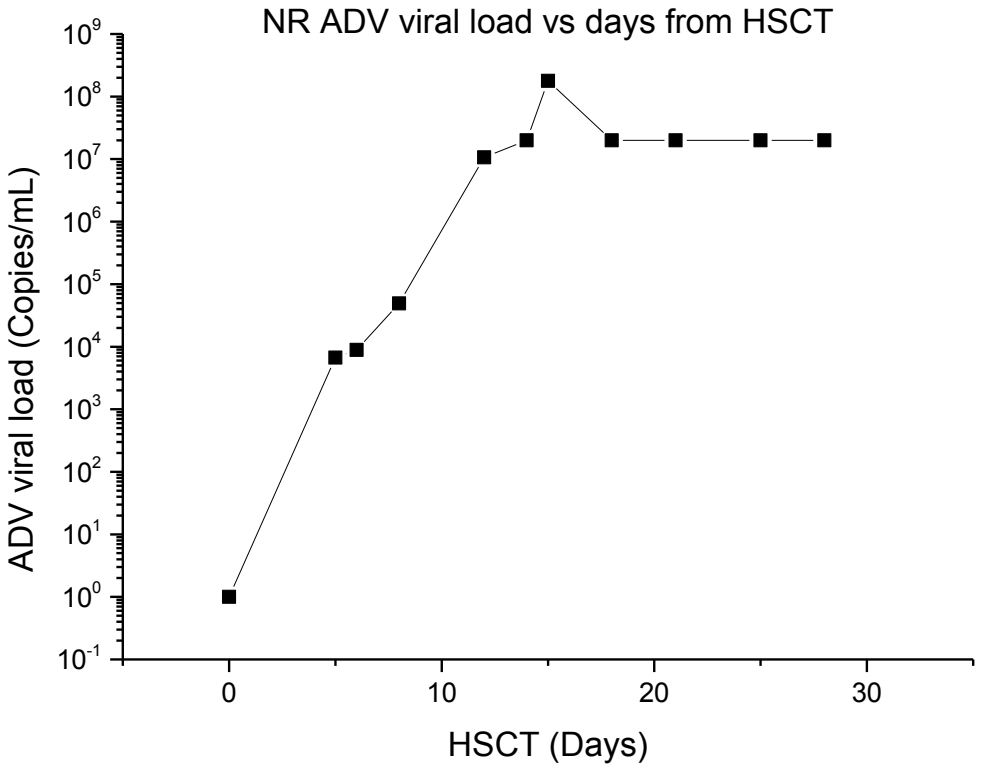
E


BJ ADV viral load and CD4 count vs days from HSCT
ADV viral load (Copies/mL)
CD4 count (Cells/microL)
HSCT (Days)
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
150
100
50
0
0
50
100
150
200
250
300

F


CD ADV viral load and CD4 count vs days from HSCT
ADV viral load (Copies/mL)
CD4 count (Cells/microL)
HSCT (Days)
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
10^{6}
0
10
20
30
40
50
0
50
100
150
200
250
300
350
400
450
500
550
600

G


NR ADV viral load vs days from HSCT
ADV viral load (Copies/mL)
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
0
10
20
30
HSCT (Days)

CHAPTER 3

EXPANSION OF ADV VIRUS SPECIFIC T-CELLS (VSTs) FROM HEALTHY VOLUNTEERS AND CHARACTERISATION OF THESE VSTs

3.1 EXPANSION OF ADV-SPECIFIC T-CELLS

Objective:

To generate ADV-specific T cells from 10 healthy volunteers using peripheral blood as starting material.

Methods:

The starting material is a voluntary donation of 50mL heparinised whole blood. Peripheral blood mononuclear cells (PBMCs) are derived from the starting material by Ficoll/Paque (Pharmacia, Uppsala, Sweden) density gradient separation and resuspended at a cell density of 10^6/mL in culture medium RPMI 1640 (LifeTechnologies, New York, USA) supplemented with 10% human AB serum (Lonza, Walkersville, USA). A total of $2x10^7$ PBMCs (20mL of cell suspension) are transferred to a cell expansion system (G-Rex 10, Wilson Wolf, New Brighton, USA) along with AdV5 Hexon PepTivator (Miltenyi Biotec, Bergisch Gladbach, Germany) (100ng/peptide) + rh IL4 (recombinant human Interleukin-4, CellGenix, Freiburg, Germany) (4µg) + rh IL7 (recombinant human interleukin-7, CellGenix, Freiburg, Germany) (0.2µg) and incubated at 37°C, 5% CO_2 for total of 10 days. Remaining PBMCs are cryopreserved at $5x10^6$ per vial in 10% DMSO and Human AB serum to be used as comparison in subsequent experiments.

At the end of incubation period of 10 days, supernatant is collected on 5 of the donors and frozen down in 1mL vials at -80°C. ADV-specific T cells are collected in 50mL falcon tube and washed with RPMI 1640. After washing cells are

resuspended in suitable volume of RPMI/10% human AB serum and cell numbers counted. Cells are cryopreserved in at $5x10^6$ in 1mL vial in RPMI/10% AB serum and 10% DMSO, and stored in liquid nitrogen tanks.

Preparation of stock solutions:

PepTivator-AdV5 Hexon - A stock solution of PepTivator-AdV5 Hexon of 30nmol (approximately 50 µg) of each peptide per mL is prepared by dissolving 6nmol PepTivator-ADV5 Hexon in 200µL of sterile water. 2µL of stock solution is added to each biopot to give 100ng/peptide per pot.

Rh IL-4 – 50 µg freeze dried stock added to 250 µL sterile water to make stock solution of 200 µg/mL. 20µL of stock solution is added to 20mL cell culture to give final concentration of 0.2 µg /mL.

Rh IL-7 – 50 µg freeze dried stock added to 200 µL sterile water to make stock solution of 250 µg/mL. To make a working solution of 10 µg/mL dilute 1:25 by adding 10 µL of stock solution to 240 µL sterile water. Add 20µL of working solution to 20mL of cell culture to give final concentration of 10 ng/mL.

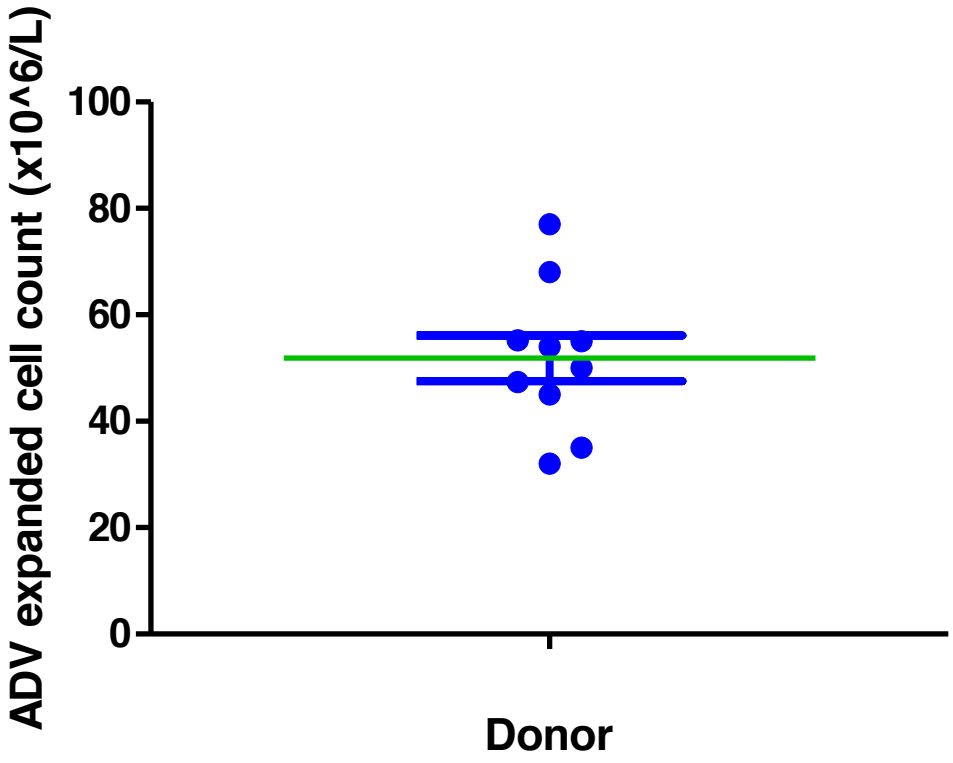
Results:

Number of ADV-specific T cells per donor (starting from $20x10^6$ PBMCs)

Donor	Expanded number of cells	Fold increase
Donor 1	54×10^6	1.7
Donor 2	68×10^6	2.4
Donor 3	32×10^6	0.6
Donor 4	35×10^6	0.75
Donor 5	45×10^6	1.25
Donor 6	47.4×10^6	1.34
Donor 7	55×10^6	1.75
Donor 8	50×10^6	1.5
Donor 9	55.2×10^6	1.76

Donor 10	77 x 10^6	2.85
Median (SD)	*52x10^6 (12.91)*	*1.6 (0.68)*

Figure 3.1 Cell count of ADV expanded cells for 10 donors



Conclusion:

ADV-specific T cells were expanded successfully from all 10 donors with a median final product of 52 x 10^6 , median fold increase is 1.6.

3.2 Th 1, 2, 17 PHENOTYPING ON EXPANDED ADV-SPECIFIC T-CELLS

CD4+ T cell subsets have been identified based on their distinctive patterns of cytokine secretion. As a signature cytokine, Th1 cells selectively produce large amounts of IFN-γ. Th2 cells selectively produce Il-4, and Th17 express high levels of IL-17A. Through secretion of IFN-γ and other effector molecules, Th1 cells activate macrophages, natural killer (NK) cells, and CD8+ T cells and are responsible for cell-mediated immunity. Th1 cells provide protection against intracellular bacteria, fungi, protozoa and viruses; and are involved in autoimmune responses. IL-4 produced by Th2 cells is involved in driving B cells to generate IgE-secreting cells which play a role in basophil/mast cell mediated immune reactions. Th2 cells mediate protection against extracellular parasites but also involved in allergic reactions. Through the secretion of Il-17A, Th17 cells recruit and activate neutrophils and mediate immune responses against extracellular bacteria and fungi. Th17 cells are also implicated in autoimmune responses.

Aim:

To characterize cell phenotypes between unexpanded and ADV-expanded T cells; and to determine if one class of T helper cells are expanded at the expense of another.

Method:

A) Stimulation of cells

Using Th1/Th2/Th17 phenotyping kit (BD Biosciences, Oxford, UK) cryopreserved cells are thawed and stimulated at a concentration of 1 – 10 million cells per ml in media (RPMI + 5% human serum) for 5 hours with PMA/Ionomycin (at 50 ng/mL and 1 µg/mL respectively) in the presence of BD GolgiStop™ Protein Transport Inhibitor. Cells are plated out in 6-well plates, 4 µL of BD GolgiStop is added for every 6 mL of cell culture.

B) Staining of cells

1. Harvesting of cells

- Collect cells from *in vitro* stimulatory cultures treated with a protein transport inhibitor. - Spin down cells at 250 x g for 10 minutes at room temperature and wash once with stain buffer (FBS) or phosphate-buffered saline (PBS).
- Count cells and transfer approximately $1x10^6$ cells to each flow test tube for immunofluorescent staining. Cells need to be protected from light throughout the staining procedure and storage.

2. Fixing the cells

- Suspend cells with 1mL of cold BD Cytofix™ Fixation buffer and incubate for 20 minutes at room temperature.
- Spin down cells at 250 x g for 10 minutes at room temperature. Be careful not to disturb the loose cell pellet after centrifugation. Do not aspirate all of the buffer but leave 50-150µL of solution in the tubes to avoid cell loss for all subsequent wash steps below.
- Wash cells once at room temperature in stain buffer (FBS) or PBS and spin

down the cells at 300 x g for 10 minutes at room temperature.

- Cells can be stored in stain buffer (1mL per tube) at 4°C for up to 72 hours.

3. Permeabilising the fixed cells

- For cells kept at 4°C, spin down cells at 300 x g for 10mins at room temperature and remove stain buffer.

- Dilute 10X BD Perm/Wash™ buffer in distilled water to make a 1x solution prior to use.

- Suspend cells in 1ml of 1x BD perm/Wash™ buffer and incubate at room temperature for 15 minutes.

- Spin down cells at 300 x g for 10 minutes at room temperature and remove supernatant.

4. Staining with the Cocktail

- Thoroughly suspend fixed/permeabilised cells in each tube in 50 µL of 1x BD Perm/Wash™ buffer and add 20 µL of cocktail or appropriate negative control:

Tube 1 – unstimulated cells unstained

Tube 2 – APC alone

Tube 3 – FITC alone

Tube 4 – PE alone

Tube 5 – PerCp Cy5.5 alone

Tube 6 – Unstimulated 4 colour cocktail

Tube 7 – Stimulated with iso antibody

Tube 8 – Stimulated cells with 20µL 4 colour cocktail (CD4 *PerCp-Cy5.5*, IL-17A

PE, IFN-γ *FITC*, IL-4 *APC*)

- Incubate with antibodies at room temperature for 30 mins in the dark.

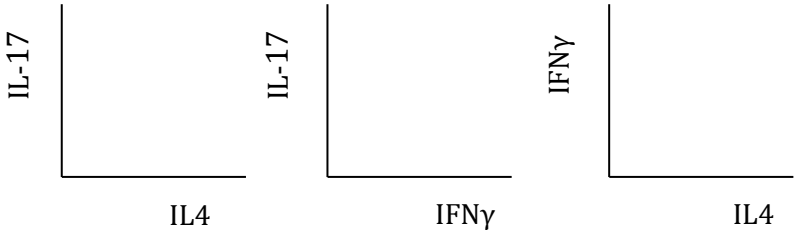
- Wash cells once with 3mL of 1x BD Perm/Wash™ buffer at room temperature.

- Suspend cells in PBS or stain buffer prior to analysis (400 µL for all tubes except for 4-colour cocktail tube – use 500 µL).

C) Flow Cytometric Analysis

- Cells are gated first on forward and size scatter to obtain lymphocyte population, then CD4+ cells. CD4 expression on T cells can decrease after activation. Acquire at least 20,000 to 30,000 CD4+ve lymphocytes. Flow cytometry was performed on a BD™ LSR II System.

Figure 3.2 Gating strategy for Th1, 2,17 phenotyping analysis



Reagents:

PMA (Promega, Madison, USA) – 5mg reconstituted in 1.62 mL DMSO to produce a 5mM stock solution and stored in 10 µL aliquots. Working solution produced by diluting stock solution 1:100 in sterile PBS. 5µL of PMA working solution is used per mL of cell culture to give final concentration of 50 ng/mL.

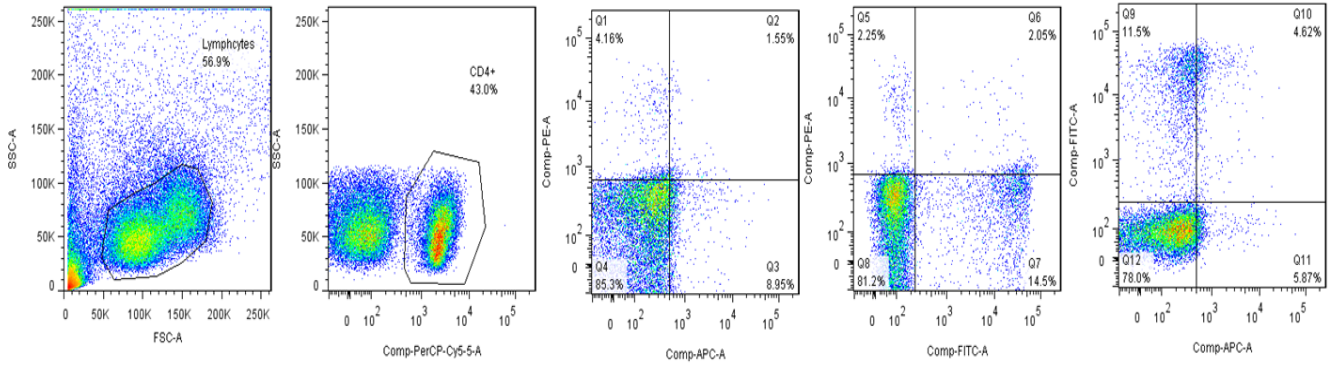
Ionomycin (MP Biomedical, California, USA) – 1mg reconstituted in 400 µL DMSO to produce stock solution of 2.5mg/mL stock and stored in 40 µL aliquots. Working solution produced by diluting stock solution 1:25 in sterile PBS. 10µL of Ionomycin working solution is used per mL of cell culture to give final concentration of 1µg/mL.

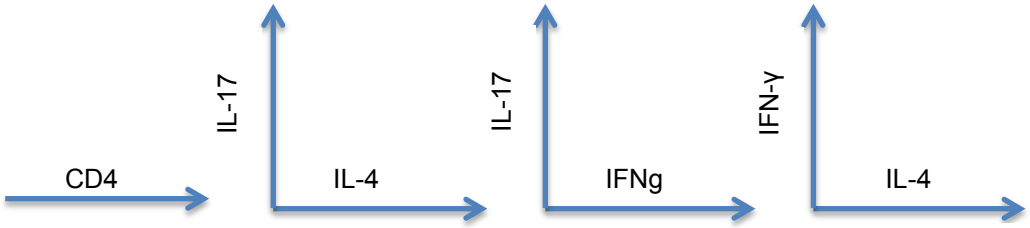
Results:

The experiment was performed on cells from 6 donors. However the ADV expanded cells from all donors died after stimulation and incubation with PMA/Ionomycin cocktail.

Figure 3.3 Representative dot plot of Th1,2,17 phenotyping from one donor

A) Unexpanded cells





CD4+ cells: 43%

(as percentage of CD4+ cells) - Th1 (IFNγ) cells: (14.5+2.05+11.5+4.62)% / 2 = 16.34% => 7.03%

- Th2 (IL4) cells: (8.95+1.55+5.87+4.62)% / 2 = 10.5% => 4.52%
- Th17 (IL17A) cells: (4.15+1.55+2.25+2.05)% / 2 = 5% => 2.15%

B) ADV expanded cells

Unfortunately ADV expanded cells died after stimulation with PMA and Ionomycin cocktail and no results could be obtained.

Conclusion:

As the ADV expanded cells died after stimulation, conclusion cannot be drawn.

3.3 CHARACTERISATION OF REGULATORY T-CELLS (Tregs) by FoxP3 STAINING

FoxP3, a 49-55 kDa protein, is a member of the forkhead/winged-helix family of transcriptional regulators, and was identified as the gene defective in 'scurfy' (sf) mice. Constitutive high expression of FoxP3 mRNA has been shown in CD4+Cd25+ regulatory T cells (Treg cells), and ectopic expression of FoxP3 in CD4+CD25- cells imparts a Treg phenotype in these cells. The 236A/E7 antibody reacts with human FoxP3 protein.

Aim:

To determine if there is regulatory T cells outgrowth in ADV expanded product.

Methods:

- The FoxP3 staining kit from eBiosceince (San Diego, USA) was used.
- Thaw out cryopreserved ADV CTLs and unexpanded PBMCs in RPMI/10% HAS and count cells.

- Resuspend 1x10^6 cells in PBS and surface stain for CD4 (PerCP-CY5.5), CD25 (PE), and CD127 (FITC).

- Add cold PBS into each FACs tube so that max volume is 200 µL each.

- Wash cells at 1500rpm for 7 min.

- Tip off supernatant and resuspend cell pellet in 1mL of freshly prepared Fixation/Permeabilisation solution; and mix well.

- Incubate at 4 °C for 60 minutes in the dark.

- Wash once by adding 2mL 1x Perm buffer then spin 350 x g for 5 min at 4°C. Tip off supernatant.

- Block with 2% normal rat or mouse serum in 1x Perm buffer (20 µL serum in 1mL buffer). Add 100µL into each FACs tube.

- Incubate at 4°C in the dark for 15 minutes.

- Without washing after blocking step add 20µL fluorochome conjugated anti-human FoxP3 antibody or isotype control in 1x Perm Buffer and incubate at 4°C for at least 30 minutes in the dark.

- Wash cells with 2mL 1x permeabilisation buffer. Spin at 350 x g for 5 minutes.

- Repeat above step.

- Resuspend in 500 µL Flow Cytometry staining Buffer or PBS and analyse by flow cytometry on BD^{TM} LSR II System. Acquire at least 50,000 lymphocytes and at least 5000 $CD4^+CD2^+CD127^{low}$ cells.

Reagents:

1. FoxP3 staining Buffer set eBioscience (cat 17-4777)

2. Antibodies:

-CD4 PerCp Cy5.5 (BD Pharmingen cat 560650)

-CD25 PE (BD Pharmingen cat 555432)

-CD127 FITC (eBioscience cat 11-1278-73)

-FoxP3 APC (clone 236A/E7 eBioscience cat 17-4777)

Preparing solutions:

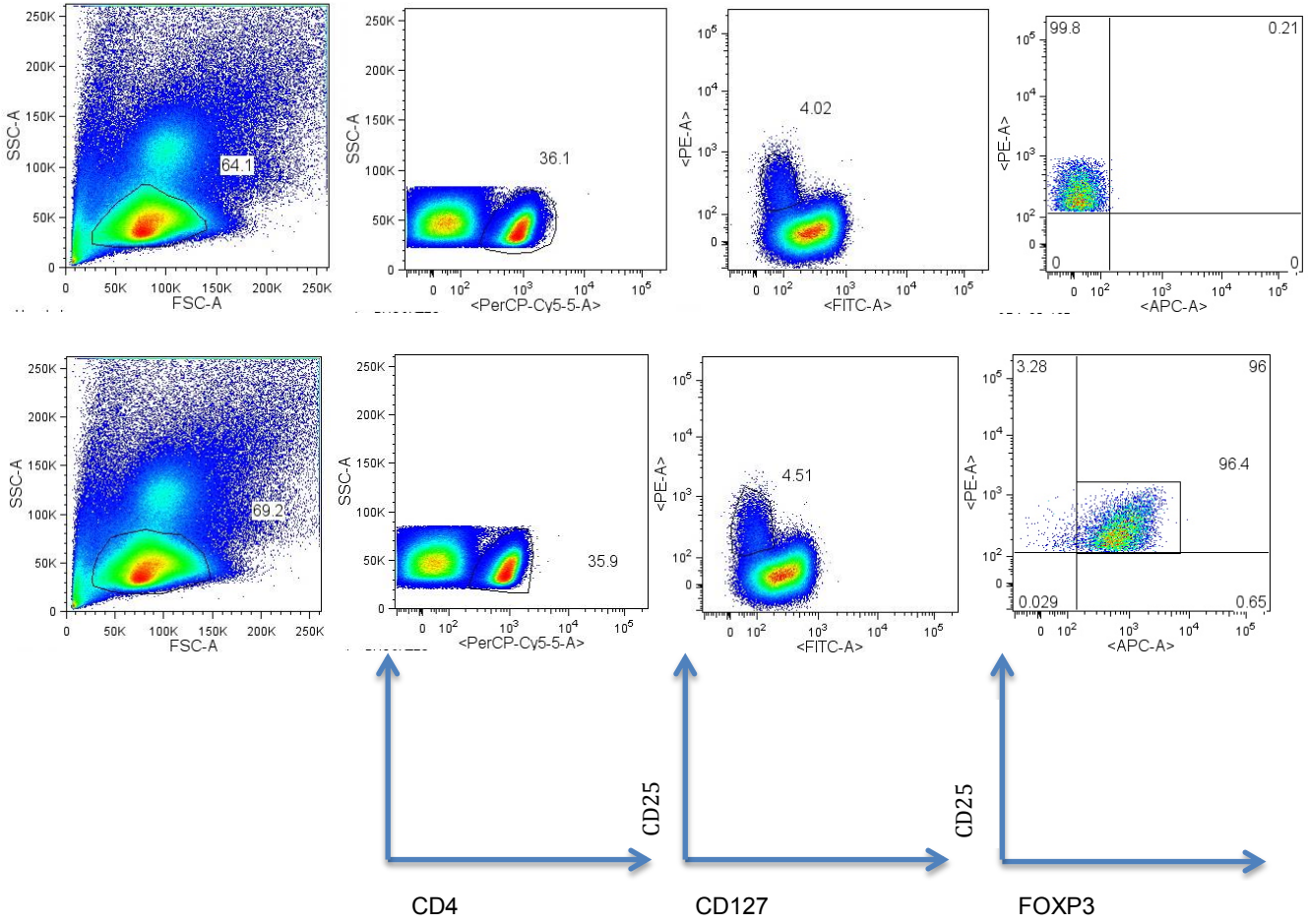
1. Dilute **Fixation/permeabilisation concentrate** (1 part) into the **Fixation/Permeabilisation Diluent** (3 parts).

2. **Permeabilisation buffer** 10x stock dilute to 1x with DI water.

Results:

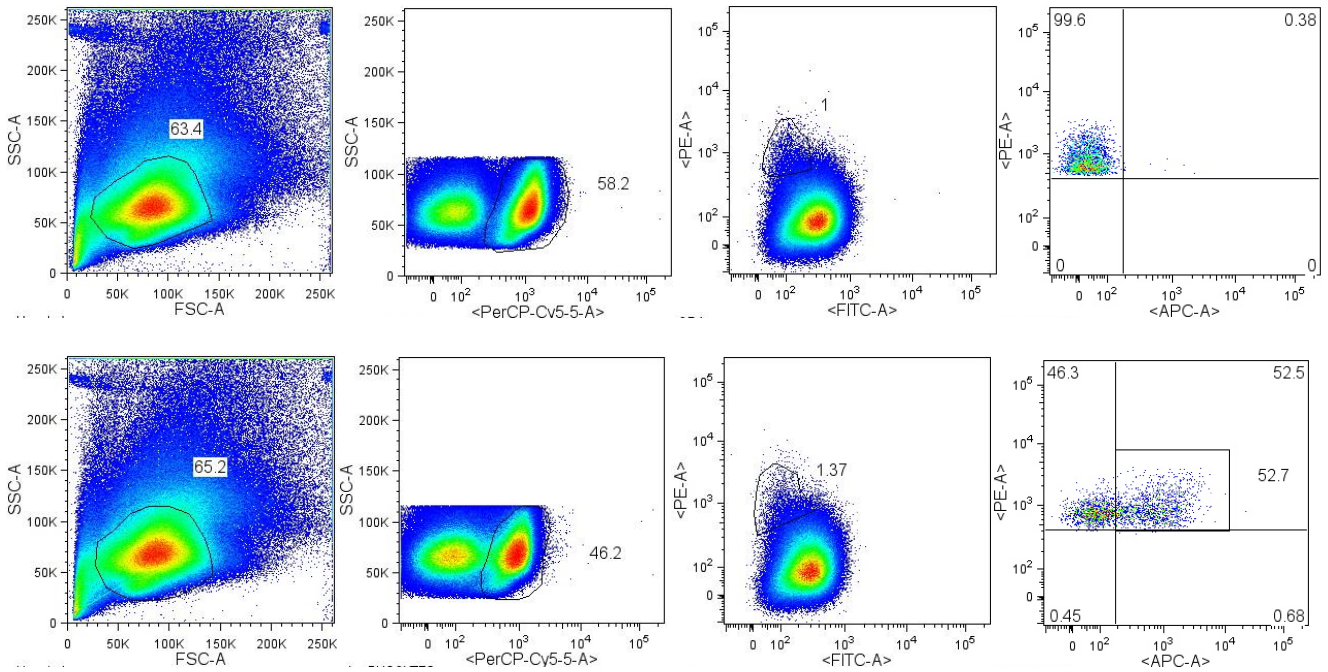
Figure 3.4 Representative plot of FoxP3 phenotyping from one donor

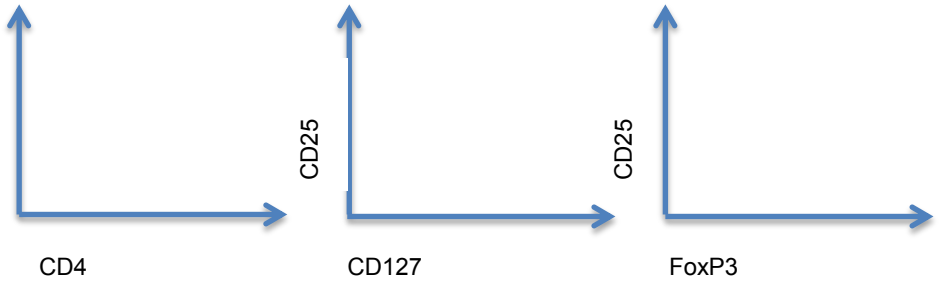
Unexpanded



Isotype control

ADV expanded



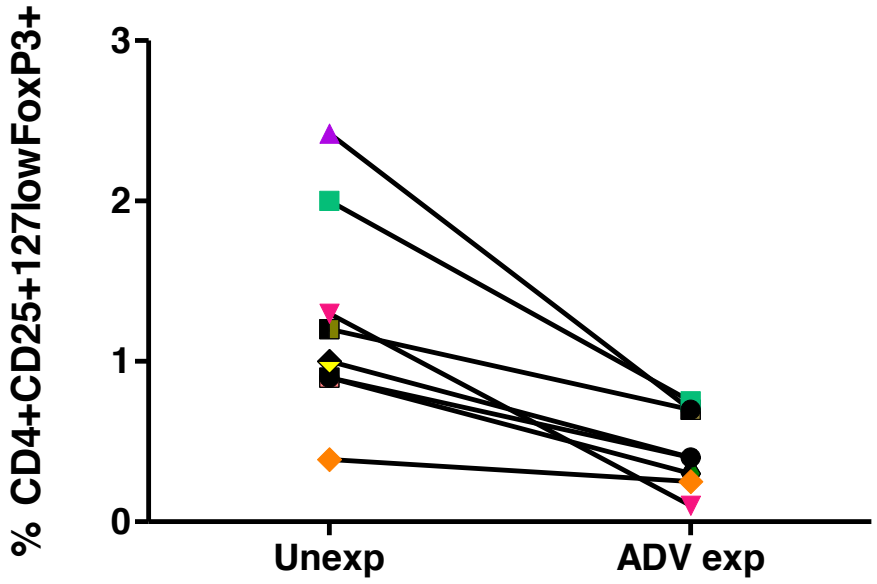


Isotype control

Results from all donors

Donor	$CD4^+CD25^+CD127^{low}FoxP3^+$ (%)	
	Unexpanded T cells	ADV expanded T cells
1	0.9	0.4
2	0.9	0.3
3	1.6	0.3
4	1.3	0.1
5	6.4	0.9
6	2	0.8
7	1	0.4
8	2.4	0.7
9	0.4	0.3
10	0.2	0.5

Figure 3.5 Proportion of Regulatory T cells ($CD4^+CD25^+CD127^{low}FoxP3^+$) in all donors



Conclusion:

There is no outgrowth of regulatory T cells in ADV expanded T cells.

3.4 CHARACTERISATION OF T-CELL SUBPOPULATIONS

Aim:

To characterise the T cell subpopulations in the virus-expanded cells compared to unexpanded T cells, in particular T memory stem cell population.

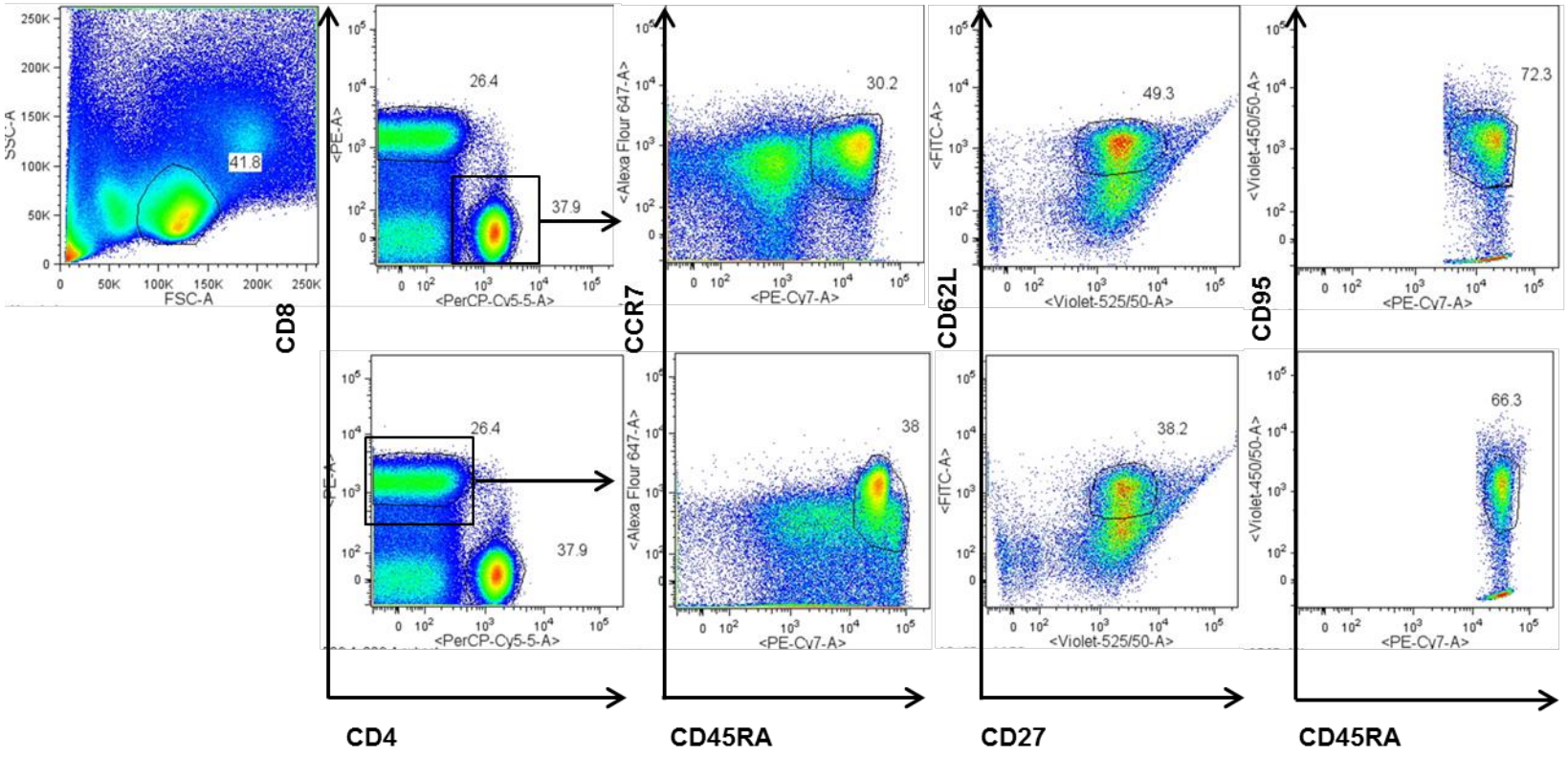
Methods:

Cryopreserved unexpanded T cells and ADV specific T cells from 8 donors were thawed in RPMI/10% HAS. Cells were washed, counted and resuspended in phosphate-buffered saline (PBS; Invitrogen Paisley, UK) and incubated for 10 min at room temperature with saturating amounts of antibody (5 µL), then washed and resuspended in PBS before going through flow cytometric analysis on Becton Dickinson LSRII using BD FACSDIVA software. Cells were stained for CD4 (PerCP-Cy5.5), CD8 (PE), CD45RA (PE Cy7), CCR7 (Alexa Flour 647), CD62L (FITC), CD27 (Violet 525), CD95 (Violet 450).

Results:

Figure 3.6 Representative dot plot of T cell phenotyping from one donor

Unexpanded



ADV expanded

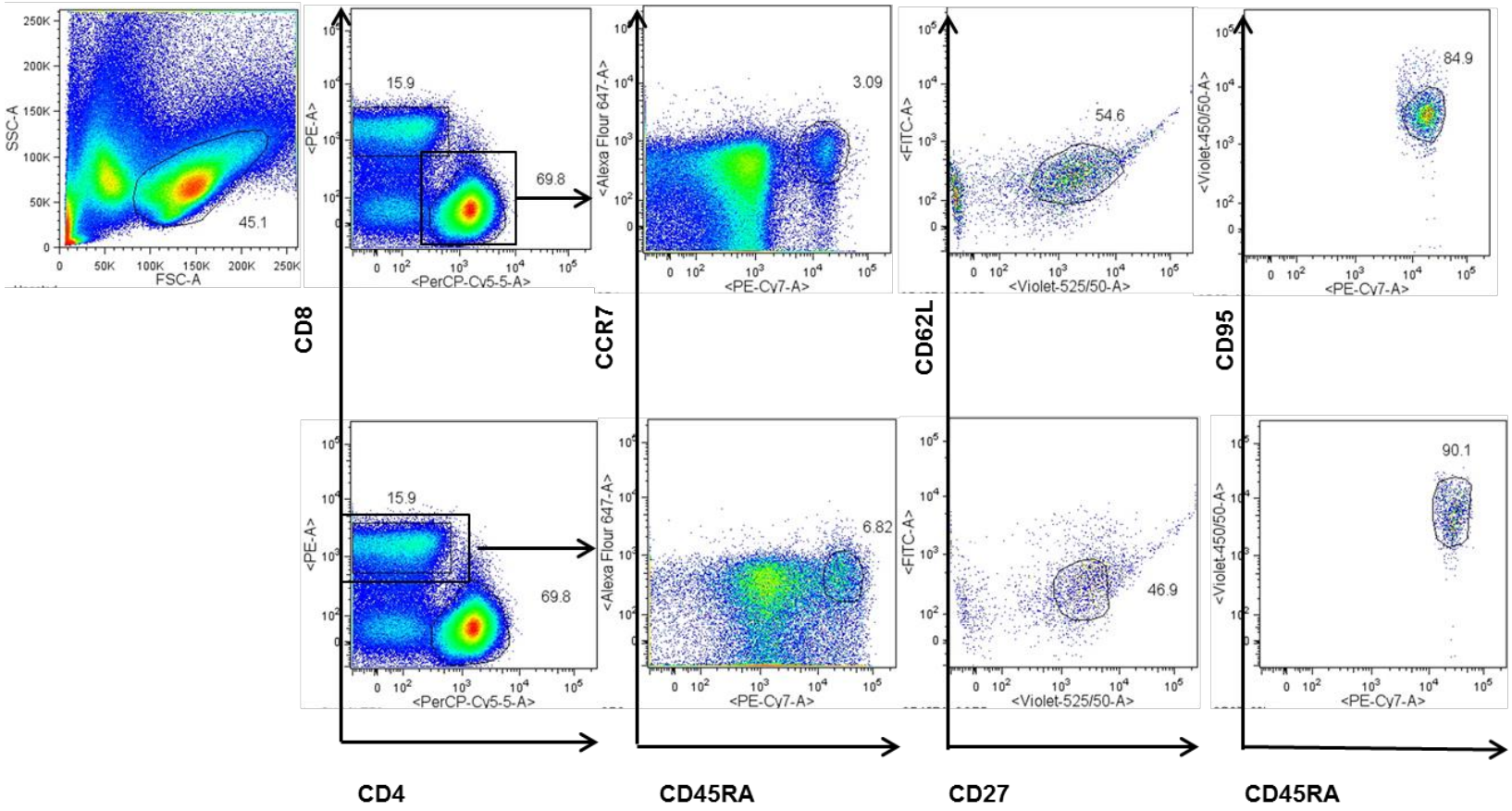
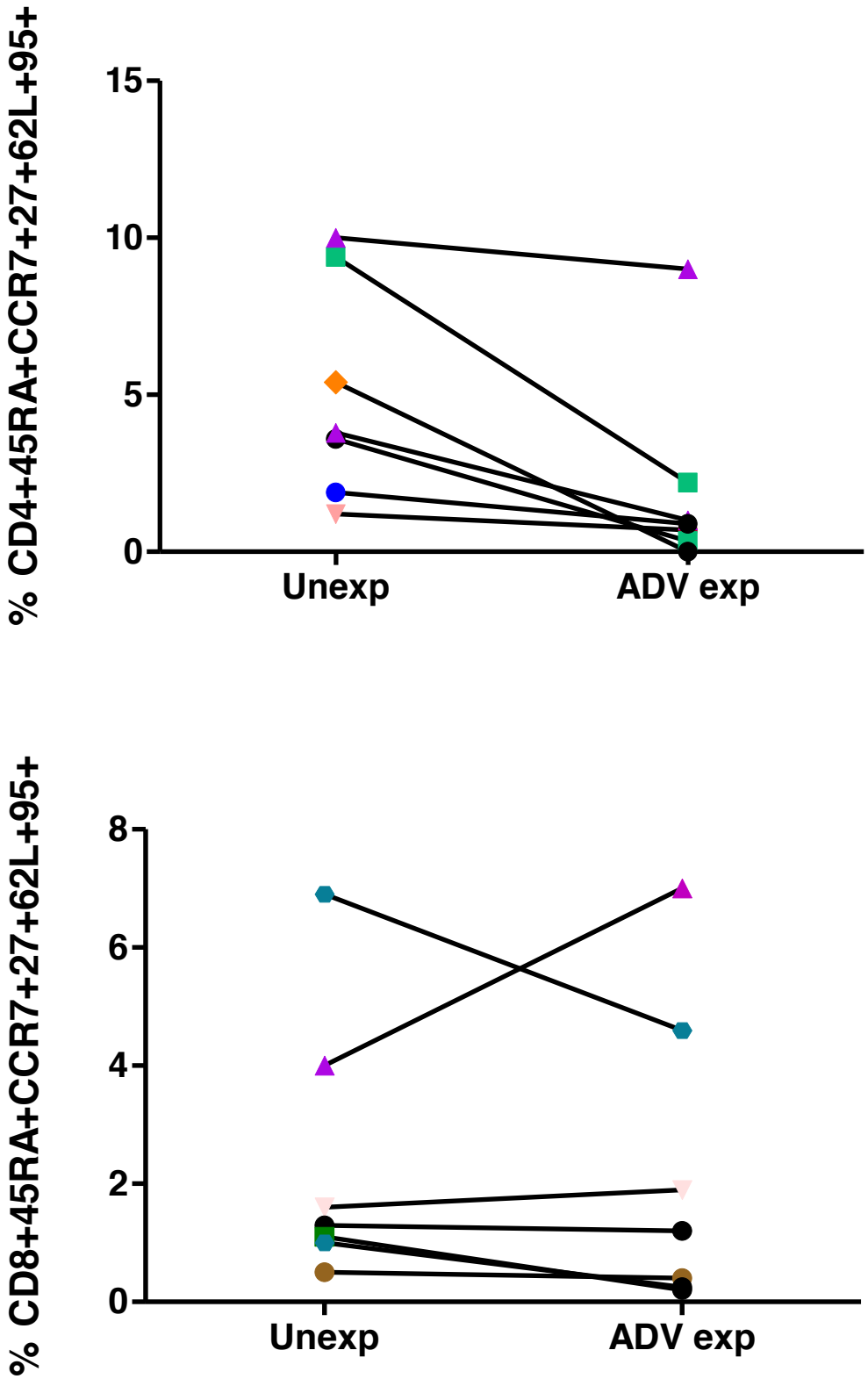


Figure 3.7 Proportion of Tscm ($CD4^+$ and $CD8^+$) in unexpanded vs ADV expanded cells



Conclusion:

There is a general trend for decrease in proportion of cells expressing stem cell memory markers (CD45RA+CD27+CD62L+CD95+) in the ADV-expanded T cells compared to unexpanded cells.

3.5 DIVERSITY OF T-CELL RESPONSES

Aim:

To assess diversity of T cell responses of ADV specific T cells by spectratyping by PCR.

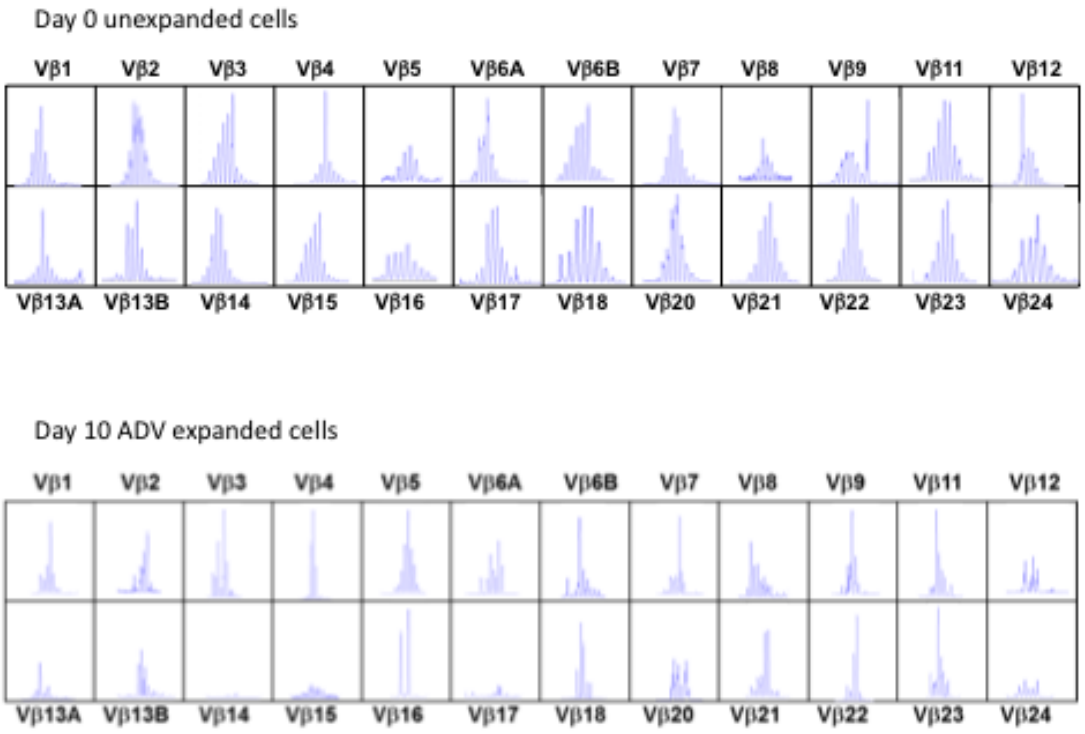
Method:

CDR3 TCR molecular spectratyping was performed for unexpanded and ADV-expanded T cells. Template cDNA corresponding to rearranged transcripts with different CDR3 lengths from specific TCR variable region genes was amplified by PCR and the products run on AB3130 Genetic Analyzer (Applied Biosystems) and analyzed with SpA Web-based software. This was performed by Dr Stuart Adam at Bone Marrow Lab, Camellia Botnar Laboratories, Great Ormond Street Hospital.

Results:

Representative result from one donor showing normal spectratype in unexpanded cells compared to the skewed spectratype with some clonal expansions in the ADV expanded cells.

Figure 3.8 Representative spectratyping plot from one donor (unexpanded vs ADV expanded cells)



Conclusion:

As expected the ADV expanded cells showed antigen-driven oligoclonal T cell expansions in response to ADV stimulation.

3.6 SPECIFIC CYTOKINE (IFNγ) PRODUCTION BY ADV-SPECIFIC T-CELLS

Aim:

To confirm that expanded CD8+ and CD4+ T cells are antigen specific and produce effector cytokines (IFN-γ) upon stimulation.

Method:

Antigen-specific T cells are analysed using the IFN-γ secretion Assay (Miltenyi Biotec, Bergisch Gladbach, Germany) starting from thawed cryopreserved PBMCs. Cells were plated at density of $5x10^6$ /cm^2 to ensure optimum stimulation.

Total cell number (cell number after suspension)	Medium volume to add per well	Culture plate	Well diameter
$0.15x10^7$	0.15mL	96	0.64cm
$0.50x10^7$	0.50mL	48	1.13cm
$1.00x10^7$	1.00mL	24	1.6cm
$2.00x10^7$	2.00mL	12	2.26cm
$5.00x10^7$	5.00mL	6	3.5cm

Cells were stimulated for 4 hours with AdV5 Hexon PepTivator (Miltenyi Biotec), Staphylococcal enterotoxin B (Sigma-Aldrich, Dorset, UK) as positive control, or unstimulated as negative control; and incubated at 37°C, 5-7% CO_2, in moist chamber.

After incubation an IFN-γ specific **Catch Reagent** was attached to the cell

surface of leukocytes. The cells were then incubated for a short time at 37°C to allow cytokine secretion. The secreted IFN-γ binds to the IFN-γ Catch Reagent on the positive, secreting cells. These cells were subsequently labelled with a second IFN-γ-specific antibody, the **IFN-γ Detection Antibody** conjugated to R-phycoerythrin (PE) for sensitive detection by flow cytometry. Non-specific background was minimised by dead cell exclusion staining with DAPI viability dye just before flow cytometry analysis and gating on viable cells.
Statistical analysis by paired T test.

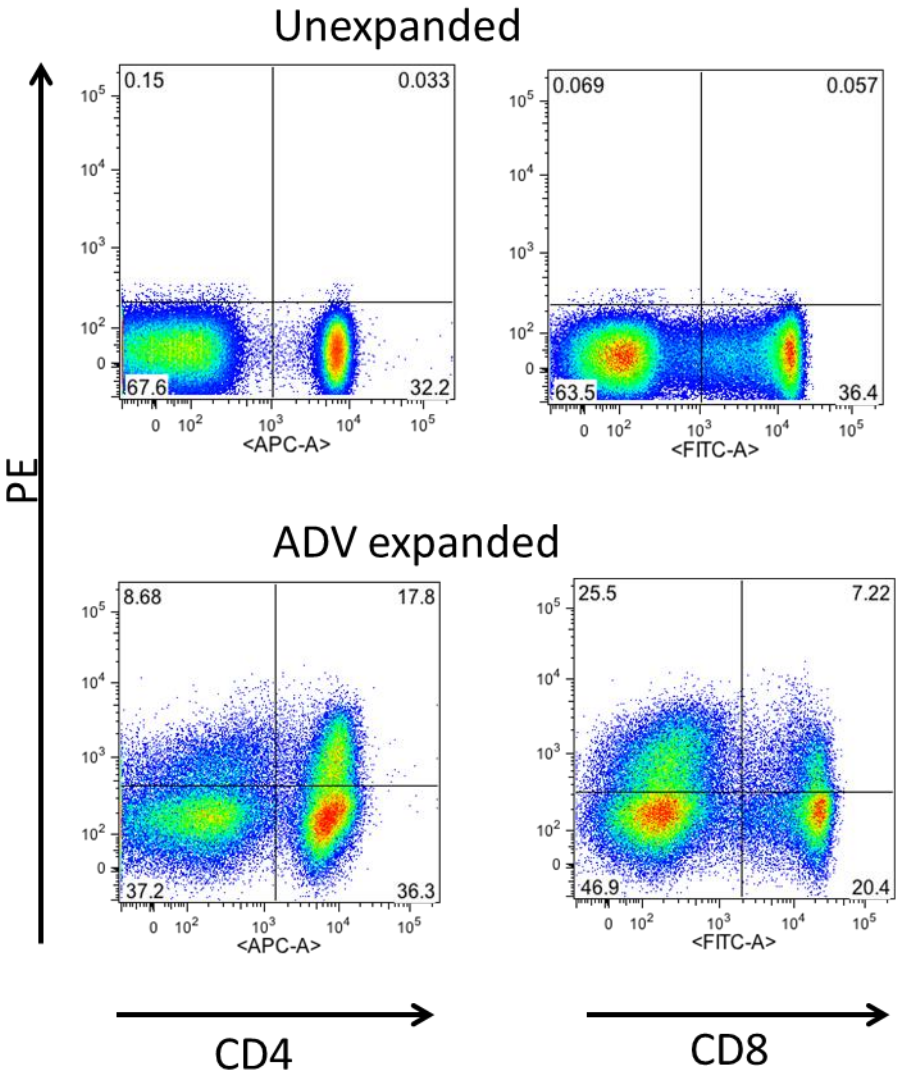
Reagents:

SEB – 1mg powder reconstituted in 1mL sterile water for injection to make stock solution of 1mg/mL. Concentration used - 10µg/mL (1µL per test).

Results:

Representative plot from one donor (PE = IFNγ, APC = CD4, FITC = CD8).
Gated on lymphocytes (forward/side scatter) and Live cells (using DAPI antibody).

Figure 3.9 Representative IFNγ plot for ADV specificity (unexpanded vs ADV expanded)



Results from all donors

	Unexpanded		Expanded (fold increase from unexpanded)	
Donor	CD4+	CD8+	CD4+	CD8+
1	0.25	0.22	16.2 (64.8)	3.78 (17.2)
2	0.04	0.16	10.8 (270)	0.98 (6.1)
3	0.11	1.02	23.1 (210)	5.45 (5.3)
4	1.62	1.22	2.77 (1.7)	1.94 (1.6)
5	0.06	0.03	8.91 (148.5)	1.11 (37)
6	0.02	0.05	0.47 (23.5)	0.05 (1)
7	0.03	0.06	17.8 (593)	7.22 (120.3)
8	0.19	0.61	1.75 (9.2)	0.37 (0.6)
9	0	0	10.1 (10.1)	2.36(2.36)
Fold increase (average)			147.9 SD: 193.3 P<0.02	21.3 SD: 38.95 p<0.1

Conclusion:

Both CD4+ and CD8+ T cells in the expanded cell line were antigen-specific and produced effector cytokines (IFNγ) at a much higher level compared to unexpanded T cells.

3.7 BYSTANDER ACTIVATION BY CFSE PROLIFERATION

Aim:

To determine if ADV specific T cells can cause bystander activation and contribute to inflammatory complications in immunotherapy.

Methods:

Fresh PBMCs are obtained from the original donor and labeled with CFSE using CellTrace CFSE Cell Proliferation Kit (Life Technologies, Paisley, UK). Peripheral blood mononuclear cells (PBMCs) are derived from fresh blood by Ficoll/Paque (Pharmacia, Uppsala, Sweden) density gradient separation and resuspended at a cell density of $1x10^6$ cells/mL in culture medium X-vivo 10 (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Lonza, Walkersville, USA).

CFSE was dissolved in DMSO to a stock concentration of 5 mM and stored in single aliquots at -20°C. 2µL of 5mM stock CFSE solution is added to each millilitre of cells for a final working concentration of 10 µM. After thorough mixing, the cell suspension was incubated at 37°C for 10 mins. The labeling reaction was then quenched with addition of 5 volumes of ice-cold culture media to the cells, and the mixture was centrifuged at 1500 rpm for 5 min. CFSE-labeled PBMC were then plated out at $1x10^5$ in 96-well round bottom plate in different

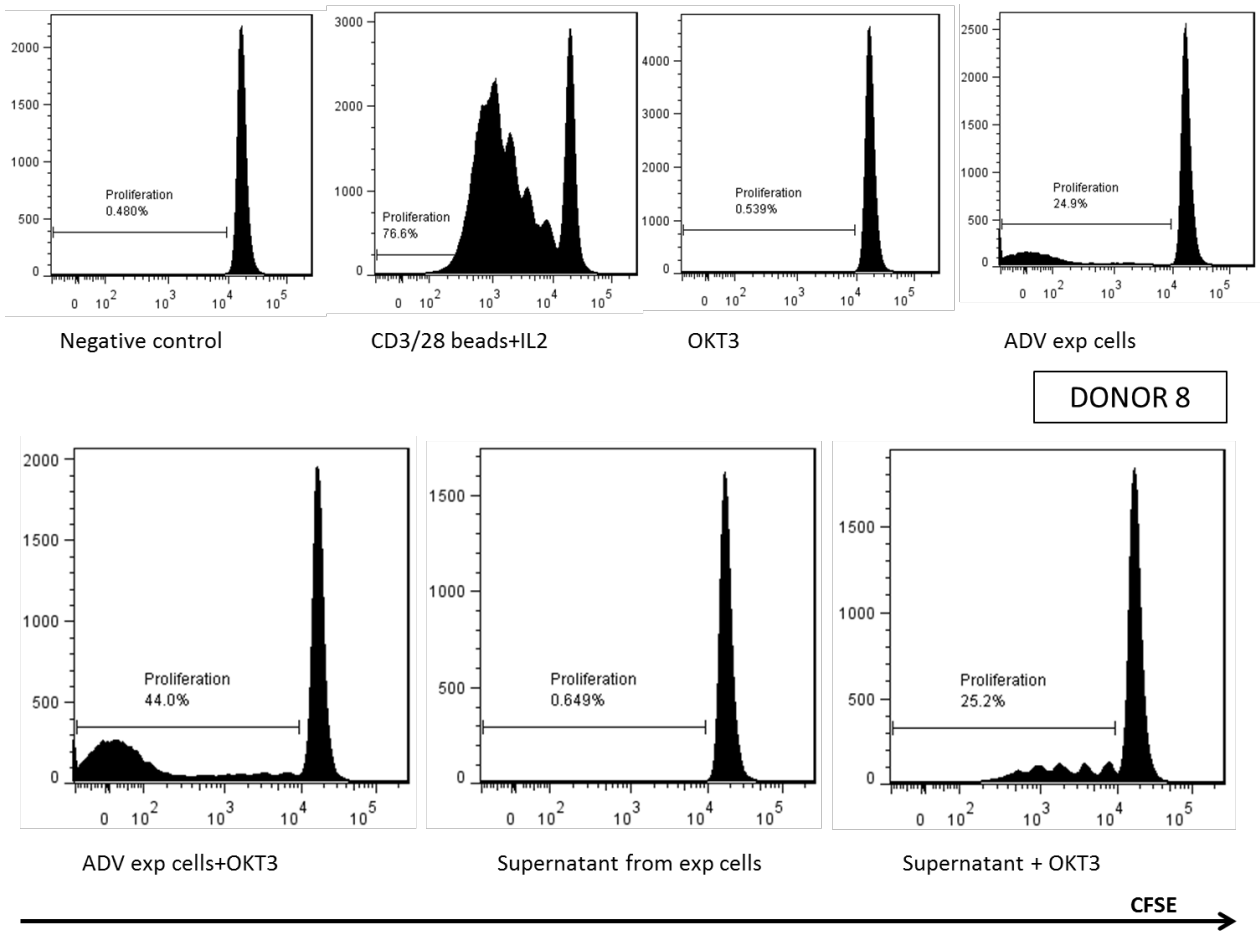
conditions: 1) culture medium alone as negative control; 2) CD3/28 beads (1:1 ratio i.e. 2µL of 10^8 in 1mL) and IL2 (200u/mL) as positive control; 3) ADV expanded cells (from the same donor, $1x10^5$); 4) ADV expanded cells + OKT3 (10µg/mL); 5) supernatant collected at the end of incubation period from ADV expanded T cells; 6) supernatant + OKT3 (10µg/mL). Cells were incubated at 37°C, 5% CO_2 for 4 days; after which cells were harvested and resuspended in PBS and analysed on LSRII flow cytometer using FACSDIVA software (Becton Dickinson).

Supernatant was not collected from donors 1, 2 and 4.

Statistical analysis by paired T test.

Results:

Figure 3.10 Representative CFSE proliferation result from one donor



Results from all donors

Percentage of proliferation (above baseline [CM])

Donor	CM	CD3/28 beads+IL2	ADV CTLs (fold increase above baseline)	ADV CTLs+OKT3	Supernatant (fold increase above baseline)	Supernatant +OKT3
1	3.38	26.92	81.62 (24)	84.12	-	-
2	6.98	9.22	31.72 (4.5)	65.32	-	-
4	0.89	55.21	20.61 (23.2)	58.31	-	-
5	1.6	81.7	47.4 (29.6)	83.6	1.62 (1)	-
6	0.85	75.95	31.75 (4.4)	33.15	1.11 (1.3)	1.15
7	0.19	71.71	22.81 (120)	48.01	0.04 (0.2)	23.41
8	0.46	81.44	23.84 (51.8)	26.54	0.05 (0.1)	5.44
9	1.09	90.41	30.01 (27.5)	48.81	1.67 (1.5)	0.57
10	0.45	90.75	49.15 (2.2)	76.95	0.66 (1.5)	0.33
Fold increase from baseline (average)			31.9 SD: 36.59 P<0.01		0.9 SD: 0.63 P<0.002	

Conclusion:

ADV CTLs were able to induce proliferation of fresh PBMCs but not supernatant collected from culture of ADV CTLs. This suggests proliferation by cell-to-cell contact rather than cytokine induced proliferation.

3.8 CYTOKINE PROFILE

Aim:

To determine cytokine profile in supernatant of ADV expanded T cells by sandwich immunoassays.

Methods:

Using MSD Multi-spot Assay system (Rockville, USA), 5 cytokines were measured using two different panels -

Proinflammatory panel 1: IFNγ, IL2, TNF-a

Cytokine panel 1: Il7, IL15

A plate precoated with capture antibodies on independent and well-defined spots is provided for each panel. Supernatant of ADV expanded T cells were collected from six donors and each sample was run in duplicates and 2 different dilutions:

6 donors x 2 samples = 12 wells x duplicate = 24 wells

1. 2-fold dilution 30microL sample + 30 microL diluent

2. 5-fold dilution 20microL sample + 80 microL diluent

Calibrator dilutions were prepared as follows:

- 1000microL of Diluent 2 to lyophilized calibrator vial (Pro); diluent 43 (cyto).

- 300microL of diluent in 7 ependorfs

- 100microL into above

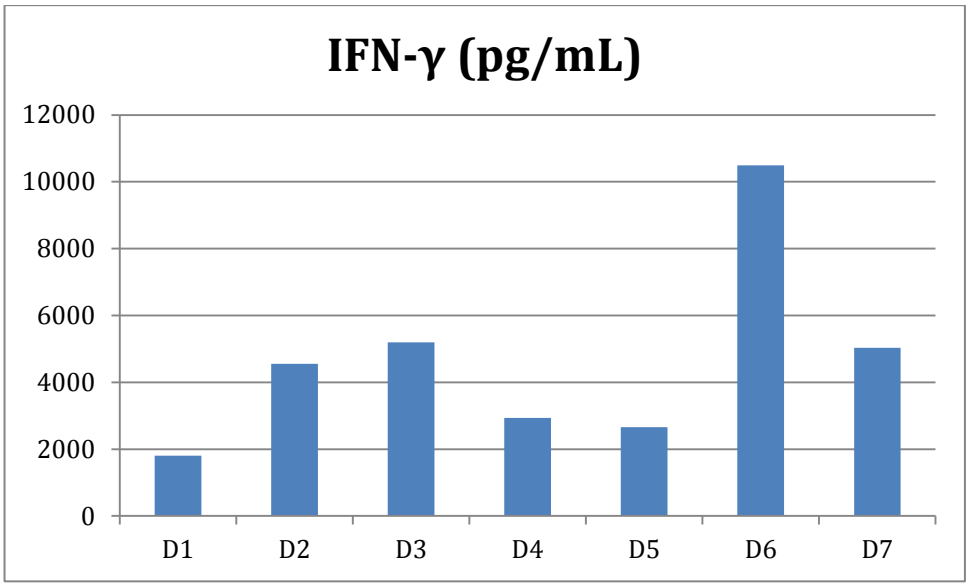
Samples were diluted with diluent in 2- and 5-fold, and 50microL of sample, calibrator and control are added to plates precoated with capture antibodies,

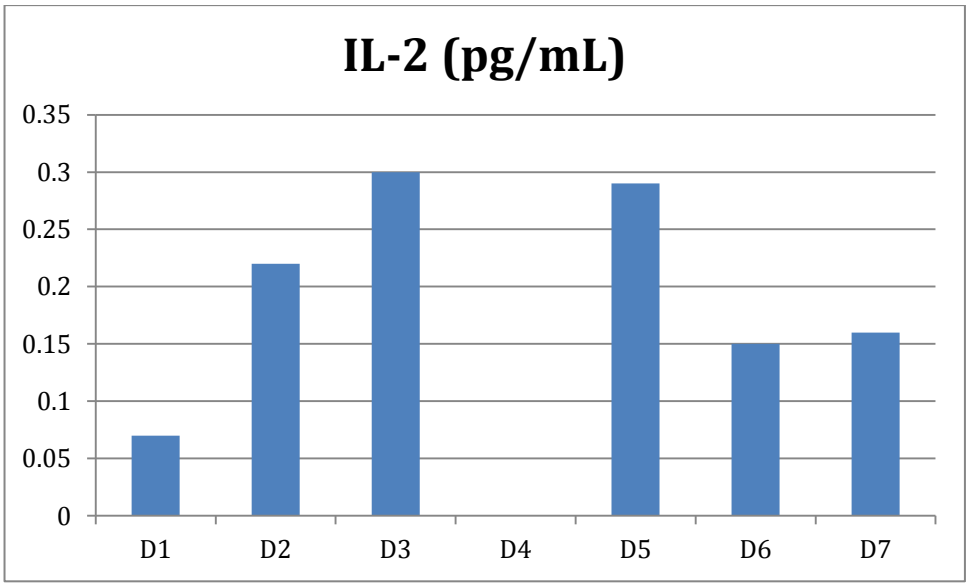
sealed and incubated at room temperature with shaking for 2 hours. Plates were then washed 3 times and read on MSD instrument.

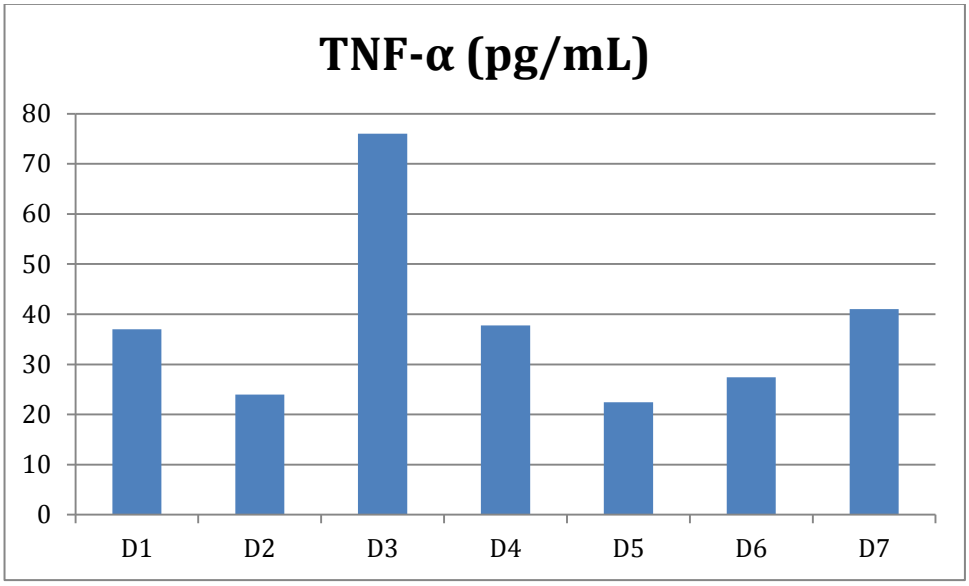
Results:

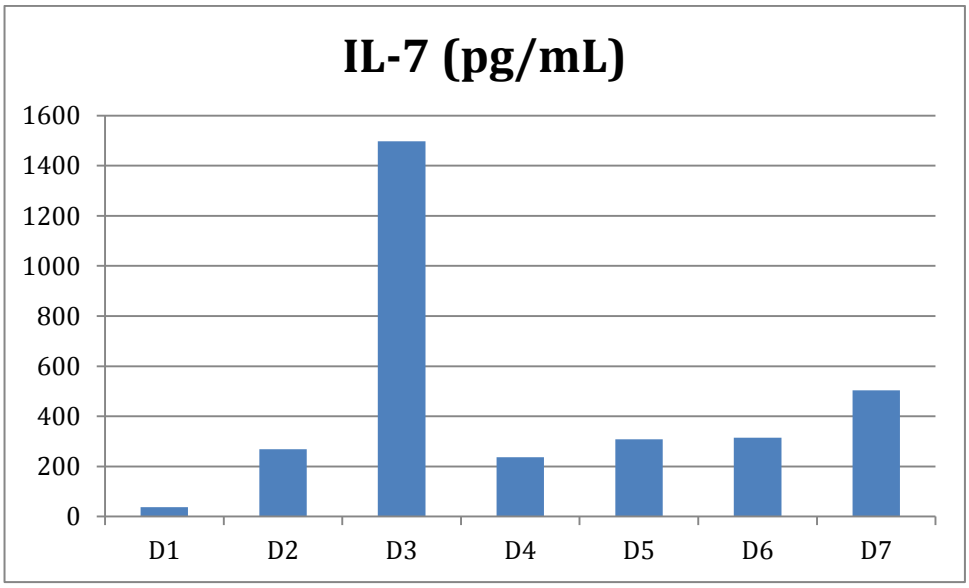
Donor	IFNγ (pg/mL 5x dilution)	IL2 (pg/mL 2x dilution)	TNF-a (pg/mL 5x dilution)	IL7 (pg/mL 5x dilution)	IL15 (pg/mL 2x dilution)
D1	**1800**	**0.07**	**36.98**	**162**	**1.3**
D2	**4555**	**0.22**	**23.95**	**269**	**1.25**
D3	**5196**	**0.3**	**76.04**	**1498**	**1.68**
D4	**2938**	**0**	**37.77**	**237**	**1.44**
D5	**2657**	**0.29**	**22.45**	**309**	**2.89**
D6	**10495**	**0.15**	**27.44**	**315**	**1.04**
D7	**5033**	**0.16**	**41.04**	**503**	**1.8**
Median	***4555***	***0.16***	***37***	***309***	***1.44***

Figure 3.11 Individual graphs for cytokine profiling (x-axis = donor; y-axis = cytokine level)






TNF-α (pg/mL)
80
70
60
50
40
30
20
10
0
D1
D2
D3
D4
D5
D6
D7


IL-7 (pg/mL)
1600
1400
1200
1000
800
600
400
200
0
D1
D2
D3
D4
D5
D6
D7

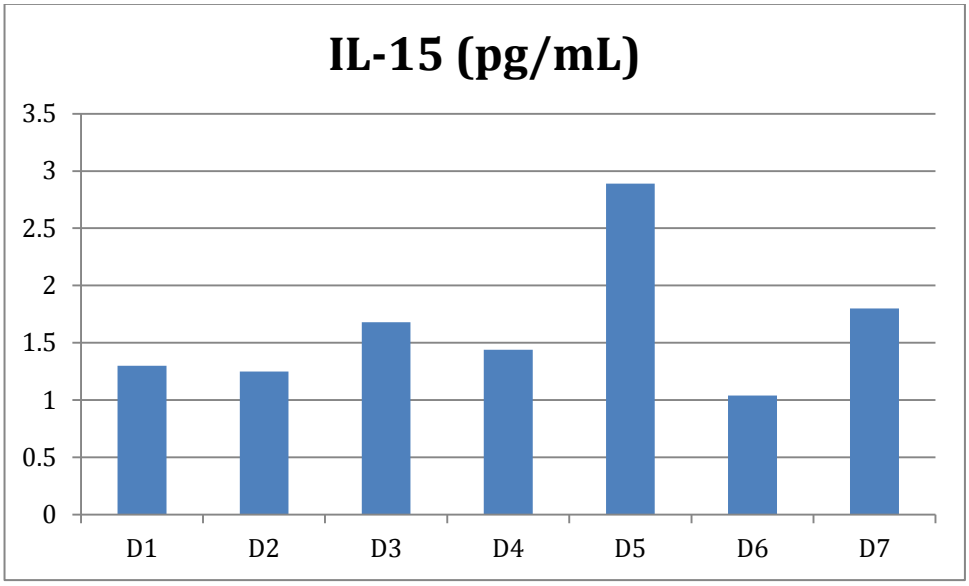
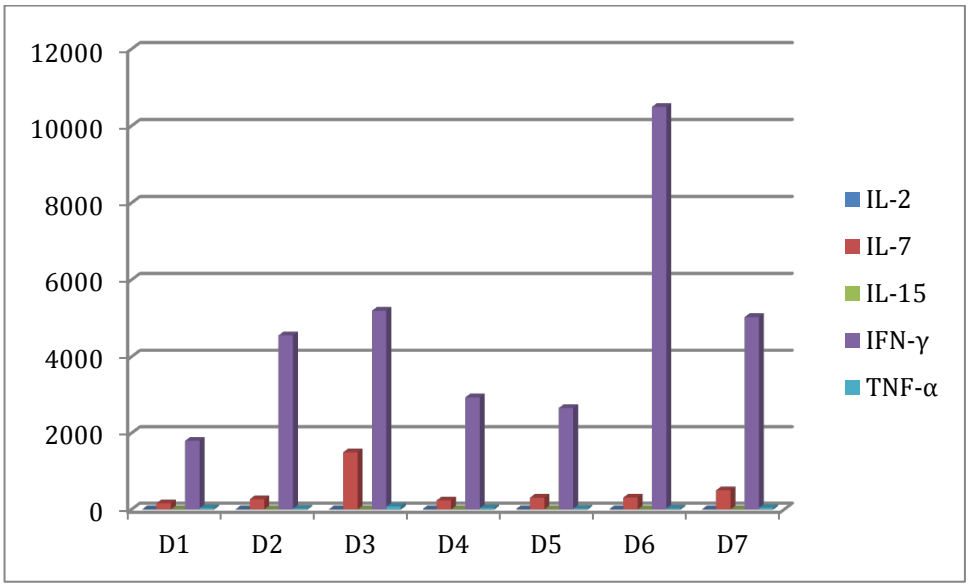

IL-15 (pg/mL)
3.5
3
2.5
2
1.5
1
0.5
0
D1
D2
D3
D4
D5
D6
D7

Figure 3.12 Combined graphs of cytokine production for each donor



Conclusion:

Supernatant of ADV-activated T cells contained mostly IFNγ and TNFα but not the other Th1 cytokine – IL2. IL7 is also present in high amounts as it is used in the culture along with IL4.

CHAPTER 4

DISCUSSION

Since the start of the ASPIRE trial in Quarter 1 2013 at Great Ormond Street Hospital, 6 patients have been treated pre-emptively with donor-derived ADV-specific CTLs (Cytovir ADV) for the treatment of adenoviraemia. All treated patients have experienced a reduction in ADV viral load in the blood. Serious adverse events were recorded in 3 patients. One patient died of astrovirus encephalitis and death was not deemed related to Cytovir ADV. One patient developed severe GvHD around 60 days after cell infusion and subsequently died 2 years later of infectious complications. One patient developed acute pancreatitis 9 days after cell infusion after experiencing a drop in ADV viral load in blood. The occurrence of pancreatitis co-incided with a rebound in ADV viral load; and patient made a full recovery.

The other three treated patients did not experience any serious adverse events at 6 months follow-up from cell infusion and were able to demonstrate in-vivo ADV specific response by ELIspot or IFNγ cytokine release assay.

Whether the reduction in viral load was due to endogenous reconstitution of immunity, effect of donor-derived CTLs or effect of antivirals cannot be ascertained. Unlike CMV immunity, ADV specific T cells tend to appear only during acute infection and tend not to be detected when disease is quiescent. The frequency of T cells reactive with adenoviral hexon epitope is usually many times lower than frequency of CMV reactive T cells (Leen et al., 2006). Also many patients were lymphopaenic at time of evaluation making flow cytometry assessment difficult. This is also a heterogeneous group of patients with both haematological as well as non-malignant genetic diseases as their underlying condition. Although all patients on the study received serotherapy, individual

conditioning regiments varied. This makes drawing any significant conclusion difficult.

However it can be argued that the outcome of a contemporaneous group of patients who developed adenoviraemia and did not receive Cytovir ADV was worse. Of the 21 patients who became viraemic during the trial period, 15 patients did not receive Cytovir ADV (7 patients were not registered on the study and did not have products made; 8 patients were registered but not eligible to receive Cytovir ADV at the time of reactivation). Of these 15 patients, mortality rate was 60% (n=9); compared to 33% (n=2) in the group of 6 patients who received Cytovir ADV. Of the 9 viraemic patients who died and did not receive ADV-specific CTLs, 6 had high adenoviral load in blood at the time of death that could have been contributory to their demise. It could be argued that in some patients where adenovirus was already causing significant disease, that administration with adenovirus CTLs would not have made a difference to the eventual outcome. However, in some patients, there was a window in which adenoviraemia was present but before virus had opportunity to cause significant organ damage. It is perhaps in these patients that treatment with ADV CTLs could have controlled viraemia and allowed more time for immune reconstitution to take place, thereby bringing about a more favourable clinical outcome.

The next section will examine different aspects of T-cell immunotherapy for the treatment of viral infections post HSCT.

REGULATORY T CELLS (Tregs)

Regulatory T cells (Tregs) encompass several distinct phenotypes of immune system cells (Maizels and Smith, 2011) that are suppressive in nature and regulate responses towards tumour, foreign and allo-antigens that constitutively express CD25 (Fehervari and Sakaguchi, 2004b; Sakaguchi, 2004). They have an important role during immune homeostasis through the maintenance of immune tolerance and prevention of inflammatory disease (Hori et al., 2003). Treg cells are essential for the tolerance of commensal microbiota in the gut, but an excessive Treg cell response may facilitate tumor growth and chronic infection by limiting anti-tumor or anti-pathogenic immune responses (Gao et al., 2012; Workman et al., 2009). Tregs constitutively express a variety of molecular markers, most significantly CD25 (IL-2Rα chain), CD62L, CD103, CD152 (CTLA4 – a molecule classically only expressed after T cell activation) and GITR (glucocorticoid-induced TNF receptor family-related protein, also known as TNFRSF18). None of these markers are uniquely associated with Tregs as they can also be upregulated upon activation of conventional T cells (Fehervari and Sakaguchi, 2004a; Fehervari and Sakaguchi, 2004b). However, the isolation of T cells that constitutively express CD25 has become a very useful functional definition for Treg cells and it is widely known that this population of cells is important in the maintenance of self-tolerance and control of immune response, as depletion of CD25+CD4+ cells induce autoimmunity or enhancement of immunity (Sakaguchi et al., 2001). Subsequent work had concentrated on finding molecular markers that are unique to this T cell lineage and breakthrough came about with studies on the Scurfy *(sf)* mutant mouse model. The Scurfy

mouse exhibits a fatal X-linked lymphoproliferative disease that is mediated by highly activated CD4+ T cells (Godfrey et al., 1991; Lyon et al., 1990). The *sf* mutation was subsequently mapped to a novel forkhead/winged-helix family transcriptional repressor termed *Foxp3,* which encodes the protein scurfin (Godfrey et al., 1991). A 2-bp frameshift insertion in the X chromosome gene *Foxp3* results in a truncated gene product lacking the C-terminal forkhead domain. Male mice hemizygous for the X-linked *Foxp3*^{sf} mutation succumb to a CD4+ T cell-mediated, lymphoproliferative disease characterised by wasting and multi-organ lymphocytic infiltrates (Fontenot et al., 2003; Lyon et al., 1990). Similarly multiple mutations have been identified in the human orthologue, *FOXP3*, as the underlying cause of the aggressive autoimmune syndrome IPEX (for Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) (Bennett et al., 2001; Wildin et al., 2002). This finding led several independent groups to demonstrate that Foxp3 mRNA and the encoded protein were specifically expressed only in naturally arising CD25+CD4+ Treg cells and were never observed in normal T cells even after activation (Fehervari and Sakaguchi, 2004b; Fontenot et al., 2003; Hori et al., 2003). Fontenot and colleagues (Fontenot et al., 2003) demonstrated that retroviral-driven Foxp3 expression in $CD4^{+}CD25^{-}$ T cells resulted in complete protection from wasting disease with accompanying colitis and inhibition of homeostatic proliferation of $CD8^{+}$ T cells. These observations led to the conclusion that Foxp3 plays a role in the development and function of Tregs and is also used as a phenotypic marker of Tregs (Fontenot et al., 2003; Fontenot et al., 2005; Fontenot and Rudensky, 2005). In addition to Foxp3-expressing Tregs are other functional

regulatory cells, which may produce IL-10 (termed Tr1) and IL-35 (Tr35) (Collison et al., 2010). The definition of the Tr1 subset is relatively fluid as IL-10 can also be produced by both Th1 (IFNγ+) and Th2 (IL-4+) effectors (Jankovic et al., 2007). T cells producing TGF-β can also act in a regulatory capacity. And while Tregs are generally found to be CD4+, CD8+ T cells can also express Foxp3 and produce the same suppressive cytokines (Maizels and Smith, 2011; Nakagawa et al., 2010)

Tregs are generated during the normal course of T cell development in the thymus and are termed thymic or natural (n)Tregs, but may also develop in the periphery in certain situations and are termed induced (i)Tregs (Shevach, 2011). Thymic Tregs are a CD4+CD25+Foxp3+ phenotype, and deficiency of these cells results in fatal autoimmune inflammation; this subset remains committed to this function over time (Maizels and Smith, 2011; Rubtsov et al., 2010). Naïve T cells differentiate into iTreg cells in the presence of IL-2, transforming growth factor (TGF)-β and stimulation through the T-cell receptor (TcR) (Chen et al., 2003; Gao et al., 2012). TGF-β is also required for IL-6-dependent expression of the transcription factor RORγt and inhibition of FOXP3 expression during the generation of Th17 cells from naive T cells (Gao et al., 2012; Mangan et al., 2006). Induced Tregs can convert from CD4+Foxp3- to CD4+Foxp3+, expanding the range of Treg specificities to exogenous antigens. However, iTregs are not irrevocably programmed and may revert to an effector phenotype, losing expression of CD25 and Foxp3 under certain situations (Maizels and Smith, 2011). In the peripheral Treg cell pool there are three further classifications: ‘central’, ‘effector’ and ‘tissue-resident’. Central Treg cells

constitute the majority of Treg cells in the circulation and secondary lymphoid organs. They express CC-chemokine receptor 7 (CCR7) and the adhesion receptor CD62L (also known as L-selectin), which direct their recirculation through lymphoid tissues. Effector Treg cells make up a minor fraction of Treg cells in the circulation and secondary lymphoid organs. They share phenotypic features with activated conventional T cells and are variously defined as $CD62L^{low}CCR7^{low}CD44^{hi}KLRG1^{+}$ (killer cell lectin-like receptor subfamily G member 1-positive) $CD103^{+}$. Tissue-resident Treg cells are those that have long-term residence in non-lymphoid tissues, as opposed to the short-term migration through non-lymphoid tissues observed by effector Treg cells. Currently, the best examples of tissue-resident Treg cells are adipose tissue Treg cells, which are marked by the expression of peroxisome proliferator-activated receptor-γ, and gut-resident Treg cells, which are marked by free fatty acid receptor 2 expression (Liston and Gray, 2014). Additional characteristics differentiate central and effector Tregs. Central Tregs are predominantly found in the T-cell zones within the spleen, whereas effector Tregs localise to the marginal zone, red pulp, and B-cell follicle within the spleen. Distinct signals required for their homeostasis and survival also differ: central Tregs depend on IL-2R signalling, whereas effector Tregs require ligation through the co-stimulatory molecule inducible T cell co-stimulator (ICOS) (Rothstein and Camirand, 2015; Smigiel et al., 2014). Central Tregs arise in the thymus and serve as a longer-lived pool of recirculating Tregs in secondary lymphoid organs. They become effector Tregs upon activation which are required for the maintenance of self-tolerance in nonlymphoid tissues, as well as in secondary

lymphoid organs (Rothstein and Camirand, 2015). The transcription factor interferon regulatory factor 4 (IRF4) appears to be a key regulator in effector Treg differentiation. It also promotes the expression of B lymphocyte-induced maturation protein-1 (BLIMP1) in effector Tregs, which is required for their function (Cretney et al., 2011).

$CD4^+$ $CD25^{high}$ T-cell subset represents only 1-2% of circulating $CD4^+$ T cells in humans (compared to situation in rodents where 6-10% of splenic $CD4^+$ T cells demonstrate regulatory function). Upon TCR cross-linking, $CD4^+CD25^{high}$ cells are anergic and totally inhibit the proliferation and cytokine secretion by activated $CD4^+CD25^-$ responder T cells in a contact-dependent manner (Baecher-Allan and Hafler, 2005).

**** Tregs and infections***

Recent work has shed more light into the role of Tregs in response to both acute and chronic infections (Boer et al., 2015; Maizels and Smith, 2011; Richert-Spuhler and Lund, 2015; Sanchez et al., 2012). Tregs are induced by a wide range of pathogens and can exert distinct effects for different pathogens and in different stages of infection. They may potentiate effector response by assisting in recruitment of T cells to the infection site to resolve infection, facilitate accelerated antigen-specific memory responses, limit pathology, and contribute to disease resolution and healing (Richert-Spuhler and Lund, 2015). On the other hand they may also facilitate chronic pathogen persistence by reducing effector immunity and clearance of infection (Boer et al., 2015).

Tregs utilise several mechanisms to suppress pro-inflammatory cells. These include secretion or generation of inhibitory soluble factors (e.g. TGF-β, IL-10, IL-35, and adenosine), modulation of antigen-presenting cells (APCs) and activity of certain Treg membrane expressed molecules (such as cytotoxic T-lymphocyte-associated antigen 4 – CTLA-4), direct killing of targets (through granzyme A/B), or deprivation of IL-2 or the amino acid tryptophan (through high IL-2R or the amino acid tryptophan (through high IL-2R expression or induction of indoleamine 2,3-dioxygenase in dendritic cells, respectively) (Boer et al., 2015; Rothstein and Camirand, 2015). Several Treg-expressed molecular markers have now been implicated directly in mediating suppression, such as CTLA-4, which modulates APCs via its ligands CD80 and CD86. Typically, APC at sites of infection upregulate CD80 and CD86 in response to signalling by TLR or other microbial pattern recognition receptors. As a result, APC increase both in number and level of costimulatory molecule expression, resulting in the initiation of T cell responses in a CD28-dependent manner. The inhibitory receptor CTLA-4 shares the same ligands with CD28 but opposes T cell responses via a cell-extrinsic pathway; it influences the cells around it rather than the cells expressing it (Walker and Sansom, 2011) (Hou et al., 2015). Tregs expressing CTLA-4 capture CD80 and CD86 ligands from APC via trans-endocytosis and degrade them intracellularly, thereby relatively depleting the APC's expression of essential co-stimulatory receptors for T-cell CD28 ligation (Qureshi et al., 2011). In addition, the ecto-enzyme CD39 (E-NTPDase1), a relatively newly discovered Treg marker, exerts its suppressive effects through breakdown of adenosine triphosphate (ATP) (Boer et al., 2015). It is now

apparent that in response to environmental cues, Treg responses adapt to the type of immune response [i.e. T helper 1 (Th1), Th2, Th17, and T follicular helper cell (Tfh)]. This allows Tregs to both gain access to inflamed peripheral tissues and to limit the immune response at hand. The specialisation of Treg function is accomplished by distinct Treg subsets. Treg also function within peripheral tissues where they contribute to tissue homeostasis and repair. Therefore complexity of Treg function now extends to the control of nonimmunological processes in nonlymphoid tissues (Rothstein and Camirand, 2015).

Using a murine model, Jost and colleagues (Jost et al., 2014) demonstrated that Foxp3+ Tregs as well as T-cell-derived IL-10 interfere with an effective anti-viral immune response during mCMV infection. Depletion of Fop3+ Tregs by using DEREG (depletion of T reg) mice resulted in elevated T-cell activation as measured by the expression of CD62L, granzyme B and IFN-γ, and was accompanied by a significant reduction in viral titers in salivary glands, the site of persistent mCMV replication and shedding. The group also identified CD4+ Foxp3- T cells to produce elevated levels of the immunosuppressive cytokine IL-10 at early time points during mCMV infection. Thus the group's data illustrated a crucial role for CD4+Foxp3+ nTregs as well as IL-10-producing CD4+Foxp3- iTregs in the regulation of appropriate T-cell responses and viral clearance during mCMV infection.

**** Tregs and immunotherapy***

Because viral infections/reactivation and GvHD are two of the more common complications arising post HSCT, there has been interest in the concept of co-infusing both Tregs and anti-viral T cells as form of immunotherapy that inhibits GvHD on one hand and promotes anti-viral immune reconstitution on the other (Litjens et al., 2012; Lugthart et al., 2012; Samuel et al., 2014). Litjens and colleagues (Litjens et al., 2012) were able to identify functional Ag-reactive natural Tregs (nTregs) cells for a range of different common viral and vaccination Ags, at a frequency similar to the frequency of Ag-reactive T effector cells within the CD4+ T cell population. Ag-reactive nTregs were identified by CD154 expression and showed a memory phenotype and shared all phenotypical and functional characteristics of nTregs. They could be expanded by specific antigenic stimulation, while their Ag-reactive suppressive activity was maintained. Previous mouse model data also suggest that the coinfusion of Tregs and conventional T cells enhances rather than abrogates recovery of virus-specific immune reconstitution through prevention of GvHD (Nguyen et al., 2008). Samuel and colleagues (Samuel et al., 2014) investigated the phenotype and function of CMV-specific T cells isolated through CD25 expression from G-CSF-mobilised PBMCs with regard to FoxP3 expression and suppressive capacity and explored the impact of removal of CD4+ CD25+ Tregs from starting populations to augment selectivity of antigen-specific T cells for adoptive immunotherapy. The group found that CD25 enrichment post CMV stimulation in G-CSF-mobilised PMBCs results in the simultaneous generation of both a functional population of anti-viral T cells and Tregs thus illustrating a potential

single therapeutic strategy for the treatment of both GvHD and CMV reactivation following allogeneic haematopoietic stem cell transplantation. It also circumvents the many problems incurred with successive donations and procurement of cells from unrelated donors, thereby simplifying the clinical application of adoptive immunotherapy and broaden the approach for manufacturing multi-functional T cells.

MEMORY T CELLS

There is increasing interest amongst research groups to determine which infused T cell population results in long-lasting viral immunity in patients. In earlier studies carried out by group in Baylor, trispecific T cells for EBV, CMV and ADV were given to patients (Gerdemann et al., 2013b). Although they were able to detect increase in frequency of T cells against latent viruses EBV and CMV, ADV specific T cells were only detected in those with recent or concurrent ADV infection. Looking more closely at CMV specific T cells and further into T cell subpopulation, Scheinberg's group (Scheinberg et al., 2009) demonstrated that less differentiated CD27+ CD57- CMV-specific memory T cells were more likely to persist in recipient after HSCT, compared with more terminally differentiated CD27- CD57+ CMV- specific memory T cells. The group also demonstrated that greater numbers of less differentiated CD8+ CMV-specific T cells in the donor appears to confer protection against viral reactivation in the recipient after HSCT. Feuchtinger and colleagues (Feuchtinger et al., 2010) treated 18 patients with CMV-specific T cells generated by ex vivo stimulation with pp65 and isolation of interferon-γ-producing cells. The group identified a mixture of naïve, central memory, and effector memory stages in the INF-γ-

selected T-cell population. The pp65-specific T-cell immunity could be detected for > 6 months in patients after adoptive T-cell transfer.

More recently a subset of human T cells called stem cell-like memory T cells (Tscm) have been shown to be self-renewing and multipotent and can sustain generation of all memory and effector T cell subsets (Gattinoni et al., 2011). Tscm express naïve markers but also have increased CD95 expression and show attributes of memory T cells (CD45RA+CCR7+CD62+CD27+CD28+CD127+CD95+). They represent about 2-3% of all circulating CD4+ and CD8+ T cells in healthy donors, and <1% in umbilical cord blood (Gattinoni et al., 2011). Similar to memory T cells, Tscm cells possess the memory capability of rapid acquisition of effector functions after TCR stimulation; and divide extensively in response to IL-15. In donors with detectable naïve-like CD8+ T cells specific to influenza or CMV epitopes, vast majority of tetramer-binding cells highly expressed CD95. By analysing TCR-β sequences of CMV-specific T cell subsets spanning a time period of 22 months, dominant persisting clonotypes in Tscm cells were found indicating that they represent a stable memory T cell population rather than merely recently activated cells transitioning from a naive to a conventional memory state (Gattinoni et al., 2011). Tscm have also shown enhanced proliferative and survival capacities compared with naïve and conventional memory subsets after adoptive transfer into NSG mice. By adoptively transferring CD8+ T cell subsets into immunodeficient mice, the group found that Tscm cells engrafted with 10- to 100- fold more progeny than central memory T cells (Tcm) or naïve T cells (Tn). Effector memory T cells (Tem), representing cell populations currently used in

adoptive immunotherapy, had a poor proliferative and survival capability resulting in negligible engraftment 1 month after transfer.

It is increasingly recognised that in the setting of haematopoietic stem cell transplant and adoptive T cell transfer, it is more desirable to have minimally differentiated cells with a high proliferative potential which can expand in the presence of virus in recipients and maintain effector functions. Adoptive transfer of long-lived, multipotent CD62L+ memory T cells might significantly improve persistence and potential therapeutic efficacy of adoptive immunotherapies (Gattinoni, 2014). To illustrate the stemness of CD8+ memory T cells, Graef's group measured self-renewal and multipotency of a single Tcm cell throughout a series of in vivo clonogenic assays (Graef et al., 2014). The group showed that after pathogen stimulation, a single Tcm cell could propagate itself while giving rise to a diverse progeny of Tem and Teff cells. The group suggested that regenerative quality rather than the quantity of transplanted T cells determine long-term persistence and success of adoptive T cell transfer; and that large numbers of cells are not necessary for therapeutic success when memory stem cell populations are employed.

It has also been demonstrated elsewhere that lowest numbers of ex vivo-selected CD8+ memory T cells can reconstitute pathogen-specific immunity in immunocompromised hosts (Stemberger et al., 2014). Using murine *Listeria monocytogenes* infection model, the group was able to show that major histocompatibility complex-Streptamer-enriched antigen-specific $CD62L^{hi}$ but not $CD62L^{lo}$ CD8+ memory T cells proliferated, differentiated, and protected against *L.m.* infections after prophylactic application. Two patients received low-dose

HLA-Streptamer-enriched CMV-specific CD8+ T cells (3750 cells and 5130 cells per kg body weight, respectively) and experienced decreasing viral load with demonstrable antigen-specific T cells. The expanding CMV A2-pp65-multimer-positive cells showed development from a less differentiated phenotype initially containing CCR7+ CD45RA- central memory phenotype cells to a mature population with a high percentage of Tem cells (CCR7-CD45RA+). The authors are in agreement with others that relatively undifferentiated human CD62Lhi memory T cells are better candidates for long-term protection against viruses in adoptive T cell transfer (Berger et al., 2008; Gattinoni et al., 2011).

Looking at plasticity of antigen-specific CD4+ T cells, CMV specific T cells that express TNF-α only predominantly displayed an early-differentiated phenotype with proportion expressing stem cell memory markers, compared to CD4+ T cells that express both IFN-γ and TNF-α which express more terminally differentiated cell markers (Edwards et al., 2014). TNF-α expressing CD4+ T cells also displayed functional plasticity by switching from TNF-α only to both IFN-γ+ and TNF-α+ upon in vitro stimulation with IE-1 CMV protein, suggesting that presence of less-differentiated populations endows T cells with functional flexibility and can reinforce immune responses in the control of viral infection, upon exhaustion of more differentiated cells.

In my own experiments looking at T-cell phenotypes of unexpanded cells compared to ADV-specific T cells, there was a general trend for decrease in proportion of cells expressing stem cell memory markers (CD45RA+CD27+CD62L+CD95+). Previous adoptive T-cell transfer based clinical trials suggest that the capability to persist in vivo is dependent on the

differentiation stage. The cells employed in these trials display terminal differentiation and, following transfer, fail to persist in the long term in vivo (Mahnke et al., 2013; Russo et al., 2012). If used in the prophylactic setting it is more desirable to transfer polyspecific Tcm cells that can survive until pathogens start to replicate and functionally differentiate after antigenic stimulation and prevent clinical manifestation. ADV-expanded cells in my experiments although showed a reduction in proportion of cells with Tscm phenotype markings, they were able to show antigen specificity and cytokine production upon stimulation with ADV peptides. This suggests that in the preemptive setting such as described in the ASPIRE trial, the more differentiated transferred antigen-specific T cells may still be able to provide antiviral killing properties until transplant recipient reconstitutes immunity.

It has been suggested that cytokines used in in-vitro culture of VSTs may enhance proportion of Tscm population. In the ASPIRE trial, donor PBMCs are supplemented with IL4 and IL7 when stimulated with ADV peptide. Several groups have reported on IL-7 and IL-15 preferentially expanding Tscm cells and not inducing excessive proliferation and acquisition of effector function (Cieri et al., 2013; Lugli et al., 2013). Effector cells expanded in vitro from early differentiated cells, such as from the Tcm cells, also correlate with improved persistence (Mahnke et al., 2013; Restifo et al., 2012).

BYSTANDER ACTIVATION

One of the concerns with T-cell immunotherapy is alloreactivity or stimulation of bystander cells. A case of third-party (haploidentical) virus-specific T cells triggering bystander GvHD has previously been reported (Qasim et al., 2011). A 7-year-old girl who had undergone a 1C mismatched unrelated donor stem cell transplant received a dose of 10^4/kg CD3+ ADV-specific T cells from a haploidentical family donor for adenoviraemia. This resulted in resolution of high level adenoviraemia along with appearance of ADV-specific lymphocytes when tested *in vitro* 6 weeks post infusion. However, skin and liver GvHD developed around the same time with presence of original donor cells only detected by FISH on liver biopsy. This suggested proliferation of a bystander T cell population stimulated by antigen-specific T cell responses. As had been shown in mouse models bystander activation can be mediated by cytokines, independent of specific TCR stimulation (Tough et al., 1996). Similarly using a transgenic mouse model the experiments of Ehl and colleagues (Ehl et al., 1997) showed that CD8+ T cells from unimmunised 'naïve" TCR transgenic mice can differentiate into LCMV-specific cytolytic effector CTL during infections with vaccinia virus or *Listeria monocytoges.* Interestingly the group also showed that the factor mediating the bystander activation was transferable in the supernatant of specifically activated T cells, suggesting presence of soluble signal generated by specific responder cells. In human subjects who had received tetanus toxoid (TT)-booster vaccination, the phenomenon of bystander activation and expansion of CD4+ memory T cells specific for two unrelated antigens, purified protein derivative of tuberculin and *Candida albicans* have

been described (Di Genova et al., 2006). More recently the same group used a mouse model to better understand the underlying mechanism. Activated transgenic $CD4^+$ T cells (OT-II) specific for a peptide from OVA were transferred to mice pre-vaccinated with tetanus toxoid (TT). When these mice were subsequently challenged with TT, not only was there cytokine production (IL2 and IFNγ) by TT-specific memory CD4+ T cells, the preactivated OT-II T cells also underwent bystander proliferation. The authors also found the bystander proliferation of peptide-activated T cells was proportional to the strength of the secondary cellular immune response to TT; and suggested IL-2 and IL-7 as potential mediators for bystander stimulation. Interestingly, co-culture with inflammatory cytokines IFN-γ, IL-1β, Il-6 and TNF-α did not induce proliferation, arguing against direct involvement of inflammatory cytokines in bystander proliferation. The authors concluded that transfer of cytokines, possibly IL-2, from an antigen-specific CD4+ T-cell memory response of significant magnitude, occurs in vivo at a tissue site. Parallel CD4+ T cells activated by other antigens can express appropriate receptors and undergo proliferation which could contribute to immunopathology (Di Genova et al., 2010).

Using an *in vitro* system within which to analyse bystander T cell activation in human Bangs and colleagues investigated specific characteristics of bystander-activated T cells and confirmed that it occurs preferentially among CD4+ memory T cells and that the mechanism for their activation involves soluble factors and is not dependent upon cell-cell contact (Bangs et al., 2009).

RECENT CLINICAL EXPERIENCE

Recently the Tubingen group in Germany published their experience using donor-derived ADV hexon-specific T cells to treat 30 patients with treatment refractory ADV infection (Feucht et al., 2015). VSTs were derived by stimulating donor PBMCs with ADV hexon protein for 16 hours followed by enrichment of IFN-γ secreting cells using *CliniMACS* (Miltenyi Biotec). Mean T-cell dose transferred was $4.1x10^3$ CD3+ cells/kg (range $0.3\text{-}24x10^3$), composed of a mixture of naïve, central memory, effector memory and effector T-cell populations with predominance of late effector stages. 14 out of 23 evaluable patients developed in vivo expansion of ADV-specific T cells with 86% clearing viraemia within the 6-month observation period. ADV-related mortality was 100% in non-responders compared to 9.5% in responders.

RECENT ADVANCES

In the last few years there has been increasing interest in therapeutic application of T-cell engineering particularly in the treatment of haematological malignancy. One strategy to bypass immune tolerance in the treatment of malignancy is by modifying native T cells with a chimeric antigen receptor (CAR) which can then be targeted against any extracellular antigen. CAR T cells are reprogrammed using synthetic biology and gene transfer techniques to attack cells expressing the target extracellular antigen, independent of MHC presentation (Miller and Maus, 2015). CAR therapies were first conceived in the last 1980s, but there has been a recent breakthrough in the application of autologous CD19-directed

CAR T cells for the treatment of adult and paediatric relapsed/refractory ALL (Humphries, 2013; Miller and Maus, 2015). However one difficulty with adoptively-transferred tumor-specific T cells is the requirement for extensive *in vivo* proliferation via stimulation through CAR or TCR, costimulatory receptors and cytokine, and most tumours do not present antigens in an immunostimulatory fashion and lack costimulatory molecules. Therefore infused tumor-specific T cells are unlikely to receive adequate stimulation after infusion. By contrast, most viral infections are highly immunostimulatory, especially in the case of EBV and CMV infection post-transplant (Sun et al., 2015). Therefore Cruz and colleagues (Cruz et al., 2013) explored the option of infusing donor-derived virus-specific T cells that have been engineered to express CD19-CAR into 8 patients with residual B-cell malignancies after HSCT. No infusion-related toxicities were observed and VSTs persisted from 1 to 12 weeks after each infusion. Two of 6 patients with relapsed disease during the period of CD19.CAR-VST persistence had objective antitumor activity and 2 who received cells while in remission remained disease free. In 2 of 3 patients with viral reactivation, donor CD19.CAR-VSTs expanded concomitantly with VSTs. The authors concluded that the inherent virus specificity of the native TCRs in the allogeneic CD19-CAR-VSTs promoted the proliferation and persistence of these CAR-VSTs in vivo in response to endogenous viruses (CMV, ADV, EBV) and associated co-stimulatory molecules presented on antigen-presenting cells infected with virus or cross-presenting viral antigens. However there was little apparent expansion of these cells in patients. Therefore in a more recent study Sun and colleagues (Sun et al., 2015) developed and optimised a GMP

compliant method for the early transduction of VSTs (instead of transducing on day 19 of culture as in Cruz's study which might have allowed enough T-cell differentiation to occur to limit subsequent proliferative capacity of transduced T-cells). Using a CAR directed to the tumor-associated antigen disialoganglioside (GD2) the group transduced VSTs directed to EBV, ADV and CMV early on day 3 of culture with prosurvival cytokines (IL-4 and IL-7) to allow rapid expansion and early cryopreservation of GD2.CAR-modified triVSTs for use in a clinical trial in patient who have received T-cell depleted HSCT for multiple relapsed neuroblastoma. The authors suggested that early-transduced T-cells should have a greater potential to proliferate, eliminate tumors and enter the memory compartment than VSTs transduced on day 19 at which time the majority of cells have lost expression of CCR7 and have an effector memory phenotype. These GD2.CAR-modified triVSTs have been used in a phase 1 clinical safety trial (NCT01460901). The primary aim of the trial is to investigate safety and persistence of the modified cells and evaluate the anti-viral and anti-tumor function of the infused GD2.CAR-modified triVSTs in patients with relapsed/refractory neuroblastoma post allogeneic stem cell transplant.

SUMMARY

In summary this thesis has demonstrated the safety and efficacy in adoptive T-cell Immunotherapy in the treatment of adenoviraemia in a small number of high-risk paediatric transplant recipients. Although it is difficult to ascribe effectiveness solely to transferred virus-specific T cells in exclusion of drug therapy and immune reconstitution, the majority of patients who developed adenoviraemia and received Cytovir ADV survived compared to the non-

survivors who developed adenoviraemia and did not receive VSTs. The results of the phase I/II2 clinical ASPIRE study support a further clinical trial enrolling a larger number of patients to further the understanding of patient and dose selection. Ultimately the wider application of virus-specific T cells beyond the realms of clinical trials, and progression from bespoke designer treatment to licenced medicinal products incorporated into standard clinical care is currently limited by manufacturing logistics. However there is increasing interest and investments from pharmaceutical companies in the field of T-cell therapy, and certainly the potential to reduce morbidity and mortality and possibility of reducing need for antivirals is an incentive for health care providers to accelerate the clinical application of virus-specific T cells beyond the super specialised centres. In the UK there are a number of transplant centres that perform paediatric allogeneic stem cell transplants. A collaboration between all these centres would be ideal in ensuring all high-risk paediatric patients will have access to virus-specific T cells. Due to the costs involved in the manufacturing process, it would be sensible to have one or two central production hub and a robust method of cell transfer out to required centres. In addition it would be helpful to develop harmonised end points and consensus between research groups on clinical trial design so to facilitate comparisons between studies. Given the explosion in the field of cell therapy, both in the treatment of viral infection and cancer, the development of a national and international consensus may ultimately make it easier for groups to obtain ethical approvals and facilitate development of harmonised cell therapy trials worldwide.

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