

**Donor T cells with inducible caspase safety switch following haploidentical
transplants.**

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A thesis submitted to The University College London

for the degree of Doctor of Medicine (Research)

'I, Reem Ahmed Elfeky, 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

Haplo-identical donors are alternative source of hematopoietic stem cells for patients without a more closely matched donor or who need an urgent allogeneic hematopoietic stem cell transplant (HSCT). Because the donor graft for such haploidentical transplants (haplo-HSCT) has a high frequency of alloreactive T cells recognizing the non-shared HLA haplotype, extensive T-cell depletion remains a fundamental prerequisite if the graft is not to cause fatal acute graft-versus-host-disease (GvHD).

While extensive T-cell removal of the graft effectively prevents GvHD, it increases the risk of graft rejection, relapse, viral and opportunistic infections. Consequently, efforts were made to retain the desired T cells while selectively depleting alloreactive T cells (Aversa et al, 2005 and Mielke et al, 2008).

Engineered T cells with safety switches have been developed to increase the feasibility of higher numbers of donor-derived T cells whilst providing a tool to control the increased risk of aGvHD that maybe associated with incomplete abrogation of alloreactivity against the recipient [Amrolia et al, 2006]. This thesis presents data from a phase I/II first- in- man use of TCR $\alpha\beta$ /CD19 depleted transplant followed by adoptive transfer of genetically modified donor T cells. These donor T cells were modified through gamma-retroviral vector that carried inducible suicide gene (inducible caspase 9; icas9) which makes cells die on exposure to a drug called AP1903. This thesis also examines the development of an alternative lentiviral vector for icas9 gene transfer, investigates the effect of immune suppressive medications on genetically modified T cells and investigates mechanism of resistance to AP1903.

Impact statement

BPX-501 (T cells transduced with gamma-retroviral vector that carried inducible caspase 9) administration improved the outcomes of TCR $\alpha\beta$ /CD19 depleted grafts with better handling of post-HSCT viral infections and low relapse rates associated with accelerated T cell immune reconstitution. Overall survival rates of 94% was achieved. These rates are equivalent to rates reported with the use of matched unrelated donor grafts. This strategy offers an effective alternative source of hematopoietic stem cells for patients without a more closely matched donor or who need an urgent allogeneic hematopoietic stem cell transplant (HSCT).

Recent years has witnessed advances in CAR T cell therapies to target B -cell malignancies with 2 anti-CD19 CAR T schemes being approved by FDA (Leyfman, 2018). However, the application of CAR-T cells is obviously hampered by the adverse effects, such as cytokines release syndrome and on-target off-tumor toxicity. Researchers have developed a number of safety strategies of CAR-T cells, including use of suicide genes (Yu et al, 2019) to limit CAR T cell mediated toxicities. This study showed the efficacy of icas9 as a suicide gene technology with rapid response in clearance of marked T cells, however, incomplete clearance represents a concern that need tackling.

While there are different possible mechanistic factors leading to resistance to killing, a mutation in icas9 transgene is one of the potential factors that can lead to poor expression of suicide gene and resistance (current study and Zhou et al, 2016). The raised question here is could we induce a change (mutation) in icas9 transgene to increase expression of the gene and thus improve the sensitivity to the dimerizer and allow not only rapid but also completed clearance of marked cells in case of toxicity.

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**Chapter 1 :
Introduction**

1.1 Introduction on haploidentical transplant

Allogeneic hematopoietic stem cell transplantation (HSCT) can be a curative treatment for children and adolescents with various malignant and non-malignant diseases. While a human leukocyte antigen (HLA)-identical sibling donor is the preferred choice; a matched unrelated volunteer donor is another realistic option for successful HSCT. However, depending on ethnicity, 30%-80% of patients lack an HLA-matched donor (Gragert et al, 2014) leading to a considerable number of deaths in patients denied transplantation. Although mismatched transplantation from related or unrelated adults and cord blood donors can be used in this scenario, such approaches are associated with a higher risk of morbidity and mortality compared to Graft versus Host Disease (GvHD) and delayed immune reconstitution (Gennery et al, 2010, Booth et al, 2016).

1.2 Advantages of haploidentical transplant:

Nevertheless, HSCT from haploidentical family donors has several advantages (Im et al, 2016 (a)): 1) virtually all patients who need HSCT can find a donor who will be willing to donate being a parent or a sibling; 2) transplantation could be performed without delay, which is critical to patients with high-risk malignant disease or very severe aplastic anaemia requiring urgent treatment; 3) further access to the donor for cellular therapy to treat relapse or infection or for additional transplantations is easy. In addition, a haploidentical family donor could rescue a patient who experienced early graft failure (after other HSCT procedures) which is a life-threatening complication requiring prompt intervention [Lang et al, 2008, Yoshihara et al, 2012, Park et al, 2014, Rådestad et al, 2014).

1.3 Early reports on haploidentical transplant

Earlier attempts involved the use of T cell replete haploidentical transplant (TCRHT) bone marrow (BM) after a myeloablative conditioning protocol. Unfortunately, these attempts were unsuccessful with patients developing hyperacute GvHD with early severe pulmonary oedema ending in multi-system organ failure (Powles et al, 1983) and extremely low survival rates of 10% (Beatty et al, 1985).

Based on these disappointing early experiences using TCR BM, attempts were made to deplete T cells from BM for the prevention of GvHD. Reisner et al. demonstrated that the galactose-binding lectins soybean agglutinin (SBA) and peanut agglutinin (PNA) selectively bind to haematopoietic precursors in the bone marrow and spleen of the mouse. They found that these stem cells could be separated from T

lymphocytes by differential agglutination with SBA and PNA, and that the agglutinated cell fraction could be used to reconstitute haematopoietic function in lethally irradiated, H-2 incompatible mice without GvHD (Reisner et al, 1978). The first ex vivo T cell depleted (TCD) graft, using soybean agglutinin and rosette formation with sheep red blood cells, were performed in an infant with acute leukaemia (Reisner et al, 1981) followed by 4 children with immunodeficiency syndromes (Friedrich et al, 1984). The patients' courses demonstrated the potential of lectin-separated marrow grafts to restore durable haematopoiesis without the development of GvHD. This approach represented a milestone in the history of haploidentical transplant where a single manipulation TCD was able to prevent both acute GvHD (aGvHD) and chronic GvHD (cGvHD). Despite of the continuous success of this method as been demonstrated in multiple studies (Pai et al, 2014, Buckley et al, 2011) however, it did not follow good manufacturing practice (GMP).

With the development of anti-CD3 monoclonal antibodies, researchers studied using anti-CD3 antibodies in mismatched grafts for TCD. One of the largest studies were carried out by Mehta et al, 2004 who used intensive total body irradiation (TBI)-based myeloablative preparative regimens with partial in vitro T-cell depletion of haploidentical BM using anti-CD3 antibodies (Ab) (T10B9 or OKT-3). This led to a 1-1.5 log reduction of T cells with an infused T cell dose between $0.05-16 \times 10^5/\text{Kg}$. In this large series of 201 patients, 98% engrafted, with 5-year cumulative incidences (CIs) of relapse and transplant related mortality (TRM) of 31% and 51%, respectively. The CIs of grades II-IV aGvHD and cGvHD were 13% and 15%, respectively. An encouraging survival probability of 20% was seen in patients with advanced hematologic malignancies. Of note, the patients who developed grades II-IV aGvHD had received a significantly higher T-cell dose than the remaining patients (median $0.8 \times 10^5/\text{kg}$ versus (vs) $0.5 \times 10^5/\text{kg}$; $p=0.04$).

1.4 The move from BM as a stem cell source to peripheral blood stem cells (PBSCs)

Reisner group (Bachar-Lustig et al, 1995) introduced the "megadose" concept based on studies in animal models. The "megadose" concept was based on the observation that the transplantation of large numbers of purified CD34 stem cells could overcome the HLA barrier of haplo-identical transplantation by veto cells; dendritic cells within the CD34 selected stem cells (Rachamim et al, 1998, Gur et al, 2002). Megadose can also elude the residual anti-donor cytotoxic T-lymphocyte activity of the recipient (Bachar-Lustig et al, 1995).

As the number of BM CD34+ stem cells is limited by the amount of BM that can be harvested, Aversa et al, 1994 originally introduced into clinical setting a strategy for combining BM-derived CD34 stem cells and CD34 stem cells purified from granulocyte colony-stimulating factor (G-CSF)-mobilised peripheral stem cells from haplo-identical donors using a soybean agglutinin and E-rosetting-based technique. With this approach, large numbers of CD34 stem cells depleted of T cells (3-log reduction) could be transplanted. Primary engraftment was achieved in 16/17 patients with end-stage leukaemia. Without any pharmacologic GvHD prophylaxis, aGvHD II-IV were <10% with severe aGvHD occurring in only one patient and no cases of cGvHD were observed.

1.5 The move to CD34 + selection using more recent technologies:

In 1995, researches were looking for a TCD technique that could allow processing of large number of stem cells in a reasonable time and under good manufacturing practice conditions. Thus, indirect TCD of PBSCs moved from soya bean agglutination technique to using positive selection based on Cellpro system Ceptrate system (Cellpro, Inc., Bothell, USA) (Berenson et al, 1996) then using magnetic-activated cell sorting (Handgretinger et al, 1998) and later using automated CliniMacs (Miltenyi biotechnology) (Schumm et al, 1999) to purify CD34 + cells. By the later method, higher degree of T cell depletion (4.5-5 log) and B cell depletion (>3 log) were obtained. In absence of GvHD prophylaxis agents, Handgretinger et al, 2001 showed in 32 patients, almost no GvHD and due to B cell depletion, Epstein Barr Virus (EBV) lymphoproliferative disease was prevented (Handgretinger et al, 2001). Subsequent studies in larger cohort of patients started to show significant drawbacks with this TCD approach (Schumm et al, 1999). A large study performed by Acute leukaemia and paediatrics Working parties of the European Blood and Bone Marrow Transplantation group (EBMT) analysed outcome of 127 children with acute lymphoblastic leukaemia (ALL) receiving a CD34 selection graft. 5y leukaemia free survival (LFS) was 30%-34% in patients with complete remission (CR) CR1, CR2, 22% in CR3 and 0% in CR4. Higher CD34+ dose above $12.4 \times 10^6/\text{Kg}$ was associated with 5y LFS of $35 \pm 7\%$ vs $17 \pm 6\%$ in lower doses (Klingebiel et al, 2010) defining a poor anti-leukaemia effect of CD34+ selection with a relapse incidence between $36 \pm 4\%$. Moreover, non-relapse mortality (NRM) was 28.3% with 61% of deaths due to infections, 25% due to GvHD and poor handling of viruses, 24% due to relapse related mortality. Further studies showed that this TCD approach led to a significant delay in immune reconstitution with lethal viral infections mainly

cytomegalovirus (CMV) and adenovirus (AdV) both in adults and children (Walker et al, 2004, Redei et al, 2002, Peters et al, 1999, Yamasaki et al, 2004).

CD34+selection does not only deplete T cells but also indirectly depletes Natural Killer (NK) cells. Mature NK cells have been shown in different studies not only to display anti-leukaemia effect (Kim et al, 2005) but also important anti-viral function of possible importance in recipients of stem cell transplant (Locatelli et al, 2009, Moretta et al, 2008).

NK cells are key members of the natural immune system with a fundamental importance in limiting or eradicating pathogens during the early phases of a primary infection; before T and B cells can mount efficient responses (Locatelli et al, 2009, Moretta et al, 2008, Moretta et al, 2002). They also play a pivotal role in the defence against transformed cells. NK cells function through surface receptors; that include an array of inhibitory as well as activating receptors. Killer Ig-like receptors (KIRs) are NK inhibitory receptors that upon interacting with peptide-major histocompatibility complex (MHC) class I molecules (KIR ligand), precludes NK mediated killing. These interactions prevent NK cells from mediating attack against normal tissue (expressing MHC class I). While the cells in which MHC class I is downregulated (viral infected cells or malignant cells) are susceptible to NK mediated killing (Moretta et al, 2002, Artavanis-Tsakonas et al, 2003, Moretta et al, 2006, Moretta et al, 2011, Pende et al, 2009). Interestingly, molecular studies have shown another mechanism for NK mediated killing of transforming cells in a mechanism called "missing self-recognition" (Ljunggren et al, 1990), provided that the donor: i) expresses a KIR-ligand which is missing in the recipient HLA genotype; and ii) expresses the specific KIR, leading to a KIR/KIR-ligand mismatch in graft-versus-host (GvH) direction thus a graft vs leukaemia effect (GvL). In HSCT, NK alloreactivity can be predicted on analysis of the HLA-class I genotypes looking for a KIR ligand being expressed in the donor but missing in the recipient (KIR ligand mismatch). The clinical relevance of NK cell alloreactivity has been initially demonstrated in adults with acute myeloid leukaemia (AML) given haplo-HSCT. In these patients, a marked reduction in the risk of leukaemia recurrence was observed when the donor showed an NK alloreactivity with respect to the recipient HLA typing (Ruggeri et al, 2002). While mediating a GvL effect, NK cells do not induce GvHD. This selectivity is related to the fact, that while malignant cells display on their surface ligands for NK activating receptors, these ligands are missing in normal tissue (Pende et al, 2005). Moreover, in mice model, it was demonstrated that (Ruggeri et al, 2002) alloreactive NK cells can kill recipient dendritic cells (DCs) and cytotoxic T cells through activating signals. In a transplant

setting, recipient DCs act as antigen presenting cells that can interact with donor T cells leading to development of GvHD. Moreover, the ability of NK cells to eliminate cytotoxic T cells that survive the conditioning regimen is relevant to prevent rejection through a HvG effect. Thus, NK cells not only preclude the occurrence of GvHD but also prevent graft rejection.

Different studies (Pende et al, 2009, Vago et al, 2008) showed that quantitative NK cell reconstitution-emerging from stem cell progenitors- takes 7-8 weeks post-allograft however functionality might be delayed for up to a year with studies showing evidence of skewing of NK compartment in favour of immature NK cells (Dulphy et al, 2008). Thus, combined NK depletion/TCD in a CD34+ selection graft will limit the capacity of the graft in dealing with high burden disease or active viral infection increasing the risk of relapse, relapse related and infection related mortality.

1.6 Engineering the ideal graft:

1.6.1 CD3/CD19 depletion:

The idea of engineering an ideal graft that harbour a 1) mega dose of CD34+ cells to facilitate engraftment and overcome the HLA mismatch barrier, 2) enough T and B cell depletion to alleviate the risks of GvHD and EBV post-transplant lymphoproliferative disease (PTLD), 3) has NK cells that should prevent relapse in malignant settings and could have potential anti-viral effect was thus brought -up between 2002-2004 using automated CliniMacs for CD3+/CD19+ depletion. Though log depletion for T cells were 3.5-4 (1 log less reduction than with CD34+ selection) yet more NK cells, DCs and monocytes were preserved. These cells were considered facilitators of engraftment and possible protector against post-transplant viral reactivation and tumour relapse. A pilot study on 29 children and adolescents with malignant disease showed 100% engraftment with this approach and evidence of early immune reconstitution with a median time to achieve more than 100 CD3 and CD4 cells/ml was 61.0 and 63.5 days, respectively. However, overall survival (OS) was 48% at a median follow-up of 282 days with the main causes of death were tumour relapse and viral reactivation. Though CD3+/CD19+ depleted grafts overcame the risk of graft rejection in a haplo-HSCT however tumour recurrence and viral relapse was still an ongoing problem post-transplant (Bader et al, 2008). Subsequent study by the same team at Frankfurt showed more promising results among 58 adults with malignant and non-malignant Again 100% engraftment was achieved after using a preparative regimen of Fludarabine/Melphalan (Mel)/Thiotepa. 3-year OS was 100% among patients with non-malignant disease. Patients with acute leukaemia (ALL and AML; n =22) who at the time of transplantation were in

hematologic remission exhibited a very low recurrence rate following haploidentical transplantation with this procedure. These patients have a survival probability of 68% at 3 years after transplantation. Patients with acute leukaemia who were not in remission at the time of transplantation (n =7) could not be saved with this procedure. Thus, other therapy options were mandated (Bader et al, 2011).Figure 1.1 summarises the pros and cons of the different T cell depletion procedure.

Figure 1.1. Pros and Cons of different graft manipulation procedure in a haplo-HSCT

	Pros		Cons
TCR	Low graft failure rate Low Relapse rate Low risk of viral infection	1983	Hyperacute GvHD induced death Poor overall survival
TCD SB:	Low graft failure rate Low rates of GvHD	1984 1989	Not follow GMP
TCD OKT3:	Less aGvHD II-IV Less cGvHD	1993	High relapse rate Poor overall survival
Mega dose PBSCs	Good engraftment Less aGvHD	1995 1999	Mega dose concept in clinical trials Infection related mortality High relapse rate especially in cases with active disease
CD3/CD19 depleted	Good engraftment Improved overall survival in non-malignant setting	2005	Poor overall survival in malignant disease
		2010	TCRαβ/CD19 depletion

Abbreviations: PBSCs: peripheral blood stem cells, GMP: good manufacturing practice.

1.6.2 TCR alpha beta/CD19 depleted grafts:

CD3/CD19 depletion leads to a loss of certain cell subsets namely gamma delta ($\gamma\delta$) T cells as well as $\alpha\beta$ T cells. $\gamma\delta$ T-Cells straddle the innate and adaptive arms of the immune system and are involved in response to pathogens [e.g., mycobacteria; CMV] and tumours. Like $\alpha\beta$ T-cells, $\gamma\delta$ T-cells develop in the thymus, but express a rearranged T-cell receptor (TCR) consisting of a TCR- γ and a TCR- δ chain (Prinz et al, 2013). However, on a genomic level, $\gamma\delta$ T cells were shown to have a polyclonal repertoire; their activation is by direct cell contact in an MHC class I or II independent pathway. In humans, most peripheral blood $\gamma\delta$ T-cells express V δ 2 TCR chain paired with V γ 9 chain (Arden et al, 1995); constituting 1-5% of T cells. V δ 2 V γ 9 T cells

recognise the non-peptide phospho-antigen on bacteria and tumor cells. Pamidronate/Zolindronic acid were noted to activate $\gamma\delta$ T cells increasing their proliferative capacity and INF γ release (Kunzmann et al, 2000). V δ 2 V γ 9 T cells have been used in several phase I clinical trials in adults with difficult to treat cancer. In 9 adults with relapsed Hodgkin lymphoma and multiple myeloma, combined IL2/pamidronate was used as a salvage therapy. In vivo activation/proliferation of $\gamma\delta$ T cells was observed in 5 patients (55%), and objective responses were achieved in 3 patients (33%). Only patients with significant in vivo proliferation of $\gamma\delta$ T cells responded to treatment, indicating that $\gamma\delta$ T cells might contribute to this anti-lymphoma effect (Wilhelm et al, 2003). A further study including 10 adults with metastatic renal cell carcinoma were recruited in a phase I trial involved multiple infusions of invitro expanded $\gamma\delta$ T cells. Repeated infusions at different dose levels (up to 8×10^9 total cells), either alone or with subcutaneous IL-2 were well tolerated and associated with disease stabilisation in 60% of the cases (Bennouna Jet al, 2008). Another important subset of $\gamma\delta$ T cells are V δ 1 that were shown to mediate CMV immunity (Godder et al, 2007). V δ 1 harbour a terminally differentiated cytotoxic phenotype upon CMV reactivation and were shown to cross-react with tumor cells of hematopoietic origin thus explaining the protecting effect of CMV reactivation on tumor relapse (Scheper et al, 2013). Moreover, $\gamma\delta$ T cells cross talk with dendritic cells and NK cells (Vantourout et al, 2013) with a possible synergistic effect on dealing with viruses and malignant cells.

While T cells carrying $\alpha\beta$ receptor chains are immuno-reactive and responsible for GvHD, the $\gamma\delta$ T cells were shown to have no allo-reactive capacity (unable to recognise antigens presented on MHC molecules) (Drobyski et al, 1999, Morita et al, 1999).

Based on these data, a move from a CD3/CD19 depletion to a TCR $\alpha\beta$ /CD19 depletion was thought favourable. With this method, a T cell reduction of 4.5-5 log, comparable to CD34+selection, was achieved (Handgretinger, 2012). This approach involves incubation of mobilised mononuclear cells with a biotinylated anti-TCR $\alpha\beta$ antibody and subsequently with an anti-biotin antibody conjugated to magnetic microbeads. The cells then run through a strong magnetic field and TCR $\alpha\beta$ are retained while all other cells pass through (Bremm et al, 2017).

The first report on children undergoing haploidentical transplant of TCR $\alpha\beta$ /CD19 depletion was presented in ASH, 2011 where 2 centres; Tübingen and Rome, recruited 23 patients with different haematological diseases including 13 cases with

advanced /active leukaemia. In this pilot study, 100% engraftment was achieved with a relapse rate of 21% and no reported gut/liver aGvHD or cGvHD. Graft manipulation resulted in a consistent efficiency of TCR $\alpha\beta$ depletion of 4.3-4.5 log depletion (equivalent to log depletion with CD34+ selection). Rapid immune reconstitution with expansion of $\gamma\delta$ T cells in the first 100 days followed by $\alpha\beta$ T cell predominance by D+100 with evidence of $\alpha\beta$ TCR polyclonality being recorded early post-HSCT (Handgretinger et al, 2011).

Following this initial report, multiple centres worldwide started using this approach in haploidentical transplant settings. Table 1.1 and Table 1.2 summarised the outcome in different studies in children using this approach whether in the context of non-malignant or malignant conditions.

Table 1.1. Outcome of TCR $\alpha\beta$ / CD19 depleted grafts in patients transplanted for non-malignant disease

Studies	Bertaina et al, 2014	Balashov et al, 2015	Shah et al, 2018
No	23	37	26
Donor	Haplo	Haplo (10) MUD (27)	Haplo MMUD (n=3)
Conditioning	MAC (30%) RIC (35%) MIC (35%)	MAC (5.4%) RIC \pm Mel (91.8%) UC (2.7%)	MAC (88.5%) UC (11.5%)
Serotherapy	rATG 12mg/kg	rATG 10mg/kg (n=35) Alemtuzumab (n=2)	rATG 15mg/kg (n=18) Alemtuzumab (n=4)
GvHD prophylaxis	0%	100%	84.6%
OS	91.1%	96.7%	83.9%
Graft loss	16.2%	27%	3.8%
aGvHD grade I-II	13.1%	21.5%	41.5% (all skin Grade I-II)
aGvHD grade III-IV	0%	2.8% grade IV UC SCID	4.1% (Grade III skin)
Viral reactivation	38%	46%	69.2%
TRM	8.6%	3.3%	16.1%

	(Disseminated CMV/Adeno)	CMV pneumonia	(50% were infection related deaths)
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Abbreviations: haplo: haploidentical transplants, MUD: matched unrelated donor, MMUD: mismatched unrelated donor, MAC: myeloablative conditioning, RIC: reduced intensity conditioning, MIC: minimal intensity conditioning, UC: unconditioned, rATG: rabbit anti-thymocyte globulin

1.6.2.1 The outcome of TCR $\alpha\beta$ /CD19 depleted grafts in malignancy setting

Different groups have reported a high overall survival rates between 84-96% with the use of TCR $\alpha\beta$ /CD19 depleted grafts (Bertaina et al, 2014, Balashov et al, 2015, Shah et al, 2018). An Italian group reported a high graft failure rate 16.6% (Bertaina et al, 2014) in comparison to a UK group (3.3%) (Shah et al, 2018). Of note, graft loss occurred mainly among patients who are well known to be difficult to engraft; patients with sickle cell anaemia, thalassemia with allo-sensitisation and osteopetrosis whereas no graft failure was reported among primary immune deficiency patients (PIDs). All patients who had primary graft loss were successfully re-transplanted either from the same donor or the other parent. Interestingly, Balashov et al, 2015 applied TCR $\alpha\beta$ /CD19 depletion not only in the haplo-HSCT but also with MUDs. There was no difference between recipients of MUD vs recipients of haplo-HSCT in terms of rates of occurrence of aGvHD II-IV, viral reactivation or graft failure. It was noted that the CI of graft failure was significantly higher among patients who received a (RIC) conditioning 36.7% \pm 9.6% versus cases who received RIC+ Melphalan 10% \pm 9%. However, despite limited rates of GvHD in both studies and lack of GvHD prophylaxis among the Italian cohort (Bertaina et al, 2014), yet patients in both studies had significant problems with viral reactivation.

Shah et al, 2018 reported a very low graft loss rate reflecting the 1) use of MAC in most of cases, and the 2) lack of difficult to engraft cases-all were PIDs. However, again a noticeable high incidence of viral reactivation was seen, being responsible for 50% of deaths. This might argue against a real benefit from preserving NK and gamma delta T cells in the graft. Noticeably in all studies no cGvHD was recorded.

Although all studies showed that the quantitative recovery of T cells was not halted by the TCR $\alpha\beta$ /CD19 depletion approach ;> 500 CD3 (cells/ul) achieved as early as 3 months post-HSCT (Bertaina et al, 2014) and median time to CD4 recovery of \geq 200 being 129 days with appearance of naïve T cells in most patients as discussed in Shah et al, 2018. However, as mentioned rates of viral reactivation were noticeably high across all studies. The generation of a broad TCR repertoire -not mentioned in any of the 3 studies was shown by Zvygan et al, 2016 to be delayed. Moreover, whilst

there might be beneficial anti-CMV effect from infused $\delta\gamma$ cells as been shown in one of the UK patients (Kharya et al, 2014) yet not all patients in the UK cohort were able to clear CMV with conventional therapies and in fact 3 patients required CMV CTLs (dose: $1 \times 10^4/\text{Kg}$) on D+34, D+31, D+7 since their viral titres remained $>10^5$ copies/ml despite anti-viral therapy and out of 6 cases with disseminated CMV and adenoviraemia (AdV viraemia), only 2 survived despite intensive anti-viral therapies.

To tackle the problem of viral reactivation among TCR $\alpha\beta$ /CD19 depleted grafts, Maschan et al, 2018 has recently published a pilot study including 53 patients with malignant and non-malignant disease who received a TCR $\alpha\beta$ /CD19 depleted grafts followed by the use of memory (naïve T Cell depleted) donor lymphocyte infusion (DLI) in 3 escalating doses at a median of 48 days post-HSCT. The planned doses were 0.25, 5 and $1 \times 10^5/\text{Kg}$ for haploidentical donors and 1, 2, $3 \times 10^5/\text{Kg}$ for MUD. Among the 31 evaluable patients with no CMV specific T cells, 12 developed CMV specific response immediately before DLI 2 and 7 before DLI 3 and 1 30 days after DLI3. Thus, the CI of recovery of CMV specific immunity after DLI was documented in 20 cases (64.5%). Moreover, the use of DLI on escalated monthly doses in haplo and MUD setting was associated with a CI of de novo aGvHD of 2%, Grade II or above of 20% and cGvHD of 6%. Though the authors highlighted the safety of this approach with low risk of toxicity; mainly GvHD yet using MUD in almost 50% of cases makes it difficult to ensure safety of this approach in a haplo-setting.

Table 1.2. Outcome of TCR $\alpha\beta$ / CD19 depleted grafts in patients transplanted for malignant disease

Studies	Im et al, 2016 (b)	Maschan et al, 2016	Laberko et al 2017	Locatelli et al, 2017
No	42 (8 NM)	33	182 (68 NM)	80
Diagnosis (numbers)	ALL (7) AML (14) Another M (13) Others (8)	All AML	AL (91) Another M (23) PID (32) Others (36)	ALL (56) AML (14)
Status at transplant	ALL AML	CR1 (22) CR \geq 2 (5) AD (6)	NM	ALL CR1 or CR2: (52) \geq CR3 (4)

				AML CR1/CR2 (14)
Donor	Haplo (100%)	Haplo (30%) MUD/MMUD (46.5%)	Haplo (27%) MUD (74%)	Haplo (100%)
Conditioning	MIC (100%)	RIC± Mel	RIC± Mel (98%)	MAC (100%)
Serotherapy	rATG	hATG	rATG/hATG	rATG
GvHD prophylaxis	100%	84.8%	81.9%	None
OS	72% (at 1y) In M: 68% (at 1y)	67% (at 2y)	68% (at 2y) 58% for M 87% for NM	72% at last follow-up
Graft loss	0%	0%	NM	2.5%
aGvHD grade I-II	19%	21%	NM	30%
aGvHD grade III-IV	11.9%	21%	40% for M 27% for NM	0%
cGvHD	(16.6%)	30% (70% after DLI)	NM	5% (skin-limited)
CI of Viral reactivation	CMV (31%) EBV (54.7%) BK cystitis (23.8%)	52% for CMV 50% for EBV	51% for CMV 53% for EBV	NM
NRM at 100 days	0%	0% €	NM	5%
Overall NRM	2.3% CMV pneumonia D/R mismatch 0% among M cases	10% All infection related	13%	5% (50%: adenoviral related)

Relapse	47%	30%	37%	24%
2y Relapse free survival	50±10.1% (M) 88±11.7(NM)	78%	NM	71% [∞] 71% for ALL 68% for AML
Follow-up	1.6y	1.76 years (among survivors)	1.1 years	3.8years

Abbreviations: ALL: acute lymphoblastic leukaemia, AD: active disease, NRM: non-relapse mortality, Mel: Melphalan, Other M: other malignant disease, NM: not mentioned, hATG: horse anti-thymocyte globulin,

€: 4 received therapeutic DLI (native unselected for mixed chimerism). $2.5 \times 10^4/\text{Kg}$ (Haplo) and $5 \times 10^5/\text{Kg}$ (MUD).

∞: 5-year relapse free survival was recorded.

1.6.2.2 The outcome of TCR $\alpha\beta$ /CD19 depleted grafts in malignant setting

In the malignancy setting, TCR $\alpha\beta$ /CD19 depletion was associated with a relapse rate of 24-37% and leukaemia free survival of 71% (Im et al, 2016(b), Maschan et al, 2016, Laberko et al, 2017, Locatelli et al, 2017). Post-HSCT viral reactivation was still a problem; reported at least among 50% of the patients and contributed significantly to NRM (Table 1.2). Multiple groups have used TCR $\alpha\beta$ /CD19 depletion in patients with malignant disease. The study by Locatelli et al 2017, represent the longest follow-up to date of paediatric cases with malignant conditions. Interestingly, in this study a comparative analysis between TCR $\alpha\beta$ /CD19 haplo-HSCT (80 cases) with historic data on TCR grafts; matched sibling donor (MSD) (41 cases) and 10/10 MUD (51 cases) was carried out. Haplo-HSCT had the lowest rate of grade III-IV aGvHD, visceral GvHD and cGvHD ($p < 0.01$, $p < 0.01$ and $p = 0.03$, respectively). There was no significant difference in the risks of any cause specific death (GvHD, infection, regimen related toxicity and leukaemia relapse) among the 3 groups and of note there was no significant difference on GvHD-Relapse free survival among haplo-HSCT (70.7%) vs MSD (63.2%) and MUD (59.1%); $p = 0.33$. One important point to mention here was the finding that the NK KIR mismatch which is mandated in the context of CD34+ selection graft (Koehl et al, 2016) to decrease incidence of relapse was shown clearly by Locatelli et al, 2017 to have no favourable influence. The authors suggested that NK mediated GvL might have been partially obscured by other cells in graft mainly $\gamma\delta$ T cells.

Maschan et al, 2016 (b) used TCR $\alpha\beta$ /CD19 depletion for both haplo-HSCT and MUD in a pilot study on patients with AML to improve the previously observed high, up to 30% TRM rate at their institute after MUD transplant. Though relapse rate was comparable to Locatelli et al, 2017 yet it is important to acknowledge that 4 cases received a therapeutic unmanipulated DLI (for patients with >10% increase in mixed chimerism in CD34+ fraction or bone marrow) at a median of 160 days post-HSCT and another 16 with high risk AML received pre-emptive TCR $\alpha\beta$ /CD19 donor DLI at a median of 46 days post-transplant. The authors did not specify if DLI led to any favourable response in terms of tumour relapse rate. However, high rates of cGvHD were recorded in comparison to Locatelli et al, 2017. This might be due to liberal use of DLI in high-risk group to prevent relapse. However, the add-back of TCR $\alpha\beta$ depleted DLI was an interesting approach in malignancy setting yet the authors did not mention the total TCR $\alpha\beta$ dose (combined graft and DLI) that was infused. Moreover, this study had many limitations making a comparison with other studies on TCR $\alpha\beta$ /CD19 depletion in malignancy setting difficult. These limitations included the use of MUD as a stem cell source (51% of cases), absence of a comparative analysis between patients who received DLI vs no DLI in terms of survival, viral reactivation or disease relapse.

In all studies – apart from Im et al, 2016- (b), intensified conditioning protocol (either MAC or RIC \pm Mel) was used with TCR $\alpha\beta$ /CD19 depleted grafts to ensure successful engraftment. Im et al, 2016 (b) used Fludarabine/Cyclophosphamide/Low dose TBI RIC conditioning protocol to limit toxicity and despite this conditioning, graft loss was 0% among malignant and non-malignant cases. Mixed chimerism was seen in only 2 patients; 2% donor engraftment in ALL 2 weeks before tumour relapse and 38-49% among a WAS patient with stable platelet counts and no disease recurrence. One of the factors that needed to be acknowledged in this scenario was that the Investigators used a fixed high dose of TCR $\alpha\beta$ of $1-5 \times 10^5$ /Kg. Viral related mortality was also low in comparison to other studies (2.3%) -probably reflecting the high fixed TCR $\alpha\beta$ dose infused with the graft in comparison to others ($<1 \times 10^5$ /Kg in Locatelli et al, 2017 and a median of 17×10^3 /Kg in Laberko et al, 2017. On the contrary, relapse was the major treatment failure with relapse rate and relapse related mortality of 47% and 32%; respectively. One of the factors that probably contributed to the high relapse rate was the use of RIC conditioning in patients with active disease (high tumour burden) or patients who received a subsequent transplant after a previous relapse (50% of the cases).

In conclusion, whether in the malignant or non-malignant setting, TCR $\alpha\beta$ TCR depletion abrogated the risk of graft failure and GvHD however, viral reactivation and infection related mortality need to be addressed more effectively. One way to solve this problem, is the use of a manipulated T cell add-back post-haploidentical transplant.

1.7 The evolution of the concept of T cell add-back

However, simple T cell add-back is unlikely to ameliorate these problems because of the frequency of alloreactive T cells to expand and induce fatal GvHD is far higher than that of either anti-viral or anti-leukemic T cells (Amrolia et al, 2005). To circumvent this problem, investigators sought 2 major approaches to selectively infuse T cells with reduced alloreactivity but possible anti-viral and anti-leukaemia properties.

Guinan et al, 1999, undertook a clinical trial of ex vivo induction of anergy in T cells present in donor marrow to recipient allo-antigens. Outcomes in 12 transplant recipients were evaluated. To induce alloantigen-specific anergy, BM from a donor mismatched with the recipient for one HLA haplotype was co-cultured with irradiated cells from the recipient for 36 hours in the presence of CTLA-4-Ig, an agent that inhibits B7:CD28-mediated co-stimulation. After conventional myeloablation and immuno-prophylaxis, the treated donor cells were transfused into the recipient. After the induction of anergy, the frequency of T cells capable of recognizing allo-antigens of the recipient in donor marrow was sharply reduced ($P < 0.001$). Among 11 evaluable patients, the haploidentical BM cells engrafted. Of these 11 patients, 3 developed aGvHD confined to the gastrointestinal tract with no deaths attributable to GvHD. However, overall survival was 41% (80% of deaths were infection -related). One possible explanation for high infection rate might be related to a possible inhibitory effect on bystander T cells produced by anergised T cells. Another alternative approach was to deplete the graft specifically of the alloreactive T cells responsible for GvH reactions. This was accomplished by deleting T cells that become activated in response to recipient antigen presenting cells (APCs). In a process to eliminate only activated T cells expressing IL2 receptor (CD25), the Necker team used immunotoxin (RFT5-SMPTdgA) consisting of a murine antibody moiety recognizing CD25. They used recipient's cells peripheral blood mononuclear cells (PBMNCs) (Cavazzana-Calvo et al, 2001). However, this approach was found to have several limitations; including the failure of retrieving recipient's PBMNCs in patients with aplastic disease or empty marrow. Moreover, in leukemic patients, PBMNCs might be contaminated with leukemic blasts or DCs that have processed leukemic antigen,

and allodepletion might then result in the loss of anti-leukemic cells and abrogation of GvL effect. Hence, Amrolia et al, 2005 tested a protocol for allodepletion using an anti- CD25 immunotoxin after stimulation of donor cells with recipient EBV-transformed lymphoblastoid cell lines (LCL) as stimulators to activate alloreactive T cells. LCL are excellent APCs which are relatively cheap to prepare, easily expanded to large numbers and have a standard phenotype, with less variability in expression of immuno-stimulatory molecules than PBMNCs. They also do not express myeloid or tumour antigen that may serve as targets for the GvL effect. Hence the allodepletion of donor T cells after stimulation with recipient LCL should allow them to retain their anti-leukemic activity, particularly in myeloid malignancies. A phase I clinical dose escalation study of add-back of allo-LCL-depleted donor T cells in the haplo-identical setting (CD34+ selection) was embarked. Initially, 9 patients received between 1-3 doses (each was $1 \times 10^4/\text{kg}$) of allo-LCL-depleted donor T cells. Five passed away, 4 of them due to disease relapse and another one from GvHD and adenoviral disease. Subsequently, another 6 patients (3 with non-malignant disease) received higher doses between 1-4 doses (each was $1 \times 10^5/\text{kg}$). Post-HSCT CMV/EBV viral reactivation was observed among 4/6 patients. All were able to clear the infection with no infection related deaths and evidence of accelerated recovery of CMV and EBV-specific immunity. One patient with relapsed JMML passed away due to disease relapse while the rest were alive and well with no significant GvHD. It was clear that a dose $3 \times 10^5/\text{kg}$ of allo-LCL depleted T cells could confer antiviral immunity. However, the limiting factor might be disease relapse in the malignancy setting. Donor lymphocyte infusions between $10^7/\text{kg}$ - $10^8/\text{kg}$ in the MHC-matched setting were shown to be mandated for development of GvL effect (Bar et al, 2013). In the haplo-HSCT, it may be difficult to infuse sufficient allo-depleted T cells to achieve clinically useful GvL without causing GvHD. Hence, the need for a safer strategy to allow enough DLI but in a controllable environment.

1.8 Suicide gene technology

1.8.1 Principal of suicide gene technology

Manipulated non-alloreactive T cells have been developed to increase the feasibility of higher numbers of donor-derived T cells whilst providing a tool to control the increased risk of aGvHD that might occur with incomplete abrogation of allo-reactivity against the recipient (Amrolia et al, 2003, Amrolia et al, 2006). Cell therapies are known to have a long half-life and might even persist indefinitely; therefore, a mechanism to safely switch them off and eliminate them in case of unwanted toxicities

was mandated. A suicide gene is a genetically encoded molecule that allows selective destruction of adoptively transferred T cell (Jones et al, 2014). Most experience with safety-switch genes to date has been in T lymphocytes. T cell immunotherapy has proved efficacious as treatment for viral infections and malignancies (Walter et al, 1995, Marijt et al, 2003).

1.8.2. The ideal suicide gene:

The ideal suicide gene should allow 1) elimination of all and only the genetically modified cells 2) safely with absent or limited toxicities (Jones et al, 2014).

1.8.3. Available suicide gene technologies:

Suicide gene technologies can be broadly classified based upon their mechanism of action in 1) metabolic (gene-directed enzyme pro-drug therapy), 2) dimerisation inducing, and 3) therapeutic monoclonal antibody mediated. The ideal agent for suicide gene activation should be biologically inert, have an adequate bioavailability and bio-distribution profiles, and be characterised by intrinsic acceptable or absent toxicity (Jones et al, 2014).

1.8.4. HSV-TK suicide gene technology

The first in vivo studies on safety switch-modified donor T cells involved the herpes simplex virus thymidine kinase (HSV-TK) gene, the product of which phosphorylated Ganciclovir to the active moiety, which interfered with Deoxyribonucleic Acid (DNA) synthesis. Earlier reports from Bonini et al 1997, and Tubiegeren et al 2001, showed that the transduced T cell had both an anti-tumor and anti-viral effect and were safely infused in patients and resultant GvHD could be controlled by Ganciclovir pro-drug. However, several studies involved the use of HSV-TK T cells as DLI in case of tumour relapse or included as a T cell add-back after MSD or MRD TCD grafts (Bonini et al 1997, Tubiegeren et al 2001, Ciceri et al, 2007, Traversari et al, 2007, Fehse et al, 2004, Borchers et al, 2011, Ciceri et al, 2009, Zhan et al, 2013). Data on the use of HSV-TK suicide gene in HSCT were shown in Table 1.3.

Ciceri et al, 2009, reported outcome of phase I-II multicentric study on 28 adults with malignant diseases who received a TCD haploidentical transplant followed by HSV-TK-modified T cells at D+28. The T cell repertoire conferred by HSV-TK-cells progressively developed from being oligoclonal to being polyclonal by 6 months post-transplant. The proportion of interferon γ producing T cells specific to CMV and EBV was nearly normalised within 3 months of transplantation. This led to a considerable drop in number of infective episodes after HSV-TK- modified T cells and a cumulative

incidence of NRM of 14% (infectious mortality was 9%). Moreover, of the 19 patients who had HSV-TK modified T cell engraftment and were in complete remission at time of transplant, 16 were in complete remission at a median of 3 years of follow-up. Ten cases developed aGvHD grade I-IV and one developed cGvHD which was controlled by induction of suicide gene. At 3 years of follow-up no acute or chronic adverse events were related to gene transfer procedure. Subsequent a pilot study in children showed equivalent results to adult studies (Zhan et al, 2013). Different groups employed either neomycin-based selection (Tiberghien et al, 2001, Ciceri et al, 2007, Fehse et al, 2004), or a magnetic bead-based selection of co-expressed truncated low affinity nerve growth factor receptor (Bonini et al, 1997, Borchers et al, 2011, Ciceri et al, 2009). Neomycin phosphotransferase encodes a potentially immunogenic protein and requires 7 days culture with potential damaging effect on T cells (Sauce et al, 2002). Though truncated NGFR had a good safety profile yet it. has a limited availability being only used in gene therapy trials. Zhan et al, 2013 used an HSV-TK suicide gene fused to a truncated splice variant of human CD34 (tCD34) and used the widely available Miltenyi CliniMacs biotechnology for sorting of transduced donor T cells. Three patients, one with Fanconi anaemia/relapsed myelodysplasia and 2 with severe combined immune deficiency and pre-existing viral complication H1N1 influenza and adenovirus patients who had no available matched donor were treated. All patients received a conditioned CD34 selected MMUD or haploidentical graft with no serotherapy. HSV-TK modified T cells were infused at 2 escalating doses of $5 \times 10^4/\text{kg}$ and $5 \times 10^5/\text{kg}$. There was no reported toxicity or severe GvHD. In all 3 patients, HSV-TK T cells infusion led to clearance of peri-transplant viral infection (Varicella zoster virus (VZV), H1N1 and AdV virus). Currently, HSV-TK is under evaluation in patients with acute Leukaemia in first or subsequent remission undergoing haploidentical transplant. The study (TK008) is currently open to accrual in EU, US, Israel (clinicaltrials.gov:00914628) (Bordignon et al, 2012).

Although HSV-TK was effective as a safety switch for aGvHD, it had significant drawbacks. Most important of which was immunogenicity, HSV-TK was a target for both CD4+ and CD8+ T cells leading to premature elimination (Ciceri et al, 2007, Traversari et al, 2007, Borchers et al, 2011, Berger et al, 2006). The presence of a host active immune system was shown to be necessary for the generation of HSV-TK-specific effectors. In patients given infusions very late after HSCT (with evidence of immune recovery), a single administration of HSV-TK was enough to induce a detrimental immune response, whereas patients given injections with a comparable number of transduced lymphocytes early after transplantation failed to develop a

transgene specific immune response (Traversari et al, 2007). Infusion of HSV-TK T cells in immunodeficient host (early post-HSCT) led to persistence of HSV-TK DLI for weeks to years' post-HSCT (Tiberghien et al, 2001, Traversari et al, 2007) Despite the clearance of HSV-TK cells by the recovering host immune system, there was no increase in disease relapse (Traversari et al, 2007). The competent immune system that cleared HSV-TK might have prevented disease relapse.

Immunogenicity to HSV-TK is not the only reported drawback. HSV-TK usage as suicide gene might interfere with the administration of Ganciclovir as an antiviral drug for cytomegalovirus infections (Ciceri et al, 2007). Moreover, HSV-TK-mediated killing used to take days to complete. Incomplete elimination with persistence of GvHD was previously reported by Bonini et al, 1997 where in one of the patients with cGvHD; lung/skin/ oral mucosa, a partial response to Ganciclovir was noted with abrogation of skin GvHD, improvement in lung function however persistence of oral mucosa cGvHD. Oral mucosal biopsy confirmed the diagnosis of cGvHD and infiltration of the tissue with HSV-TK T cells (confirmed by PCR) (Bonini et al, 1997). Further studies reported partial response in patients given Ganciclovir for GvHD (Tiberghien et al, 2001, Ciceri et al, 2009). A ganciclovir-resistant truncated HSV-TK form might be responsible for the incomplete response (Garin et al, 2001). Hence, the search for alternative suicide genes was proposed.

Table 1.3. Use of HSV-TK T cells in allogenic HSCT

Study	Clinical application	Treated Patients (number)	Evaluable Patients (number)	Total dose Infused (X10 ⁶ /kg)	Clinical response	GvHD	Response to switch off medicine	Immunogenicity to transgene
Bonini et al, 1997	HSV-TK-DLI for EBV PTLD/tumor relapse	12	8	0.5-38.6	5/8 GvL response ▲	3/8	2/3 CR 1/3 PR	0/8
Ciceri et al, 2007	HSV-TK-DLI to treat disease relapse	23	17	0.5-300	6/17 CR 5/17 PR	4/12	3/3 CR	6/17
Traversari C et al, 2007	HSV-TK-DLI for tumor relapse post-HSCT	23	16	0.5-140	NM	NM	6/16 CR	7/16 θ
Tiberghien et al, 2001	TCD MSD BM HSV-TK infused D0	12	12	0.2-2	4/12 CR ▲	5/12	4/5CR 1/4 PR	4/12
Fehse et al, 2004	CD34+MRD HSV-TK infused D+0	3	3	5	1/3 CR ▲ (Received DLI for MiRD) 2/3 graft failure	1/3	1/1	NM ∞
Borchers et al, 2011	CD34+ MSD HSV-TK infused D+60	9	9	10	7/9 CR ▲	1/9	1/1	1/9
Ciceri et al, 2009	Haplo-HSCT HSV-TK infused D+42	28	28		22/28 CR	11/28	4/10 CR 6/10 PR	0/28
Zhan et al, 2013	CD34+ selection	3	3	Dose 1 <5X10 ⁴ /kg	NA	1/3 β	NA	NM

	Haplo/MMUD graft HSV-TK D+1/D+28			Dose 2 <5X10 ⁵ /Kg				
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Abbreviations: HSV-TK-DLI: Herpes simplex virus-Thymidine kinase- donor lymphocyte infusion, PR: partial response (complete response was achieved after addition of steroids or other immunosuppressive medications), MRD: matched related donor, TCD: T-cell deplete graft, MiRD: minimal residual disease. NA: not applicable

∞: One patient has cleared HSV-TK-T cells during HSV infection. B: one patient developed aGvHD grade I that spontaneously resolved.

▲: complete response measures clinical response to tumour.

θ Immune response to HSV-tk epitopes was recorded in 7 cases. CD4>200 cells/μl was noted among 5/7 cases with immune response to HSV-TK vs 2/9 of patients who did not mount a response.

1.8.5 Development of markers for gene transfer

Investigators established different techniques to insure adequate detection and tracking of the gene modified cells using what is called gene markers.

The initial attempts of gene markers (classical markers) relied on the transfer of a selected advantage to the target cells. Genes encoding resistance to Neomycin, Hygromycin and Puromycin (cytotoxic drugs) have been widely used in vitro and in vivo to aid identification of genetically modified cells (Keller et al, 1985, Yang et al, 1987, Paulus et al, 1996). The efficiency of gene transfer into target cells was measured by counting the number of drug-resistant colonies that the gene-modified cells generated when grown in semisolid media (Phillips et al, 1996). Neomycin phosphotransferase was the most used gene marker. Though, very sensitive where it could identify one positive transduced cell out of millions of non-transduced cells. However, this system had multiple disadvantages with reports on toxicity due to its immunogenicity being a bacterial derivative (Brenner, 1996, Berger et al, 2006, Jensen et al, 2010) and prolonged culture necessary to achieve selection; 5 days for Neo^R (Brenner, 1996). Quantitative polymerase chain reaction (qPCR) was used as another approach for determination of gene transfer. In qPCR assays, DNA extracted from genetically modified cells is amplified with primers specific for the vector or the vector transgene (Phillips et al, 1996). However, very sensitive, even when small number of cells are available, but it is a laborious process that does not provide information regarding the level of gene expression. Moreover, it is not possible to determine whether the amplified signal results from a small number of transduced cells containing multiple copies of the vector or from a larger number of cells with a single copy of the vector (Phillips et al, 1996).

To overcome the limitations of previous gene markers, in situ staining methods were developed. In this approach, a marker gene is cloned into the vector that encodes a novel protein and can be detected using appropriate staining methods.

Old methods used in situ staining with substrates for intracellular enzymes such as bacterial β -galactosidase or the human placental alkaline phosphatase. The disadvantages to this approach include a high background of endogenous staining in some cells and, occasionally, unstable gene expression (Bagnis et al, 1994, Fisher-Adams et al, 1996). The most recent methods relied on the use of fluorescence-activated cell sorting (FACS) for detection of surface antigens. Flow cytometry not only facilitates rapid determination of transduced cells, but also the analysis of the

strength of the expression of the transgene in the transduced cells. In addition, transduced cells can be selectively enriched using fluorescence-activated cell sorting or immuno-adhesion techniques involving magnetic beads.

The use of truncated LNGFR was one of the most frequently used surface marker in clinical trials (Fehse et al, 1997, Bonini et al, 1997) with high; >90% immunomagnetic selection of transduced cells with anti-LNGFR antibody (Fehse et al, 1997) and no reported toxicity (Bonini et al, 1997). However, one drawback is the limited availability of technique for selection worldwide.

Researchers investigated the possibility for production of an optimum surface marker that 1) is human in nature, 2) presented only in engineered cells without major leak into extracellular space or presentation on cells that have not been genetically modified, 3) does not interfere with physiological function of target cells and most importantly 4) compatible with an approved technology that allows enrichment of modified cells to high purity under GMP compatible conditions (Fehse et al, 2000). In the year 2000, truncated CD34 (tCD34) was considered an optimum marker that fulfilled at least 3 of the 4 criteria (criteria 1, 2 and 4) for an attractive cell marker. Fehse et al, 2000 analysed 3 versions of CD34 molecule; full length CD34 protein (fCD34), truncated CD34 (tCD34) which is a naturally occurring splice variant that has a partial deletion of the cytoplasmic domain for signal transduction and an engineered variant that is completely deprived of its cytoplasmic tail. All 3 variants allowed enrichment of gene-modified cells using commercially available magnetic cell sorting Miltenyi biotechnology. However, stable high expression of CD34 on genetically modified cell was associated with the use of either fCD34 or tCD34 while low expression of surface marker was seen with the usage of dCD34. The short cytoplasmic domain of 16 amino acid present in the natural splice variant tCD34 allow the anchoring of the transmembrane protein at the cell surface and prevent CD34 shedding. Moreover, tCD34 was found to be superior to fCD34 as it lacks most of the cytoplasmic domain that mediate cell signalling (Kraus et al, 1996, Hu et al, 1998). However, one concern remained in that aberrant expression of tCD34 might affect cell physiology through hetero-dimerisation with other surface molecules, thereby altering homing, differentiation or effector cell functions. However, mouse model (Healy et al, 1995) and subsequent clinical trials using tCD34 as a gene marker demonstrated no interference of tCD34 with the normal physiology of target cells (Zhan et al, 2013). Other markers similar to tCD34 has developed since then including tCD19 as been discussed in Section 1.3 (page 14).

1.8.6 Development of monoclonal antibodies (mabs) in suicide gene technology

1.8.6.1 CD20 suicide gene technology

Introna et al, 2000 constructed a retroviral vector that contains the human CD20 cDNA under the control of the Moloney murine leukaemia virus Long Terminal Repeat (LTR). Their strategy relied on the possibility of introducing into T lymphocytes a single human gene (CD20) that permits identification of the transduced cells, their purification by immuno- selection, and their killing with Rituximab. Being human in origin it avoided immune responses but manufacturing on scale was problematic. To optimise the setting for the usage of CD20 suicide gene in clinical settings, Philips et al, 2004 created a highly compact marker/suicide gene for T cells combining target epitopes from both CD34 and CD20 antigens (RQR8). This construct allows selection with the clinically approved CliniMacs CD34 system (Miltenyi). Further, the construct binds the widely used pharmaceutical antibody rituximab, resulting in selective deletion of transgene-expressing cells. The translated protein was found to have a stable expression on the cell surface following retroviral transduction. It is bound by QBEnd10 (a mouse monoclonal anti-CD34 antibody) analogously to full-length CD34, allowing clinical-grade sorting with off-the-shelf reagents and easy in vivo tracking. Further, the construct binds rituximab; the dual-epitope design engendered highly effective complement mediated cytotoxicity (CDC) and antibody dependent cell cytotoxicity (ADCC) and consequently could render T cells to be highly susceptible to in vivo rituximab-mediated depletion. Due to the small size of RQR8, it can easily be co-expressed with a wide range of T-cell engineering components. However, the main disadvantage of this strategy is the concomitant depletion of the patients B cells. RQR8 suicide gene technology is currently being used in combination with Chimeric antigen receptor (CAR) T cell technology (Qasim et al, 2015, Qasim et al, 2017). One study clearly reported a discrepancy between a limited RQR8 expression and high expression of CAR19 on T cells despite linked transcription and translation through a self-cleaving 2A peptide configuration (Qasim et al, 2015). To date, there are no clinical evidence of the feasibility of this system in case of toxicity.

1.8.6.2 Other MABs used in suicide gene technology

Wang et al, 2011 described the use of a truncated human epidermal growth factor peptide (tEGFR) as a surface marker for in vivo tracking of adoptively transferred CAR T cells using flow cytometry and a target for cetuximab (mab) mediated dependent cellular cytotoxicity and in vivo elimination.

Human epidermal growth factor was selected because 1) it is not expressed by cells of the hematopoietic and lymphopoietic systems, 2) can be truncated with the removal of Ligand binding extracellular domains I and II of (epidermal growth factor) EGFR and receptor tyrosine kinase signalling associated with intact EGFR (Ferguson et al, 2008) while 3) retaining extracellular domain III ; the binding site of a commercially FDA-approved cetuximab (an IgG1 chimeric antibody licensed by the FDA for the treatment of metastatic colorectal cancer and head and neck cancer) (Li et al, 2005). Human T cells were subsequently transduced with a lentiviral vector that co-ordinately express tumour specific CAR in conjunction with tEGFR allowing easy tracking of tumour specific CAR cells and effective selection (>90% purity) of gene modified cells using GMP grade anti-biotin microbeads (Miltenyi Biotec). Together with, capability of removal of genetically modified cells using cetuximab mediated ADCC. Unlike the suicide strategy based on CD20 expression and the use of rituximab (Griffioen et al, 2009, Serafini et al, 2004), this suicide system would not ablate B cells and would be compatible for T-cell transfer in patients who are receiving rituximab as part of the lymphoma/leukaemia therapy. However, one significant drawback was the delayed clearance of genetically modified cells using cetuximab; between 4-6 days making this suicide gene technology unsafe where rapid elimination is mandated to switch off fatal toxicity (Wang et al, 2011).

1.9. Apoptosis and suicide gene technology

1.9.1 Introduction to apoptosis

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called “caspases” and a complex cascade of events that link the initiating stimuli to the final demise of the cell (Elmore et al, 2007). Caspases are divided into initiator caspases (caspase 8, 9) and executioner caspases (caspases 3, 7). The initiator caspases-8 and -9 normally exist as inactive procaspase monomers that are activated by dimerisation and not by cleavage (Chang et al, 2003, Renatus et al, 2001). Inappropriate activation of the executioner caspases-3, -6, and -7 is prevented by their production as inactive procaspase dimers that must be cleaved by initiator caspases. This cleavage between the large and small subunits allows a conformational change that brings the two active sites of the executioner caspase dimer together and creates a functional

mature protease (Riedl et al, 2004, Yeh et al, 1998). Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an accelerated feedback loop of caspase activation. There are 2 main pathways for apoptosis in T cells; the extrinsic and intrinsic (mitochondrial) pathway (figure 1.2)

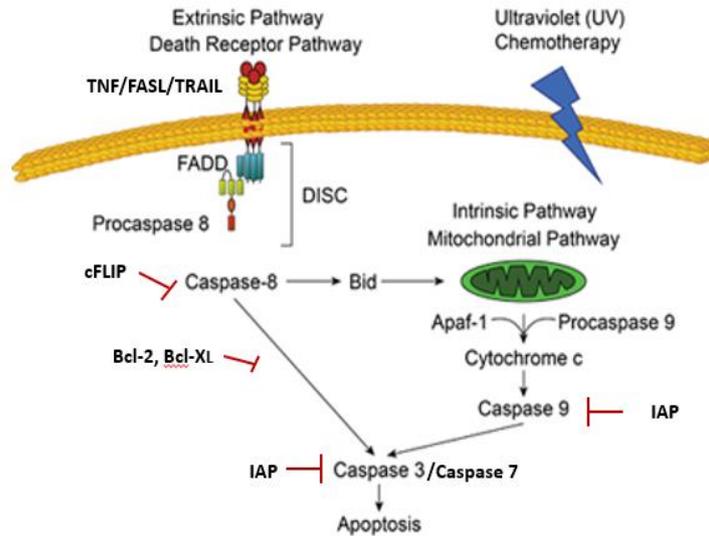
1.9.1.1 The extrinsic pathway of apoptosis

Extrinsic apoptosis is triggered by extracellular stimuli delivered in the form of ligands binding to death receptors. Death receptors include the tumor necrosis factor (TNF) superfamily and include TNF receptor-1 (TNFR1), Fas, TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-R1) and TRAIL-R2. Death receptor ligands include TNF, CD95-ligand (CD95-L; also called Fas-L) TRAIL (also called Apo2-L), and TNF-like ligand 1A (TL1A). The binding of a death receptor ligand to a death receptor causes the monomeric procaspase-8 protein to be recruited to the death-inducing signalling complex (DISC) formed at the cytoplasmic tail of the engaged death receptor that also includes the adapter protein FAS-associated death domain (FADD) or TNFR-associated death domain (TRADD). Recruitment of caspase-8 monomers results in dimerisation and activation (Elmore et al, 2007).

1.9.1.2 The intrinsic pathway of apoptosis

Intrinsic apoptosis (mitochondrial apoptosis) depends on factors released from the mitochondria. This pathway is activated by a vast array of cellular stresses, including growth factor deprivation and DNA damage upon exposure to stress, cell first releases cytochrome c from the mitochondria. Cytochrome C and deoxyadenosine triphosphate (dATP) bind to the adapter protein apoptotic protease-activating factor-1 (APAF-1) to form a multimeric complex that recruits and activates procaspase 9 (Loreto et al, 2014), which is activated by dimerisation induced when the caspase-9 -caspase activation and recruitment domain (Caspase 9-CARD) binds to (APAF1) (Chang et al, 2003, Loreto et al, 2014). Both APAF1 and caspase-9 exist in a resting cell as cytosolic, inactive monomer.

Figure 1.2. Main pathways of apoptosis in T cells



Quoted with modification from

(<https://www.nexcelom.com/applications/cellometer/fluorescent-assays/apoptosis/>)

Red arrows represent inhibitory steps in the pathway.

1.10 Development of inducible apoptotic suicide gene

Spencer et al, 1993 developed a method to control T cell signalling through ligand-mediated dimerisation of intracellular proteins. They used ligands that bind to FK506 binding protein 12 (FKBP12) whose function is to bind and inactivate calcineurin thus impairing T-cell receptor signalling. Clarkson et al, 1998 re-designed the ligand-FKBP12 interface to allow a cellular control switch without the undesired physiological or toxic effect of inhibiting calcineurin. They have substituted the bulky phenylalanine with a smaller valine residue creating a conformational change in FKBP12 with the development of a binding socket. In 2001, Juliucci et al used a small molecule- a chemical inducer of dimerisation (CID) - to create the dimer form of the ligand. That was subsequently tested on healthy volunteers with no reported significant adverse effects (Juliucci et al, 2001).

Earlier reports of dimerisation induced apoptosis involved inducible Fas and inducible death effector domain (DED) of FADD. FKBP12 was placed at the aminoacid terminal of FAS protein or DED and the construct was then cloned into a retroviral vector and used to transduce Jurkats 6.1 and T cells. Using a CID; a synthetic drug that can

cross-link with FKBP12 dimerisation of Fas or DED was initiated activating the apoptosis pathway (Spencer et al, 1996, Belshaw et al, 1996). In pre-clinical experiments, up to 90% of T cells transduced with inducible death molecules (Fas or FADD domain) were cleared by apoptosis after administration of CID (Spencer et al, 1996, Fan et al, 1999, Thomis et al, 2001, Berger et al, 2004, Junker et al, 2003). However, 2 main drawbacks precluded the use of these inducible death molecules in clinical trials. Unfortunately, inducible Fas at levels enough to confer sensitivity to CID was found to be auto-toxic. Moreover, both Fas and DED lie in the upstream of apoptotic pathway and thus cells are liable to have an escape mechanism from the effect of CID by upregulation of inhibitors of apoptosis, c-FLIP, bcl2 and bcl-X1.

Thus, using death molecules that act in the downstream of apoptosis was investigated. MacCorkle et al, 1999 developed novel pro-apoptotic molecule based on caspase 1 and caspase 3. Intracellular cross-linking of caspase-1 or caspase 3 was able to trigger rapid apoptosis in a Bcl-XL independent manner, suggesting that these conditional pro-apoptotic molecules could bypass intracellular check point genes such as Bcl-XL. However, to achieve a therapeutic response to CID, modified caspase 1 was found to be auto-toxic to cells. Fan et al, 1999, screened caspase 3, 7 and 9 for their suitability as inducible safety switch molecules both in transfected 293T cells and in transduced T cells and only inducible caspase 9 was found to be expressed at levels sufficient to confer sensitivity without autotoxicity.

Based on these previous studies, Straathof et al, 2005 devised a safety switch for T cells that exploited dimerisation of a modified caspase 9 molecule, which was part of the intrinsic apoptotic pathway. Activated caspase 9 would then activate caspase 3 and 7 and the other terminal effector molecules, leading to apoptosis.

1.11 Inducible caspase 9 structure

The inducible caspase 9 molecule (iC9) consisted of an FKBP12-F36V domain linked, via a flexible Ser-Gly- Gly-Gly-Ser linker, to a truncated caspase 9; a caspase 9 deleted from its endogenous (CARD) to ensure a control over caspase 9 activity. To allow easy detection of the presence of iC9 in T cells, a marker was mandated. A truncated CD19 (Δ CD19) was used as a marker after removal of all conserved tyrosine residues in addition to a shortened intracytoplasmic from 242 to 19 amino acids to prevent any endogenous effect. 2A-like sequence was used as a cleavage peptide linking iC9 to Δ CD19 (Zhou et al, 2015 (a)). The 2A-like sequence encoded an 18-aminoacid peptide from *Thosea Asigna* insect virus (Donnelly et al, 2001),

which mediated >99% cleavage between a glycine and terminal proline residue resulting in 17 extra amino acids in the C terminus of iCasp9, and one extra proline residue in the N terminus of CD19

1.12 Optimisation of expression and function of inducible caspase 9

CARD domain is responsible for physiological dimerisation of caspase 9 molecules, by a cytochrome C and dATP driven interaction with Apaf-1. Thus, CARD domain appears superfluous in this context and its removal might reduce basal activity. Straathof et al, 2005 produced 4 constructs of icas9; one with FKBP12, one with both CARD and FKBP12 and one with CARD and 2 FKBP12. All constructs were cloned into a retroviral vector where 5'LTR directed transgene expression and enhanced Green Florescent Protein (eGFP) was co-expressed from the same mRNA by utilisation of an internal ribosomal entry site (IRES). Inducible caspase 9 protein expression in transfected 293T cells were assessed using western blot for caspase 9 and flowcytometry for mean fluorescent intensity (MFI) of eGFP. Overexpression of icas9 was recorded in the construct with 2 FKBP12 and one CARD however this overexpression led to spontaneous dimerisation and toxicity of the construct.

Removal of CARD, one FKBP12 or both resulted in a progressive increase in the expression of icas9 and eGFP and correspondingly enhanced sensitivity to dimerizer drug due to elimination of basal spontaneous dimerisation (toxicity).

Thus, the construct containing FKBP-linker-icas9-T2A-icas9 (icas9) was shown to provide the optimum expression of icas9 in transduced cells. Transduction of EBV LCL with retroviral vector with icas9 showed a high transduction efficacy >70% and evidence of stability of transduced LCL in culture for at least 4 weeks. Moreover, proliferative capacity, T cell phenotype and cytokine production was not altered by icas9 expression. CID effectively cleared, in one hour, 91.3% of transduced cells (Straathof et al, 2005). Tey et al, 2007 demonstrated the same findings in allo-depleted (by CD25 immunotoxin) modified T cells (transduced with icas9). After 8 days of transduction, the final T cell product contained both CD4+ and CD8+ cells though CD8+ cells predominated, and most of the cells expressed CD45RO and CD62L denoting a central memory phenotype. Allo-depleted modified T cells retained both antiviral repertoire and functionality and were able to be maintained in culture medium with low dose IL2 (50 IU/ml) for 24 days after transduction (Tey et al, 2007).

1.13. Structure of Rimiducid (AP1903) and mechanism of action

Rimiducid or AP1903 is a small molecule supplied by Bellicum pharmaceutical for trial use. Administration of AP1903, a non-toxic synthetic homo-dimerizer was shown to

bind to FKBP12-F36V domain dimerizing and activating caspase 9; thus, activating downstream caspases, leading to apoptosis within 24 hours. Pharmacokinetic data showed plasma concentrations of 10- 1275ng/mL (equivalent to 7-892 nM) was attained over a 2-hr infusion of 0.01mg/kg to 1.0mg/kg dose range. Rapid clearance of AP1903 was demonstrated with plasma levels falling to 18% and 7% of maximum at 0.5 and 2hrs post dose. Pre-clinical intravenous toxicity studies in rodents and non-human primates established a favourable safety profile. No evidence of serious adverse events was reported in large animal models exposed either to high doses of AP1903 for 30 days consecutively or to long-term administration of the agent (Richard et al, 2004, Okazuka et al, 2011). AP1903 has been evaluated as an Investigational New Drug (IND) by the FDA and has successfully completed a phase I clinical safety study. No significant adverse effects were noted when AP1903 was administered over a 0.01mg/kg to 1.0 mg/kg dose range. During the assessment many mild adverse events has occurred including chest pain, flu syndrome, halitosis, headache, injection site pain, vasodilatation, increased cough, rhinitis, rash, gum haemorrhage, and ecchymosis. These events were considered by the investigator to be unrelated or to have improbable relationship to the study drug. Only one adverse event was considered possibly related to AP1903. This was an episode of vasodilatation, described as “facial flushing” for 1 volunteer at the 1.0 mg/kg AP1903 level. This event occurred at 3 minutes after the start of infusion and resolved after 32 minutes’ duration (Iulucci et al, 2001). Another possible adverse event was reported by Zhou et al, 2016 where one of the patients; included in a phase I clinical trial of CD34+ selection with genetically modified T cell add-back (transduced with retrovirus-icas9) had evidence of aGvHD and was given 3 infusions of AP1903. After each infusion of AP1903, a mild (grade 2) and transient pancytopenia was noted that resolved spontaneously after 48 hours. No other toxicities were recorded (Zhou et al, 2016). Table 1.4 summarise the clinical studies on Rimiducid (or AP1903)

Table 1.4. A summary of clinical studies on the use of AP1903

Study	Study objective	Study design	Dose regimen and duration	Total number of recruits	Characteristics of enrolled patients	Number of patients received AP1903	Number of infusions given for each patient	Reported adverse event
Phase I, safety, tolerability and PK study (Luliucci et al, 2001)	Evaluate safety, tolerability and PK	Single centre, single blind, placebo-control randomised ascending single dose study	Single dose (0.01, 0.05, 0.1, 0.5, 1mg/kg) 2hrs. intravenous infusion	28	Healthy adult volunteers	28	1	Facial flushing in 1/28 recruits
Phase I, safety and efficacy (Di Stasi et al, 2011,	Evaluate safety and efficacy of dose escalation in	Single centre, ascending dose of icas9 T cells and a	Single dose of AP1903 (0.4 mg/kg)	10	Children who had received a CD34+ haplo-HSCT followed by icas9 T cells add-back	4	1	None

Zhou et al, 2014)	patient who received icas9 T cells and developed GvHD	single dose of AP1903.	2 hrs. infusion					
Phase I Safety and efficacy of icas9 T cells (Zhou et al, 2015 (b), Zhou et al, 2016)	Assess safety and efficacy of infusion of escalating doses of icas9 T cells	Single centre ascending dose of icas9 T cells.	Single dose of AP1903 (0.4 mg/kg) 2 hrs. infusion	12	Children and adults who had a CD34+ selection HSCT followed by escalating doses of icas9 T cell add-back	3	1 dose for 2 patients 3 doses for 1 patient (116)	Transient pancytopenia in 1/3 patients
Phase I/II safety and efficacy study (Sonpavde et al, 2011)	Evaluation of safety of increasing dose of gene modified icas9 DCs with fixed	Single centre, 3 dose levels (of icas9 DCs) with a fixed dose of AP1903	6 cycles AP1903 at 0.4mg/kg	12	Adult males with mCRPC	12	6 doses	CRS in 1/12 patients

	dose of AP1903							
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Abbreviations: PK: pharmacokinetics, icas9: inducible caspase 9, mCRPC: metastatic castration-resistant prostatic cancer, CRS: cytokine release syndrome, DCs: dendritic cells

1.14 Phase I clinical trials with CD34+ selection + T cell add back having icas9:

1.14.1 Results from CASPALLO Trial:

10 cases 1-17 years old received a CD34+ haploidentical transplant for malignant diseases. All were in CR1 or CR2 apart from 1 patient who had active disease at time of transplant. This was followed by icas9 T cell add-back between D+30-D+90. T cells were allo-depleted in vitro by the usage of CD25 immunotoxin followed by genetic modification by adding the icas9-CD19 construct in a retroviral vector (icas9 T cells). Escalating doses of icas9 T cells $1 \times 10^6/\text{Kg}$, $3 \times 10^6/\text{kg}$ and $1 \times 10^7/\text{Kg}$ were evaluated. Six patients received a single T cell infusion and 4 patients received a second T cell infusion in an effort to eradicate mixed chimerism. There were no immediate toxicities related to the infusion, but 4 patients developed Grade I-II aGvHD from 2-6 weeks after their first icas9 T cell infusion and received a single dose of AP1903. with resolution of GvHD in 24-48 hours with no subsequent recurrence (Di Stasi et al, 2011). At a median follow-up of 3.5 years, modified T cells were still persistent and associated with endogenous T cell recovery (Zhou et al, 2014). The median absolute counts of CD3+CD19- T cells were greater than 500 at 3 months post-HSCT in comparison to delays up to 12 months in a historic cohort of patients who received haplo-HSCT with no T cell add-back. Interestingly, the kinetics of endogenous T cell immune reconstitution appeared identical in patients who received AP1903 to that of patients who did not develop aGvHD and did not receive AP1903. Six patients had evidence of viral reactivation during the per-HSCT period that was resistant to medical treatment. All 6 patients were able to clear viraemia (CMV, AdV, EBV and BK) within 2 weeks to 1-month post-icas9 T cell infusion with evidence of viral-reactive T cells (detected by flow cytometry for intracellular INF- γ). These viral reactive T cells were present in both the infused CD3+CD19+ icas9 T cells and the endogenous CD3+CD19- T cells. In the 3 patients who received AP1903 and had viral reactivation, a subsequent mild rise of viraemia was noted in 2 patients after AP1903 administration followed by sustained control thereafter. The 3 patients had AdV enteropathy resistant to Cidofovir and Foscarnet. Resolution of symptoms associated with a decline in viral load was noted 3 weeks post-icas9 infusion. Viral levels remained low even after AP1903 infusion with evidence of AdV- specific icas9 and endogenous T cells pre- and post-AP1903 infusion. Hence, the ability of icas9 T cells to respond to viral infection was not compromised by AP-1903 mediated alloreactive T cell killing in patients who developed GvHD. Four patients had disease relapse. All received either a single (n=2) or 2 doses of icas9 T cells (n=2). Three out of the four

patients had received AP1903 to switch off modified T cells (one of them had active disease). A fourth case had received 2 doses of T cell add-back $1 \times 10^6/\text{kg}$ and then $1 \times 10^7/\text{Kg}$ and didn't develop aGvHD yet had disease relapse that was salvaged by a successful 2nd allo-stem cell transplant (Zhou et al, 2014). The high relapse rate among recipients of AP1903 might be a concern that elimination of GvHD could have also eliminated GvL effect by donor T-cells. Four patients passed away, 3 due to disease relapse and another from respiratory failure secondary to refractory autoimmune haemolytic anaemia (Zhou et al, 2014).

1.14.2. Results from DOTTI trial

This phase I trial included 12 patients who were between 2-50 years old. All received a MAC conditioning preparative protocol apart from 3 patients. This was followed by a CD34+ selection followed by icas9 T cell add-back. However, on this occasion T cells were not allo-depleted in vitro and were only genetically modified by transduction with retrovirus with icas9-CD19 construct. For safety reasons, due to the omission of the in vitro allo-depletion procedure, icas9 T cells were given on escalating doses; increased from $1 \times 10^4/\text{kg}$ to $5 \times 10^6/\text{kg}$. Due to limited proliferation of icas9 T cells noted in 2 patients who had received a dose of $1 \times 10^4/\text{Kg}$, the remaining 10 cases were infused a dose which is at least a log higher. Eleven patients received one single dose of icas9 T cells, and one patient received an additional T cell infusion in an effort to eradicate EBV and persistent mixed chimerism. There were no immediate toxicities post-icas9 T cell infusion. However, 3 patients developed aGvHD grade I-III that responded within 6-48 hours to AP1903 infusion with no recurrence of aGvHD within 90 days post-AP1903 administration. In one of these patients, (at 16 days post-icas9 T cell infusion) the allo-reactivity looked like a cytokine release syndrome with fever, rash and extensive diarrhoea with elevated cytokines-though mild elevation in IL6. A fourth patient with relapsed AML had 3 transplants and 6 months of chemotherapy before his fourth transplant as part of DOTTI trial. Thirty-one days post-icas9 T cell infusion, the patient developed acute encephalopathy without proven infectious, drug or vascular associated toxicity. His encephalopathy was thought to be a cerebral localised GvHD and thus a dose of AP1903 was infused with a significant drop in icas9 T cells in the periphery yet progression of his encephalopathy within death in one-week time. Post-mortem brain biopsy revealed 552 copies/ μg of icas9 T cell DNA in his frontal lobe denoting possible icas9 T cell toxicity. However, due to the low number of detectable donor cells- the authors considered this event not to be icas9 T cell related toxicity. Among the 10 patients who had evidence of icas9 T cell engraftment, 8 had active viraemia or viral reactivation post-HSCT including a patient

with VZV meningitis. Viral-reactive T cells were recorded in all tested patients (n=7). As previously reported in CASPALLO trial, these viral reactive T cells were present in both the infused CD3+CD19+ icas9 T cells and the endogenous CD3+CD19- T cells. Icas9 T cell add-back was associated with the clearance of active viral infection (Zhou et al, 2016, Zhou et al, 2015 (b)). One patient had disseminated VZV at time of icas-9 T cell infusion and subsequently developed VZV meningitis with a viral load >5 million copies/ml and 6900 copies/ml in CSF and peripheral blood; respectively. Within 1-week of icas9 T cell infusion, viral load was undetectable in blood. CSF analysis-55 days post-icas9 T cell infusion showed an expansion of icas9 T cells (25% of total T cells in CSF). This patient subsequently developed aGvHD and was given a dose of AP1903. Repeat CSF (14 days after AP1903 infusion) showed clearance of icas9 T cells equivalent to its levels in peripheral blood. This patient was alive at last follow-up (D+674) with no recurrence of viral reactivation or GvHD however with disease relapse. Of note, that icas9 T cells were still detected at low levels in peripheral blood after AP1903 infusion. One patient left the study at D+166. Five patients had disease relapse. Four out of five had difficult to treat leukaemia (2 in CR3 and 2 in active diseases). Only one patient had disease relapse post-AP1903 administration. Noticeably 4/5 patients who had disease relapse received icas9 T cell infusion of $1 \times 10^4/\text{kg}$ or $1 \times 10^5/\text{kg}$ versus 1/6 evaluable cases with no disease relapse. Patients who were given icas9 T cell infusions between $1-5 \times 10^6/\text{kg}$ were in remission. Five deaths were reported; 3 due to disease relapse, 1 out of encephalopathy and one because of fungal infection (Zhou et al, 2015 (b)).

Figure 1.3. Schematic representation of icas9 suicide gene and its dimerisation upon binding to CID; AP1903

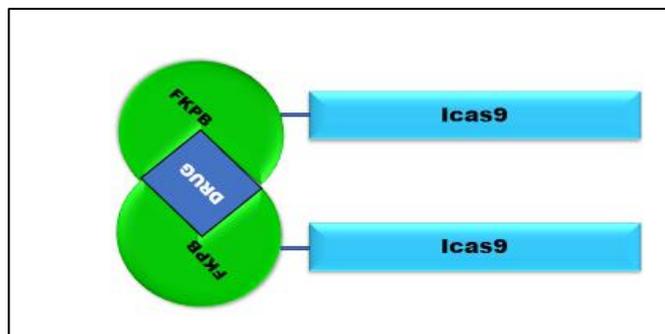
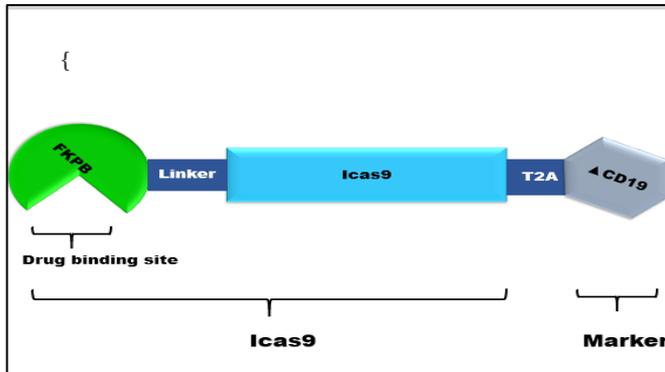


Table 1.5. Clinical trials on the safety of icas9 suicide gene in T cells

Study	Type of the study	Number of enrolled patients	Age group (years)	Icas9 T cell infusion dose	GvHD Number of patients, grade and organ involvement	Response to AP1903	Any other reported toxicity	Medical intervention and response
CASPALLO trial (Di Stasi et al, 2011, Zhou et al, 2014)	Phase I safety	10	1-17	3 escalating doses $1 \times 10^6/\text{Kg}$ $3 \times 10^6/\text{Kg}$ $1 \times 10^7/\text{Kg}$	4 Grade I-III (skin/liver)	4/4 CR	None	NA
DOTTI trial (Zhou et al, 2015(b))	Phase I safety	12	2-50	5 escalating doses $1 \times 10^4/\text{Kg}$ $1 \times 10^5/\text{Kg}$ $5 \times 10^5/\text{kg}$ $1 \times 10^6/\text{kg}$ $5 \times 10^6/\text{Kg}$	3 Grade I-III (Skin/gut/liver)	3/3▲ CR	Encephalopathy	One infusion of AP1903 Disease progression

⊖: icas9 T cells were allo-depleted by CD25 immunotoxin. ▲: One of these patients had a mixed picture of GvHD and cytokine release syndrome

1.16. Potential superiority of icas9 over other pro-caspases:

1.16.1. icas9 versus iFas

A comparative experiment testing the effect of 10 nM CID in elimination of icas9 LCL versus iFas LCL showed rapid and more effective clearance of icas9 versus iFas9 T cells. Moreover, they compared the function of iCasp9 and iFas in 2 malignant T-cell lines: Jurkats 6.1, an apoptosis-sensitive T-cell leukaemia line, and MT-2, an apoptosis-resistant T-cell line, due to c-FLIP and Bcl-XL expression. Jurkat cells and MT-2 cells were transduced with iFas and iCasp9 with similar efficiencies (92% vs 84% in Jurkat, 76% vs 70% in MT-2) and were cultured in the presence of 10 nM CID for 8 hours. Annexin-V staining showed that although iFas and iCasp9 induced apoptosis in an equivalent number of Jurkat cells (56.4% 15.6% and 57.2% 18.9%, respectively), only activation of iCasp9 resulted in apoptosis of MT-2 cells (19.3% 8.4% and 57.9% 11.9% for iFas and iCasp9, respectively). These results demonstrate that in T cells overexpressing apoptosis-inhibiting molecules, the function of iFas can be blocked, while iCasp9 can still effectively induce apoptosis. With the potential use of suicide gene technology in cancer (Straaford et al, 2005).

1.16.2. icas9 versus icas3 and icas7

While Caspase 3 or caspase 7 as terminal effectors in the apoptotic pathway should be the ideal suicide genes, however, there are conflicting data about their expression in T cells. Straaford et al, 2005 clearly demonstrated a failure in transferring icas3 or icas7 transgene to primary cells while icas9 was successfully expressed in T cells. This was attributed to the observation that caspase 3 and 7, unlike caspase 9, make poor substrates for themselves and require high cellular concentration for vector cleavage.

1.16.3. icas9 versus HSV-TK and CD20 suicide switch

Marin et al, 2012 compared effectiveness of 3 suicide genes in vitro in EBV-LCL. HSCV-TK, icas9, and human CD20. Codon optimised genes were cloned in frame with 2A-truncated codon optimised CD34 in a retroviral vector. After codon optimisation, all suicide genes were expressed at high levels. Activation of both icas9 and CD20 led to a rapid elimination of transduced T cells. HSV-TK T cells required 3 days of exposure to Ganciclovir to reach full effect (slow but equivalent response to either icas9 or CD20 response to their activator

However, HSV-TK had a long heritage and considerable clinical response. However, there are multiple drawbacks as been discussed above. One potential threat about the use of HSV-TK in clinical setting is failure of rapid and timely clearance of the T

cells in case of toxicity. icas9 T cells showed efficacy in phase I clinical trials with encouraging data and can be switched off in 30 minutes after application of activator (Di Stasi et al, 2011, Zhou et al, 2014, Zhou et al, 2015(b)). In malignant setting, icas9 suicide gene might prove superior to HSV-TK. In Comparison to CID/icas9 system, Ganciclovir/HSV-TK system precludes DNA synthesis and is thus cell cycle dependent. This means that resting malignant cells could escape clearance by this system.

Aim of the thesis

To coordinate a phase II clinical trial sponsored by Bellicum pharmaceutical (USA)- involving the use of TCR $\alpha\beta$ /CD19 depleted grafts followed by the adoptive transfer of genetically modified donor T-cells transduced with inducible caspase 9 suicide genes (icas9) (BPX-501) aiming at testing the efficacy of this approach in eliminating alloreactivity while sparing anti-viral and anti-tumor effect. In case of alloreactivity or uncontrolled GvHD, a safety switch (Caspacid or AP1903) can be used to eliminate these T cells without affecting endogenous T cell population.

Thus, the aims of my research were

1. Investigation of the feasibility and safety of BPX-501 therapy in children undergoing haplo-HSCT.
2. Testing AP 1903 for treatment of GvHD and investigating the feasibility of removing alloreactive T cells while not affecting immune reconstitution. Investigation of the mechanism of resistance to AP1903 when encountered
3. Development of lentiviral configuration for gene transfer in primary T cells.

Chapter 2
Trial protocol, Materials and Methods

Section 1: Trial protocol

2.1 Study synopsis:

Study title	Using CaspaCid (AP1903) as a safety switch in patients with haploidentical transplants.
Protocol number	15IC05
EudraCT number	2014-000584-41
Sponsor	Bellicum pharmaceutical
Investigational drugs	<ol style="list-style-type: none"> 1- Genetically modified T cell add-back with inducible caspase 9 (BPX-501). 2- Switch off drug for genetically modified T cells with icas9 (CaspaCid; AP1903)
Aim	<ol style="list-style-type: none"> 1. Testing the feasibility of a single T cell manipulation to eliminate alloreactivity while sparing antiviral and anti-tumor T cells through the usage of BPX-501. 2. Testing the possibility of a new drug; AP 1903 for treatment of GvHD with the ability to remove alloreactive T cells while not affecting immune reconstitution. <p>Thus, offering patients lacking an MHC-matched donor a successful/safe hematopoietic stem cell transplant.</p>
Objectives	<p><u>Primary objective:</u></p> <ol style="list-style-type: none"> 1. Safety of BPX-501 at the dose 1×10^6/Kg. 2. Safety of AP1903 at a dose of 0.4 mg/kg. <p><u>Secondary objective:</u></p> <ol style="list-style-type: none"> 1. Incidence of non-relapse mortality at 6m and 1year post-transplant. 2. Kinetics of donor engraftment, immune reconstitution and viral clearance. 3. Efficacy of AP1903 in switching off BPX-501 cells and clearing aGvHD.
Study design	Non-randomised open label study
Planned number of cases to be recruited	10 subjects to be recruited for the study from Great Ormond Street Hospital (GOSH)

Subject population	Patients requiring a hematopoietic stem cell transplant to cure malignant or non-malignant diseases and who failed to have a matched donor or who require rapid hematopoietic stem cell transplant for life threatening progressive disease not permitting enough time to identify a matched donor.
Treatment regimen	Patient who met eligibility criteria were consented to the study. BPX-501 was infused 21+/-14 days post-haploidentical sibling/parent stem cell transplant. AP1903 was available for patients who developed aGvHD grade II or above or who developed Grade I/II (skin only) and progressed or did not respond within 7 days to standard of care treatment.
Monitoring	Patients were monitored during the infusion of BPX-501 and AP1903 and had regular follow-ups at 30 days, 60 days, 90 days, and 180 days for evaluation of development of adverse event or severe adverse events. After 6 months, patient had 9m assessment, 1 year and then 6 monthly assessments for 2 years. For patients who received AP1903, regular assessment was carried out in the first 7 days post-infusion to monitor response of aGvHD to treatment.
Endpoints	<u>Primary Endpoint:</u> Toxicity: <ol style="list-style-type: none"> 1. Development of aGvHD after BPX-501 infusion. 2. Development of grade 3-4 toxicity (according to CTCAE criteria described below under section 2.8) post BPX-501 and AP1903 infusion. <u>Secondary Endpoint</u> Number of reported serious adverse events (SAEs) (Suspected, Unexpected Serious Adverse Reactions [SUSARs] and Suspected, Expected Serious Adverse Reactions [SESARs])
Statistical analysis	Descriptive analysis of patients enrolled in study.

	<p>Statistical analysis was performed using SPSS version 24. Descriptive analyses were performed using median, mean, minimum and maximum. Graphpad Prism 7 was used for plotting of T-cell/B-cell immune reconstitution amongst both groups.</p>
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2.2 Introduction:

The safety of BPX-501 was investigated in a phase I trial in Italy. Data were presented in ASH meeting 2015 (Locatelli et al, 2015). By that time, 23 children have been infused with BPX-501 cells at Day 14 \pm 4 days. 9 children were included in the phase I portion of the study and were given 2.5×10^5 , 5×10^5 , and 1×10^6 BPX-501 cells/kg, respectively, while 14 were included in the phase II received 1×10^6 BPX-501 cells/kg. Data on 16 patients with a minimum follow-up of 90 days; 7 children had acute leukaemia and 9 non-malignant disorders were presented. There were no reported toxicities from BPX-501 infusion and moreover, early immune recovery with $CD3 \geq 500$ cell/ μ l and appearance of newly synthesised immunoglobulin A and M by 3 months post-transplant were observed. Analysis of the function of T cells showed that the proliferative response to a polyclonal mitogen or to CMV lysate was comparable to that of a healthy control in 50% of patients as early as day+60 after a haplo-HSCT with BPX-501 infusion and that on day+150, all patients had a normal proliferative response. Response to both EBV and AdV antigens was slightly delayed, but progressively improved overtime. The study was open for recruitment in GOSH since August 2015. More sites started to open afterwards including another site in the UK; Great North Children hospital, a site in Spain; Hospital del Niño Jesús and 10 sites in the USA.

2.3 Recruiting patients based on subject and donor eligibility criteria

Patients and donors underwent initial screening including detailed medical history, clinical examination and screening investigations.

Subjects screening investigations included full blood count, biochemistry screen, bone marrow aspirate (in malignancy and on the discretion of BMT team), lymphocyte subsets including assessment of $CD3+CD19+$ cells together with virology screen including CMV, EBV, adenoviral PCR, hepatitis B sAg, Hepatitis B and hepatitis C PCR, human immunodeficiency virus (HIV) 1 and 2 antibody, anti- (human T-lymphotropic virus) HTLV I/II, Toxoplasma antibody and Treponema antibody.

Donor screening investigations included full blood count, biochemistry screen together with virology screen including CMV, EBV PCR, hepatitis B sAg, Hepatitis B

and hepatitis C PCR, HIV-antigen (p24) anti-HTLV I/II, HBs-antigen, anti-HCV, CMV status, EBV status, anti-HBc, Toxoplasma and Treponema serology.

In both donor and recipient, high-resolution molecular typing of HLA loci A, B, C, DRB1 and DQB1 was performed. Recipients were screening for presence of HLA antibodies.

2.3.1 Subjects eligibility criteria

1. Patients requiring a hematopoietic stem cell transplant to cure malignant or non-malignant diseases.
2. Patients who failed to have a matched donor or who require rapid hematopoietic stem cell transplant for life threatening progressive disease not permitting enough time to identify a matched donor.
3. Parents or legal guardians agreed to enroll their son/daughter into the study.

2.3.2 Subjects exclusion criteria

1. Subjects who had acute or chronic severe GVHD at time of transplant due to a previous allo- stem cell transplant.
2. Patients with severe concurrent medical problems.
3. Subjects who had HLA antibodies against donor.

2.3.3 Donor eligibility criteria

1. Donor had to be informed of the investigational BPX-501 and consented for a second leukapheresis.
2. Donors should be at least 5/10 HLA matched to the patient by high molecular resolution for HLA loci A, B, C, DRB1 and DQB1.
3. Donors should be in good health with no risks anticipated from 2 leukapheresis.
4. Should more than one "equally" MHC compatible donor be identified, other selection criteria to include gender, age, CMV status, health status and body weight of donor. The physician treating the subject will make the final decision.

2.3.4 Donor exclusion criteria

1. Donors who had active medical condition that would make him/her not suitable for leukapheresis as coronary heart disease, pregnant women or sickle cell trait.
2. Donors who were HIV or hepatitis C or Hepatitis B positive by PCR at time of leukapheresis.

2.3.5 Donor screening investigations

The following virology screening was undertaken during the donor assessment: anti-HIV-1, anti-HIV-2, HIV-antigen (p24), anti-HTLV I/II, HBs-antigen, anti-HCV, CMV status, EBV status, anti-HBc, EBV status, TPHA. Positive HIV, Hepatitis B and C DNA and TPHA serology is an absolute contra-indication to stem cell donation.

The virology screening was repeated at the time of leukapheresis for BPX-501 and again upon recipient admission to confirm the donor's status before carrying out the mobilisation and stem cell leukapheresis procedure.

2.4 BPX-501 manufacture

Donors who met eligibility criteria were consented for the study. Donors had 2 leukapheresis; an initial unstimulated leukapheresis (for the manufacture of BPX-501) followed after 1-2 weeks by G-CSF stimulated leukapheresis (for TCR $\alpha\beta$ /CD19 depletion using Miltenyi biotechnology as a stem cell source) (**Error! Reference source not found.**).

2.4.1 Ideal apheresis product for BPX-501 manufacture

A minimum of 1×10^9 mononuclear cells had to be collected for the unstimulated leukapheresis. In case the apheresis cut off level was not met; a second apheresis product might be required on the following day.

2.4.2 Process of BPX-501 production

Leukapheresis product was then shipped fresh in an "NanoCool" shipper box after activation of a temperature monitor to the manufacture site (MaStherCell, Belgium). In a GMP facility, T cells was be activated by Miltenyi CD3/CD28 beads. After 24 hrs, T cells were transduced with a retrovirus with icas9. T cells were kept in culture for 9 days followed by CliniMacs column selection.

2.4.3 Product release criteria:

BPX-501 release criteria included a minimum of CD3+CD19+ cells of 85% with a B cell percentage of less than 1%.

2.4.4 Product returns to GOSH

Products were released if it met the product release specification and were cryo-preserved and shipped back to GOSH in a “Cryoport” shipper with a temperature monitor. BPX-501 was to remain on site in liquid nitrogen until the planned day of infusion.

2.5 TCR $\alpha\beta$ /CD19 haploidentical transplant followed by infusion of manipulated T cells (BPX-501); Error! Reference source not found.

Patients received appropriate conditioning regimen to ablate the host immune system and open a space in bone marrow and thymus for stem cell engraftment. Conditioning regimen used was based on his/her primary medical condition followed by a TCR $\alpha\beta$ /CD19 haploidentical sibling/paternal transplant.

Manipulated T cells (BPX-501) were administered within 21 \pm 14 days after transplantation. 30 minutes pre-infusion of BPX-501, patients received pre-infusion medications which included paracetamol and antihistamine IV or oral therapy. The product bag of BPX-501 was transferred to a 37 \pm 1°C water bath (with no squeezing or shaking during the thawing process). When the cells were approximately 75% thawed, pea- size pieces of ice were left in the product bag, the bag was allowed to complete thawing at room temperature. Thawing took between 4-5 minutes.

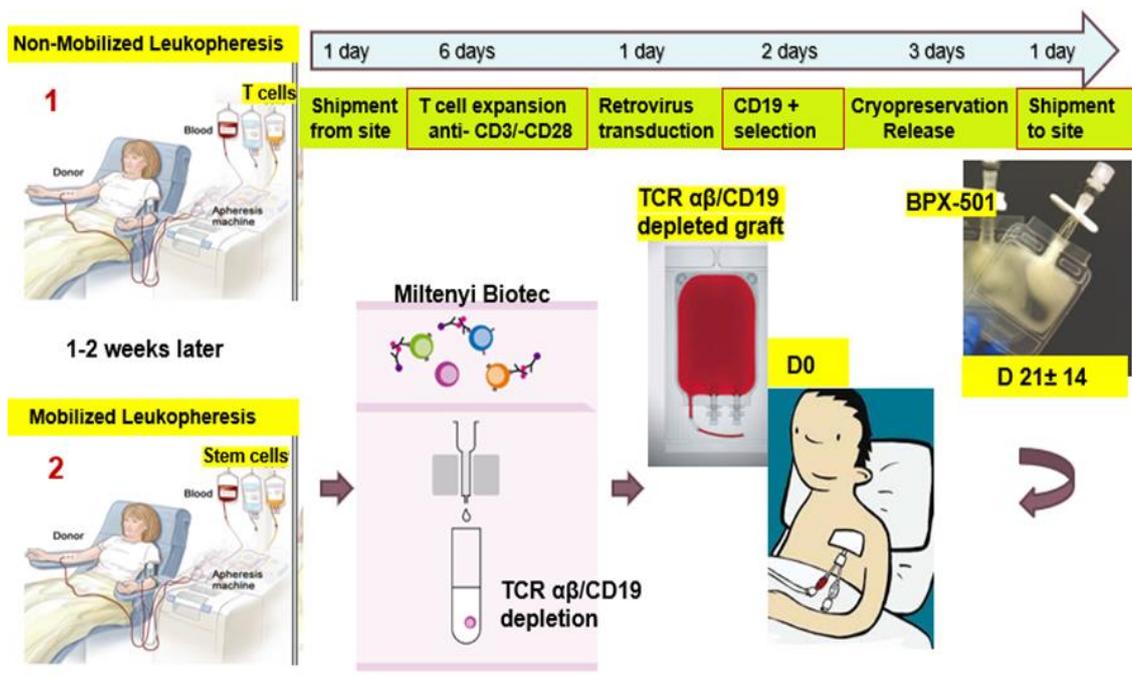
2.5.1 Monitoring of patients during BPX-501 infusion:

Vital signs were checked within 1 hour prior to the infusion and then at 15 minutes, 30 minutes and 120 minutes post-infusion.

2.5.2 Expected adverse events to BPX-501

Expected adverse effects from T cell add-back included possible development of acute GvHD or chronic GvHD.

Figure 2.1. TCR $\alpha\beta$ /CD19 haploidentical transplant followed by BPX-501 infusion



2.6 CaspaCid (AP1903) as a switch off medicine

2.6.1 Packaging and Formulation

The AP1903 for injection was packaged in 10 mL Type 1 clear glass vials. The contents of each vial composed of the labeled content (40 mg) of AP1903 drug substance dissolved in a sterile, endotoxin free, 24% solutol HS 15/water for Injection solution at an AP1903 concentration of 5 mg/mL and at pH 5.0 – 7.5.

2.6.2 Storage

The AP1903 for injection vials were stored at 5°C \pm 3°C (41°F \pm 5°F) in a limited access, qualified refrigerator, without light.

2.6.3 Preparation for Treatment

For use, the AP1903 was diluted prior to administration. The contents of the AP1903 vial was added directly to the 100ml saline bag provided with the drug, for a final volume of 108ml. It was recommended to infuse AP1903 in 2-4 hours after preparation (dilution).

2.6.4 Administration and Dosing

The AP1903 was administered via intravenous infusion at a dose of 40mg diluted in 100 mL physiological saline over 2 hours at a rate of 50 mL per hour using a DEHP-free saline bag. Patients were administered one dose of AP1903. Based on clinical response and on the discretion of principal investigator and bone marrow transplant team at GOSH, patients were given a second dose of AP1903.

2.7 Infusion of dimerizer drug, AP1903

Subjects who receive BPX- 501 and develop Grade III-IV aGvHD, as well as to those subjects with Grade II gut/liver aGvHD or with Grade I/II aGvHD (skin only) who progressed or did not respond within 7 days to standard of care treatment were administered a dose of AP1903 0.4mg/kg. A second and a third dose were considered based on clinical response and laboratory parameters on the discretion of the principal investigator and BMT team at GOSH.

2.7.1 Monitoring pre- and post-AP1903 infusion

Recommendations were to administer pre-medication (paracetamol and anti-histamines) 30 minutes pre-infusion to prevent hypersensitivity to Solutol HS or AP1903. Vital Signs were taken within 1 hour prior to start of AP1903 infusion and at 15, 30, 60, 120 and 240 minutes after the start of the infusion. Regular follow-up examination was carried out for 7 days after AP1903 infusion to document response of aGvHD to AP1903 infusion.

2.7.2 Investigational bloods prior and post-AP1903 infusion

Bloods including full blood counts, biochemistry and lymphocytes subsets with assessment of CD3+CD19+ cells were evaluated within 4 hours of infusion, at 4 hours post infusion and then at 24hours post-infusion. Expected adverse events to BPX-501 infusion

2.7.3 Expected adverse events to AP1903 infusion

Expected adverse events include facial swelling- previously reported in healthy volunteer- and possible transient neutropenia and thrombocytopenia.

2.8 Monitoring of patients enrolled to the study

Enrolled candidates were clinically, and laboratory assessed according to a certain time-based schedule (Table 2.1).

Data collected included:

1. **Days to neutrophil and platelet engraftment.**

Neutrophil engraftment was defined as the first of 3 consecutive days with absolute neutrophil count ≥ 500 cells/ μ L. Platelet engraftment was defined as the first of 7 consecutive days with platelet counts $>20 \times 10^9/L$.

2. Kinetics of donor cell engraftment, immune reconstitution and viral clearance

T and B cell enumeration used standard flow cytometry markers; CD3, CD4, CD8 and CD19, CD45 RO and CD45 RA. Molecular spectratyping and assessment of TRECs were assessed. Serological vaccine response to tetanus and pneumococcal antigen were assessed where indicated.

The presence of viral infection in blood (CMV, EBV, Adenovirus (AdV)), nasopharyngeal aspirate (NPA) and stool were recorded weekly until hospital discharge.

3. Incidence of both primary and secondary graft failure.

Primary graft failure was defined as failure to achieve Absolute neutrophil count (ANC) $\geq 500/\mu$ L after 28 days of transplant and absence of donor engraftment. Lineage specific chimerism was assessed by polymerase chain reaction amplification of specific polymorphic DNA sequences (short tandem repeats) in circulating lymphoid and myeloid cells

4. Timing and severity of acute and chronic GVHD.

Grading of aGvHD was performed according to Seattle criteria (Glucksberg et al, 1974). cGvHD was assessed and scored according to National Institute of Health (NIH) criteria (Filipovich et al, 2005).

5. Response to AP 1903 and effect on immune reconstitution.

6. Any reported adverse event to BPX-501 or AP1903.

All adverse events were recorded for the first 6 months after BPX-501/ AP1903 infusions. Adverse events were divided into grades 1-5; mild, moderate, severe, life threatening, led to death, based on CTCAE criteria (version 4.03; June 14, 2010). Serious adverse events including life threatening infections, prolonged hospitalisation or recurrent hospitalisation or death, were recorded all through the study. Any serious unexpected severe adverse event

was reported to the Medicines and Healthcare products Regulatory Agency (MHRA).

Table 2.1. Monitoring schedule for patients enrolled in the study

	D-10-D0	1m	2m	3m	4m	5m	6m	9m	12m	18m	24m
Screening for eligibility to be enrolled	x										
Physical examination	x	x	x	x	x	x	x	x	x	x	x
Full blood count	x	x	x	x	x	x	x	x	x	x	x
Biochemistry	x	x	x	x	x	x	x	x	x	x	x
Viral PCR-blood (CMV/EBV/Adeno)	x	x	x	x	x	x	x	x	x	x	x
LSS (including CD3+CD19+ cells)	x	x	x	x	x	x	x	x	x	x	X
Immunoglobulin Levels		x	x	x	x	x	x	x	x	x	x
Blood chimerism		x	x	x	x	x	x	x	x	x	x
Adverse events		x	x	x	x	x	x				
Serious adverse events		x	x	x	x	x	x	x	x	x	x

Section 2: Materials & Methods for clinical laboratory

A. Flow cytometry

Whole EDTA blood (a minimum of 100 U) was incubated with fluorochrome-conjugated monoclonal antibodies specific to white blood cell markers; BD multiset 6 colour TBNK reagent (BD bioscience; catalogue number 644611/337166) and CD45RA FITC (BD bioscience 335039). After incubation, the red blood cells were lysed and the resulting sample washed and fixed before acquisition using the FACSCalibur or FACSCanto flow cytometers. BD FACS diva 8.0.1 was used for analysis of results through gating on CD45+ cells - lymphocytes, followed by setting a gate to identify CD3+CD19+ cells. When CD3+CD19+ cells were identified, these were then gated and CD4+CD3+CD19+ and CD8+CD19+CD3 positive cells were enumerated as

a percentage of the CD3+CD19+ cells. Further analysis included identification of NK cells (CD3-CD16+), naïve CD4 (CD4+CD45RA+) and naïve CD8 cells (CD8+CD45RA+).

B. Chimerism

Cells were sorted from whole blood or bone marrow using a magnetic bead approach with the AutoMACS Pro Cell Separator (Miltenyi Biotec Ltd) . Whole blood, whole bone marrow or sorted cells were then used directly in a multiplex PCR reaction for 23 short tandem repeats (STRs) and an X/Y specific marker (Powerplex Fusion Kit, Promega UK Ltd). Fluorescently labelled PCR products were run on 3500xL Genetic Analyzer (Applied Biosystems, UK) and analysis was carried out using ChimerMarker (SoftGenetics) and Genemapper software (Applied Biosystems).

C. Molecular Spectratyping

Cells were sorted from whole blood using a magnetic bead approach with the AutoMACS Pro Cell Separator (Miltenyi Biotec Ltd). RNA was then extracted using the Qiagen whole blood kit (Qiagen UK) and reverse transcription was carried out using the GoScript RT Kit (Promega) to create single-stranded cDNA. PCR was then carried out in 24 separate tubes for 24 variable region primers used in conjunction with a single constant region primer. A second run-off PCR reaction was then carried out using a fluorescently-labelled constant region primer. PCR products were size separated using fragment analysis on a 3500xL Genetic Analyzer (Applied Biosystems, UK) and analysis was carried out using Genemapper software with an in-house scoring system

Special thanks to Dr. Kimberly Gilmour-consultant immunopathologist and Dr. Stuart Adams- lead health care scientist at Great Ormond Street hospital for running the flowcytometry, chimerism and molecular spectratyping.

Section 3: Materials and Methods for laboratory-based experiments

2.9. Production of a lentiviral vector for inducible gene (iCas9) expression:

To produce a lentiviral vector for icas9 transgene transfer in primary cells, multiple steps were carried out as follows:

2.9.1 Amplification of icas9 transgene:

Description: A polymerase chain reaction was set to amplify "icas9" transgene from retrovirus plasmid with icas9 (MMLV. iCas9.T2A.CD19) – given as a kind gift from Bellicum pharmaceuticals- using icas9 forward primer; *ctccttctctaggcgcccc* and icas9 reverse primer; *cttcccgaaggggagct*.

Oligonucleotides primers for icas9 transgene were designed using snapgene software (version 3 GSL Biotech LLC).

Material:

To carry out the PCR reaction, Q5® High-Fidelity DNA Polymerase kit (NEB, catalog number M0491) was used. This kit included 5 X Q5 reaction Buffer, 5 x Q5 High GC enhancer, Q5 High-Fidelity DNA polymerase.

Reaction components:

Based on manufacturer protocol, a 25ul reaction was set on ice using the following concentrations:

Component	25ul reaction
5 XQ5 reaction buffer	5ul
10nM dNTP	0.5ul
10uM forward Primer	1.25ul
10 uM Reverse Primer	1.25ul
Template DNA (Retroviral plasmid with icas9)	1ng
5x Q5 High GC enhancer	5ul

Q5 High-Fidelity DNA polymerase	0.25ul
Nuclease free water	to 25ul

The reaction was gently mixed and quickly transferred to the thermocycler machine. Thermocycling steps included initial denaturation at 98°C for 30 seconds followed by 35 cycles at 98°C for 10 seconds, 55-65 °C for 10 seconds and 72 °C at 30 seconds/Kb (Based on the size of icas9 transgene (2.2 Kb), this cycle took 1 minute and 30 seconds) and then a final denaturation at 72 °C for 2 minutes.

Following the thermocycling step, the success of the reaction was tested by running on a gel and checking the size of the icas9 transgene; 2.2kb.

2.9.2 Developing a Lenti-viral backbone:

Materials:

Bam H1(NEB, England; Catalog number: R0136S)

NEB Buffer 3.1

Bam H1 enzyme

Calf intestinal alkaline phosphatase (CIP) (NEB; catalog number: M0290)

Description:

A lentiviral backbone was retrieved from a Lenti-viral plasmid (PCCL1.hPGK.HSV-TK.CD34 plasmid) through a restriction digest using Bam H1 and Calf intestinal alkaline phosphatase (CIP). Bam H1 as a restriction enzyme induced a dual cut in the Lenti-viral plasmid (PCCL1-hPGK-HSV-TK-CD34) retrieving a Lenti-viral backbone. CIP was used to dephosphorylate the 5'-ends of DNA in restriction enzyme reaction to create sticky ends allowing a subsequent ligation reaction between icas9 (insert) and Lentiviral backbone (vector).

Setting the reaction:

Reaction components

All reaction components were set at room temperature followed by proper mixing. Reaction components (25ul) included 500ng of DNA template, 2.5ul of buffer 3.1, 1ul of BamH1 enzyme and nuclease free water to complete 25ul

To allow effective cutting by BamH1, the reaction components were incubated at 37°C for 1 hour followed by running the reaction on gel to check the success of restriction digest - the vector backbone by checking the size of the band; 7Kb.

2.9.3 Setting a gel extraction and gel purification:

Description:

In a preparation for a ligation reaction between the insert (icas9) and vector backbone (PCCL1-hPGK-), DNA bands were extracted from the gel.

Materials:

Gel extraction was carried out using QIAquick Gel Extraction Kit (catalog number 28706) which included QIAquick spin column, buffer QG and buffer PE.

Gel purification was carried out using QIAquick PCR purification (catalog number 28106) including buffer PB and buffer PE

Collection tube

Isopropanol

3M sodium acetate produced by ThermoFisher scientific; catalog number R1181

Procedure:

DNA band was excised from the agarose gel with a clean, sharp scalpel and weighed in a colourless tube. 3 volumes Buffer QG was added to 1 volume gel and incubated at 50°C for 10 min to dissolve the gel. This was followed by addition of 10 µl 3 M sodium acetate, pH 5.0 and 1 gel volume isopropanol to the sample and mix. A QIAquick column was placed in a 2ml collection tube. The sample was then applied to QIAquick column and centrifuged at 13,000 rpm for 1 min to allow the binding of DNA to the column. Flow through was then discarded. 2 washing steps were carried out using 500ul of buffer QG and 750 µl Buffer PE to QIAquick column simultaneously. In both steps, centrifugation was carried out at 13,000 rpm for 1 min followed by the discarding of the flow-through. To remove any residual wash buffers, an empty spin at 13,000 rpm was carried out for 1 minute followed by placement of QIAquick column into a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of water was added to the center of the QIAquick membrane and the column was left to stand for 1 min and then centrifuged at 13,000 rpm for 1 minute. To ensure removal of any residual traces of gel from the eluted DNA, gel purification was carried out using QIAquick PCR purification Kit protocol. First, 50ul of BP buffer was added to 50ul of eluted DNA (1:1). A QIAquick spin column was placed in a provided 2 ml collection tube. The

sample was then applied to the column membrane and centrifuged at 13,000 rpm for 1 minute to allow binding of the DNA to the membrane. Flow through was discarded. A washing step using 750ul of buffer PE was carried out followed by an empty spin to ensure removal of all buffer residues. QIAquick column was then placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 25 µl of water was added to the center of the QIAquick membrane and the column was left to stand for 1 min and then centrifuged at 13,000 rpm for 1 minute. 25 ul was used in this step to allow DNA concentration. DNA yield was then checked using nanodrop (ND 1000 version 3.7.1).

2.9.4 Setting a ligation reaction

Materials:

T4 DNA ligase (NEB, England; M0202S).

10X T4 DNA Ligase Reaction Buffer (NEB England; Catalog number: M0202S).

Description: To ligate icas9 transgene to Lenti-viral backbone, a ligation reaction was set off using T4 DNA Ligase which catalysed the formation of a phosphodiester bond between juxtaposed 5' phosphate of the vector backbone and 3' hydroxyl termini in insert (icas9).

Reaction components

Components	20ul
DNA insert	To be calculated in a molar ratio 1:3 (vector/insert)
DNA vector	50 ng
10 X Buffer reaction	1ul
T4 DNA Ligase	1ul
Nuclease free water	To 20ul

Reaction conditions

Following the manufacturer protocol, a ligation reaction was set in a minicentrifuge tube using the above icas9 transgene (insert) and PCCL1-hPGK (vector backbone) using T4 DNA Ligase using a molar ratio of 1:3 vector to insert. NEBicalculator (<https://nebiocalculator.neb.com/#!/ligation>) was used to calculate the molar ratio

based on insert DNA mass and DNA lengths and vector DNA mass. Reaction was set at room temperature 20-25°C followed by proper mixing. Ligation reaction was then left for 10 minutes to allow ligation between insert and vector. For proper assessment, a negative reaction was set in another minicentrifuge tube including all reaction components apart from the insert. Lentiviral plasmid (PCCL1-hPGK-icas9-CD19) (Figure 4.2) was then transformed into component cells; One Shot Stable 3 chemically competent E-coli (One Shot™ Stbl3™) as per manufacturer protocol.

2.9.5 Setting a transformation reaction:

One Shot™ Stbl3™ (produced from Invitrogen, ThermoFisher scientific, England; Catalog number:C737303).

S.O.C medium (ThermoFisher scientific; Catalog number: 15544034).

LB agar powder (ThermoFisher scientific; Catalog number: 22700025

Agar plates (Petri dishes).

Milli-Q water.

Kanamycin 100mg/100ml.

Preparation:

S.O.C medium was pre-warmed to room temperature. S.O.C medium is a rich media used primarily in the recovery step of Escherichia coli competent cell transformations. Use of SOC maximises the transformation efficiency of competent cells.

LB agar plates with Kanamycin were prepared for bacterial growth following the transformation step. 15 g of LB agar was added to 1 litre of MilliQ water in 500ml bottle. The bottle was then sterilised by autoclaving and Left for few minutes to slightly cool down. 1 mL of 100 mg/mL Kanamycin was then added to the mixture and 20-25 ml of the mixture was then distributed over petri dish plates and left to cool down.

Methods:

One vial of One Shot™ Stbl3™ chemically competent cells for each transformation was thawed on ice. 5 µL of the ligation reaction was then added to a a vial of One Shot™ cells and mixed gently without pipetting up and down. 5 µL of the negative reaction was added into another vial of One Shot™ cells and mixed gently (negative control). 1ng of plasmid PCCL1-hPGK-HSV-TK-CD34 was added into a third vial of One Shot™ cells and mixed gently (positive control). All 3 vials were incubated on ice for 30 minutes followed by heat-shocking of the cells for 45 seconds at 42°C to create pores in the plasma membrane of the bacteria and allow for plasmid DNA to enter the

bacterial cell. All vials were then placed on ice for 2 minutes. To allow recovery of competent cells, 250 μ L of pre-warmed S.O.C medium was added to each vial and vials were tightly sealed and shaken horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator. 50-200 μ L from each transformation was then plated on the prepared LB agar plates with Kanamycin and incubated overnight at 37°C.

Two different volumes were plated (50ul, 200ul) to ensure that at least one plate will have well-spaced colonies. Next day, all plates (ligation plate, negative and positive plates) were checked for the presence of colonies. The success of ligation and transformation reactions would be expected when many colonies grew on the ligation plate and positive plate while few, if any colonies, to grow on the negative plate (on some occasions the cut ends of the vector can stick together in the presence of T4 DNA ligase). To confirm the success of cloning, four colonies from the ligation plate were then picked up and left each in a sterile bottle in 5mls LB broth medium containing the appropriate selective antibiotic-Kanamycin to be incubated overnight at 37°C with vigorous shaking. This should allow growth of bacteria containing the new plasmid (PCCL1-hPGK-icas9 plasmid).

2.9.6 Miniprep of the clones using QIAprep Spin Miniprep Kit as per manufacturer protocol.

Materials:

LB broth powder (Catalog number: 12780052).

Milli-Q water.

Kanamycin 100mg/100ml.

QIAprep Spin Miniprep Kit (catalog number:12123 and 12125):

QIAprep 2.0 spin column

Buffer P1

Buffer P2

Buffer N3

Buffer PB

Buffer PE

LyseBlue reagent

RNase A solution

Preparation:

LyseBlue reagent was added to Buffer P1 at a ratio of 1 to 1000 followed by the addition of RNase A solution. Buffer P1 was then stored at 2–8°C.

1.5 ml of bacterial overnight culture was added to a 1.5 ml minicentrifuge tube and spun at 8000 rpm for 3 min at room temperature (15–25°C) to obtain a bacterial pellet. Pelleted bacterial cells were then re-suspended in 250 µl Buffer P1 with RNase A solution. 250 µL of Buffer P2 will then be added to allow damage of bacterial cell wall and release of the plasmid. To ensure proper lysis, the colour of the solution should turn blue (effect of LyseBlue reagent). To stop ongoing lysis, 350 µl Buffer N3 (neutralizing buffer) was added and mixed immediately and thoroughly by inverting the tube 4–6 times until the solution turned colorless. The solution was then centrifuged for 10 min at 13,000 rpm in a table-top microcentrifuge. Supernatant (with released plasmid) was then pipetted into the QIAprep 2.0 spin column and spun at 8000 rpm for 1 minute to allow the attachment of the plasmid to the membrane of the spin column. Spin column was then washed with 500ul of PB buffer with centrifugation at 8000 rpm for 1 minute. Flow-through was discarded and the QIAprep 2.0 spin column was then washed with 750ul of Buffer PE followed by centrifugation at 13,000 rpm for 1 minute and discarding of the flow-through. An empty spin at 13,000 rpm for 1 minute was carried out to ensure removal of all residual wash buffers. The QIAprep 2.0 column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µL of Milli-Q water was added to the center of the QIAprep 2.0 spin column, let to stand for 1 min, and then centrifuged at 13,000 rpm for another min. DNA yield was then checked using Nanodrop (ND 1000 version 3.7.1). The prepared minipreps were then checked for the presence of the desired construct using BamH1 (NEB, England; Catalog number: R0136S) in a test digest reaction- as described previously- to ensure a dual cut in the plasmid releasing the insert (icas9 transgene) and the vector backbone (PCCL1-hPGK). Test digest reaction was then run on a gel to detect the presence of 2 bands; 2.2 Kb band (icas9 transgene) and 7 kb band (PCCL1-hPGK; vector backbone).

2.9.7 Sequencing of lentiviral plasmid with icas9:

Based on the test digest results, minipreps with the suspected correct construct were sent for sequencing using primers that span the icas9 transgene. Primers used were designed using Snapgene software version 3 GSL Biotech LLC. Forward primers used were *atcccaccacatgccactct,cccaggacatcgtgtcctac, aacaggtcctcagggcca*. Reverse primers used were *cactcttgggtccctccag and ccgccagattccagtttagaagc*.

2.9.8 Preparation of a maxiprep of Lentiviral plasmid with icas9:

Materials:

Isopropranolol.

70% ethanol.

Milli-Q water.

QIAGEN Maxi-prep Kit (Catalog number:12863) included QIAGEN tip, Buffer P1 (added to it RNase A), Buffer P2, Buffer P3, Buffer QBT, Buffer QC and Buffer QF

Steps:

500ul of the miniprep with the correct construct (PCCL1-hPGK-icas9-CD19) was left in culture in 500 ml of LB medium containing Kanamycin. They were incubated overnight at 37° C with vigorous shaking. Overnight bacterial culture was then poured into a sterile bottle and harvested by centrifuging at 6000 x g for 15 min at 4°C. Supernatant was then discarded and the bacterial pellet was then resuspended in 10ml Buffer P1. 10 ml Buffer P2 was then added and mixed thoroughly by inverting the tube 4-6 times to allow lysis of bacterial cell wall with the release of the plasmid. Mixing was continued until the solution turned blue (LyseBlue buffer 's effect). 10 ml of prechilled Buffer P3 (neutralizing buffer) was then mixed thoroughly by vigorously inverting the tube 4–6 times until the solution was colorless. The bottle was then incubated on ice for 20 minutes followed by centrifuge at 15000 x g for 30 min at 4°C. During this step, QIAGEN-tip was equilibrated by adding 10 ml of buffer QBT and allowing the column to empty by gravity flow in a resin. After the centrifugation, bacterial cell wall remnants stuck to the bottle edges leaving the supernatant with the desired plasmid. This supernatant was then added to QIAGEN-tip and allowed to enter the resin by gravity flow. This step allowed the plasmid to attach to QIAGEN-tip membrane allowing any other residues to pass through. QIAGEN-tip was then washed 3 times with 30 ml with buffer QC. To elute DNA, the tip column was placed in a falcon tube and 15ml of pre-heated Buffer QF (to 65°C to increase DNA yield) was added to the tip membrane. To precipitate DNA, 10.5 ml of isopropanol was added to the eluted DNA followed by centrifugation at 15,000 x g for 30 min at 4°C. The supernatant was then carefully decanted, and DNA pellet was retrieved into a 1.5ml minicentrifuge tube and washed with 1ml of 70% ethanol. The pellet with ethanol was then centrifuged at 13,000 rpm for 10 min and supernatant was carefully

discarded. Air-dry the pellet was then left to air-dry in a heat block at 50⁰ C for 5 min (to allow complete evaporation of the volatile ethanol). DNA was then re-dissolved in 500uL of Milli-Q water and left overnight in 4⁰C to allow enough time for even distribution of DNA in the solute. DNA yield was then checked using nanodrop (ND 1000 version 3.7.1).

2.9.9 Five days lentivirus production as per institutional protocol:

Materials:

Opti-MEM (reduced serum medium) produced by ThermoFisher, UK (Catalog number 31985070).

DMEM solution produced by ThermoFisher, UK (Catalog number:11965118). A solution containing 500mls of DMEM, 50 ml of fetal calf serum (FCS) and 5mls of Pen-strep was prepared to be used as a medium for 293T cells (10% DMEM).

Third generation Lenti-viral packaging plasmids;

- pMD.G2 (expresses HIV 1-VSV glycoprotein as an envelope) produced by Addgene (Catalog number: 12259).
- pMDLg/pRRE (expresses HIV 1 GAG-POL) produced by Addgene (Catalog number 12251).
- pRSV/REV (expresses HIV 1 Rev) produced by Addgene (Catalog number 12253).
- Polyethylenimine 10mM, branched (PEI) produced by Sigma Aldrich, UK (Catalog number 408727).

0.22µM filter cups

0.45uM filter cups.

Polyallomer ultra-centrifuge tubes (33mLs tube for Surespin rotor).

Phosphate- buffered saline (PBS), produced by ThermoFisher scientific, UK (Catalog number: 10010031).

Trypsin (0.25%), phenol red produced by ThermoFisher scientific, UK (Catalog number: 15050065).

FACs buffer prepared by adding 1mls of FBS to 50 ml of PBS.

CD19 APC stain from Miltenyi Biotec, Catalog number; 130-110-351.

Preparation

In preparation for virus production, 293T cells were allowed to grow in T175 flasks for a week before the start of virus production. 293 T cells were split into 1 in 2 or 3 flasks every 2 days (based on their rate of growth) using several steps. First, media was

aspirated and then attached cells were carefully washed with 10 ml of PBS buffer. PBS buffer was then aspirated, and 5 ml Trypsin was added directly to cell wall to detach the cells from the wall of the plate. Reaction was left for 5 minutes then 10 ml of 10% DMEM was added to neutralize the trypsin. The detached cells (of one flask) were then transferred into two Ficoll tubes and spun at 1200 rpm for 5 mins to pellet cells. Supernatant was then discarded, and pelleted cells were re-suspended in 10% DMEM solution. 293T cells were then counted using hemocytometer and 10-15 million cells were seeded / T175 flask. 10% DMEM was added to complete 25 ml of media and cells. Flasks were then left horizontally in humidified incubator at 37°C overnight to allow 293T cells to attach to the flask wall and start to grow.

Steps of viral production (6 flasks/construct)

Viral production was carried out over 5 days. On day 1, 5 flasks of 293T confluent cells were then into 12 flasks. 20 million cells/T175 flask was used to ensure adequate viral production (reference). On Day2, cells were checked for being 80-90% confluent. Per flask, 25ug of transgene vector, 17.5Ug of pMD.G2 VSV envelope vector, 32.5ug of pMDLg/pRRE gag-pol vector and 25ug of pRSV/REV vector were incubated for 5 minutes at room temperature in 5ml of Opti-MEM. To this, another 5mls of Opti-MEM containing 1ul of PEI was added, filtered and incubated at room temperature for 20 minutes. 10 mins before transfection, 293T cells were washed with 10 ml of Opti-MEM and medium was gently aspirated. After 20 minutes incubation period, 15mls transfection medium with Opti-MEM (9mls of transfection mixture + 6 ml plain Opti-MEM) was added per flask. Cells were then incubated in a humidified incubator (5% CO₂) at 37 C for 4 hrs. After 4hrs, the transfection mixture was aspirated, and cells were supplemented with 25 ml of 10% DMEM. On day 3, the medium was changed, and cells were supplemented with new 10% DMEM medium. On day 4, the cultured medium (with shed virus) (harvest 1; H1) was collected and filtered through a 0.45um filter and a new medium was added to the cells. The filtered supernatant was then aliquoted into the labeled centrifuge tubes and left to spin for 2 hours at 4°C using a speed of 23,000 rpm for Superspin rotor to concentrate the virus. After spinning, the medium was carefully removed, and the tubes were put upside down on the tissue (two layers of tissue) to dry. 150 µLs of opti-MEM was added into each tube and tubes were sealed with parafilm and left on ice for 20 minutes. Concentrated virus was then aliquoted into several vials and freeze at -80 C. The same procedure was repeated on day 5 to concentrate the virus from harvest 2 (H2)

Viral titre was assessed 72 hours post-293T transduction through assessing CD19+ expression by flowcytometry. First, 1ml of 293 T was dispensed in 24 well plate at 1×10^5 of 293 T cells/well. The plate was left in a humidified incubator (5% CO₂) at 37°C overnight to allow 293 T to attach to plate walls and start to grow. 13 wells were prepared; 12 for virus and another for untransduced 293 T cells. Next day, serial dilutions; 10, 2, 0.4, 0.08, 0.016, 0.0032 ul of concentrated viral stock (harvest 1 and harvest 2) in 10% DMEM was prepared and added to labelled wells. The plate was left in a humidified incubator (5% Co₂) at 37°C for 72 hours. After 72 hours, cells were harvested and stained by CD19 FITC to check CD19 expression- surface marker of iCas9 using Cyan ADP flow cytometer from Beckham Coulter. Based on the flow results, using the lowest dilutions (0.08, 0.016 or 0.032), infectious unit was calculated using this formula: $(\text{Percent of CD19+cells} \times 100,000 \times 1000) \div (\text{dilution titer} \times 100)$. All flow data analyses were carried out using Flowjo version 10.

2.10 Transduction of Jurkats 6.1 and Peripheral Blood Mononuclear cells (PBMNCs) with Lenti-icas9

Objective:

Assessing the multiplicity of infection (MOI) required for effective transduction of 2 cell lines; Jurkats 6.1 and peripheral blood mononuclear cells (PBMNCs) with Lenti-icas9.

2.10.1 Transduction of Jurkats 6.1 with Lenti-icas9 virus:

Materials:

RPMI produced by ThermoFisher scientific, UK (Catalog number: 11875093). A solution containing 500mls of RPMI, 50 ml of fetal calf serum (FCS) (produced by ThermoFisher scientific, catalog number: Catalog number: 16000044) and 5mls of Penicillin (5000IU/ml)-Streptomycin (5000ug/ml) (Pen-strep) (produced by ThermoFisher scientific, UK, catalog number: 15070063) was prepared to be used as a medium for Jurkats 6.1 (10% RPMI).

Procedure:

Jurkats 6.1 were plated (1 million cells/ml) in 3 wells of a 24 well plate. An MOI of 5 and 10 were calculated to transduce the Jurkats 6.1 using the following formula:

MOI of 5: $5 \times \text{number of Jurkats 6.1} \div \text{viral infectious unit.}$

MOI of 10: $10 \times \text{number of Jurkats 6.1} \div \text{viral infectious unit.}$

Lenti-icas9 virus was then added at MOI of 5 and 10 to the first 2 wells respectively. No virus was added to the third well (untransduced Jurkats 6.1). Platted cells were then left to grow in a humidified incubator (5% CO₂) at 37°C. After 72 hours of incubation, 100 ul of the transduced and 200 ul of the untransduced Jurkats (split into 2 tubes) were collected into labelled tubes and spun at 12,000 rpm for 5 minutes. The supernatant was then discarded, and cell pellet were re-suspended in 1ml of FACs buffer. Re-suspended cells were centrifuged again at 12,000 rpm for 5 minutes. Supernatant was discarded, and cells were stained with suspended in 100ul of 10% RPMI. 3ul of CD19 APC stain was added to suspended cells apart of one tube of untransduced cells which was left as unstained. Cells were then incubated at 4°C in the dark for 15 minutes to ensure proper staining. 1ml of FACs buffer was then added to each falcon tube and spun at 12,000 rpm for 5 minutes. The supernatant was discarded, and cells were suspended in 250ul of FACs buffer. Using CyAn ADP flow cytometer from Beckman Coulter, cells were then checked for CD19 expression.

Transduction efficacy (%) was calculated (%) using this formula:

Percent of transduced cells =

% of CD19+ cells among transduced Jurkats 6.1 minus % of CD19+ cells among untransduced/stained Jurkats 6.1.

2.10.2 Transduction of PBMCs with Lenti-icas9 virus:

Materials:

1. Volunteer's blood.
2. X-VIVO 15 produced by Lonza (Catalog number: 04-744Q). A solution of 47.5 ml of X-VIVO 15 with 3.5 ml of human serum albumin is used a T cell culture media (5% X-VIVO).
3. Ficoll-Paque premium produced by GE Healthcare Life Sciences (catalog number: 17-5442-02).
4. CD3/CD28 T cell activation dynabeads produced by ThermoFisher scientific (catalog number: 11131D).

Procedure:

Isolation of mononuclear cells

10 ml of blood were collected on an EDTA tube from a healthy volunteer after consent. The collected blood was then poured in a 50 ml falcon tube. Blood was diluted by adding 10 ml of PBS. The blood and buffer were then mixed by drawing the mixture in and out of a pipette. 15 ml of ficoll paque was then added in a separate 50 ml falcon tube. Diluted blood was then carefully poured on the ficoll paque without mixing followed by centrifuged at 10,000 rpm for 25 minutes at 25°C (brake should be turned off). Ficoll plaque allows separation of blood components into layers based on their densities (red cells at the bottom then a ficoll layer followed by a buffy coat (mononuclear cell layer) followed by plasma). The layer of mononuclear cells was then carefully pipetted to a sterile centrifuge tube using a sterile pipette. At least 3 volumes of PBS were added to 1 volume of transferred mononuclear cells to allow proper cell washing. Cells were centrifuged at 12,000 rpm for 5 minutes. Supernatant was then discarded, and cells were suspended in 5% X-VIVO. 1 million cells /ml were plated in 4 wells of a 48 well plate. CD3/CD28 T cell activation beads were added at a ratio of 1: 1 ratio to 1ml of plated cells. 100 IU of IL2 (T cell activation cytokine) was added to 1ml of cells. The plate was then left in a humidified incubator (5% CO₂) at 37°C for 24 hours.

Transduction of PBMNCs with Lenti-icas9

24 hours after activation of the cells with CD3/CD28 beads, lenti-icas9 virus was added to 2 wells of mononuclear cells at an MOI of 5 (using the same method of calculation used with Jurkats). No virus was added to 2 wells; untransduced mononuclear cells.

$$\text{MOI of 5} = 5 \times \text{number of PMNCs} \div \text{viral infectious unit}$$

Assess transduction efficacy of mononuclear cells with Lenti-icas9

After 72 hours of transduction:

Activation beads were removed using DynaMag-15 Magnet (produced by ThermoFisher scientific; catalog number 12301D) after 72 hours of transduction (Day 4 of activation) in the following steps. First, transduced and untransduced cells were collected in falcon tubes. These falcon tubes were then placed in DynaMag-15 and left to stand for 5 minutes with possible gentle tilting (this would allow the magnetic

beads to be attached to tube walls). Suspended cells were then collected carefully (without touching tube walls). Pipetted cells were then centrifuged at 12,000 rpm for 5 minutes. Supernatant was discarded, and cells were re-suspended in fresh 5% X-vivo media. Cell count was then checked by hemocytometer and cells were plated at 1 million cells/ml in each well of a 48 well plate. 100 IU IL2 /ml was added to plated cells. 100 ul of the transduced and 200 ul of the untransduced mononuclear cells (split into 2 tubes) were collected into labelled tubes and spun at 12,000 rpm for 5 minutes. The supernatant was then discarded, and cell pellets were re-suspended in 1ml of FACs buffer. Re-suspended cells were centrifuged again at 12,000 rpm for 5 minutes. Supernatant was discarded, and cells were suspended in 100ul of 10% RPMI. 3ul of CD19 APC stain was added to suspended cells apart of one tube of untransduced cells which was left as unstained. Cells were then incubated at 4°C in the dark for 15 minutes to ensure proper staining. 1ml of FACs buffer was then added to each tube and spun at 12,000 rpm for 5 minutes. The supernatant was discarded, and cells were suspended in 250ul of FACs buffer. Using CyAn ADP flow cytometer from Beckman Coulter, cells were then checked for CD19 expression.

Transduction efficacy (%) was calculated (%) using this formula:

Percent of transduced cells = percentage of CD19+ cells among transduced mononuclear cells minus percentage of CD19+ cells among untransduced stained mononuclear cells.

This experiment was carried out 6 times using different donors.

2.11 Test the effect of AP1903 on the transduced Jurkats 6.1 and primary cells with Lenti iCas9:

Objective: To test the killing effect of AP1903 on transduced Jurkats 6.1 and primary cells with Lenti- icas9.

Two assays were used; Cell counting kit (CCK8) and apoptosis/necrosis assay using flow-cytometry.

2.11.1 CCK8 assay

Background:

Cell counting kit (CCK8) assay was used to test the effect of AP1903 on transduced Jurkats with Lenti-icas9. CCK8 is a colorimetric assay based on the dehydrogenase activity detection of viable cells. A highly water soluble tetrazolium salt (in CCK8) can

be reduced by dehydrogenase (in viable cells) to a yellow-coloured dye called formazan that dissolves in cell culture media. The amount of formazan produced is proportionate to the number of viable cells.

Material:

CCK8 produced by Sigma-Aldrich; catalog number 96992.

AP1903 (5mg/ml) given as a kind gift from Bellicum pharmaceuticals.

10% RPMI as previously described.

Methods:

100 ul of cell suspension (25,000/well) was dispensed in triplets in a 96 well plate; these include untransduced cells; UT (as a control) and transduced cells with Lenti-iCas9.

AP1903 was added in increasing concentrations (from 0-3000 nM)- method of calculation is given below. The plate was then incubated for 24 hours in a humidified incubator (at 37°C and 5% CO₂). 10ul of CCK8 was added to the wells in the tested plate. The plate was then left for 4 hours in a humidified incubator (at 37°C and 5% CO₂). Absorbance at 450nm was measured using a microplate reader (FluoStar OPTIMA) to assess cell viability upon exposure to AP1903.

2.11.2 Apoptosis/necrosis assay using Annexin V/Topro3 stain:

Background:

Apoptosis/necrosis assay using Annexin V/Topro3 stain was used to test the effect of AP1903 on T cells with Lenti-icas9. An early event in apoptosis is the flipping of phosphatidylserine of the plasma membrane from the inside surface to the outside surface. Annexin V binds specifically to phosphatidylserine and thus labelled Annexin V can be used to detect apoptotic cells. Topro-3 is a stain for nucleic acid that can detect cell death. This assay was used to count the number of cells that have undergone apoptosis/necrosis by flow cytometry where early apoptotic cells were (Annexin V+ve /Topro3 -ve) and late apoptotic/necrotic cells were (Annexin V+ve/Topro-3+ve). This assay was considered more informative than CCK8 assay as it could discriminate between cell death from apoptosis (induced by AP1903- Annexin V+/Topro-3 +) and cell death from any other cause (necrosis- Annexin V-/Topro-3 +).

Materials:

5% X-Vivo 15 as previously described.

AP1903 supplied by Bellicum pharmaceutical.

Annexin V stain produced by Miltenyi Biotec; catalog number: 130-092-052.

Topro-3 stain produced by ThermoFisher; catalog number: T3605.

CD3 FITC stain produced by Miltenyi Biotec; catalog number: 130-080-401.

CD19 APC stain produced by Miltenyi Biotec; catalog number: 170-078-090.

Methods:

Sorting of transduced T cells with Lenti-icas9:

10 ml blood on EDTA were collected from a healthy volunteer followed by Ficoll plaque separation of mononuclear cells. Mononuclear cells were then suspended in 5% X-vivo+IL2 100 IU/ml and plated in a 48 well plate 1million cell/ml and activated by CD3+/CD28 activation beads 1:1 ratio. On the next day the cells were transduced with Lenti-icas9 at MOI of 5. Transduction efficacy was checked after 72 hours of transduction as been described above. Four days after transduction cells were stained for both CD3+ FITC and CD19+ APC. Untransduced T cells were used as a control. Using Aria cell sorter, transduced T cells were then sorted for CD3+CD19+ population (reflecting genetically modified T cells). Sorted cells were further divided into a high expressing and low expressing population based on median fluorescent intensity (MFI) for CD19+ as shown in

Figure 4.5. A purity check was carried out at the end of the sorting. T cells with Lenti-icas9 high and Lenti-icas9 low were also checked for efficacy of sorting by staining for CD3 FITC and CD19 APC. Analysis was carried out using Cyan flow cytometer. Flow data were extracted from Cyan and analysed using Flowjo version 10.

24 hr. incubation of sorted cells:

Sorted cells were then dispensed in a 48 well plate ;1 million cell /well were cultured in 1ml of 5% X-Vivo media and 100 IU of IL2. Wells with untransduced T cells were used as a control. Cells were then incubated for 24 hours in a humidified incubator (5% CO₂) at 37^oC to allow cells to rest after sorting.

Add serial dilutions of AP1903 to sorted T cells:

500 uL of cell suspension (100,000 cells/well) were dispensed in duplicates in a 48 well plate) included untransduced T cells, T cells with Lenti-icas9 high and T cells with lenti-icas9 low. AP1903 was added in increasing concentrations (from 0-3000 nM).

Check the effect of AP1903 on transduced T cells with Lenti-icas9:

Cells were collected after 3.5 hrs. and 24 hrs. in labelled tubes. 1ml of 1X Binding buffer was added to each 10*5 cells followed by centrifugation at 12,000 rpm for 5min.

Supernatants were discarded, and the cell pellets were re-suspended in 100 ul of 1× Binding Buffer per 10^5 cells. 5 ul of Annexin V-FITC was then added per 10^5 cells and cells were incubated for 15 minutes in the dark at room temperature. Cells were then washed by adding 1 mL of 1× Binding Buffer per 10^5 cells and centrifuged at 12,000 rpm for 10 minutes. Supernatants were completely aspirated, and cell pellets were resuspended in 200 ul of 1× Binding Buffer per 10^5 cells. 10ul of Topro -3 stain solution was added immediately prior to analysis by flow cytometry.

Calculation of AP1903 dose to be added to cell culture media:

Calculation was carried out using Graphpad molarity calculator; <https://www.graphpad.com/quickcalcs/molarityform.cfm> using the following steps:

Calculate molarity from mass and volume:

Based on the given information about AP1903; drug concentration of 5mg/ml, and drug molecular weight of 1144.63 KD, drug molarity was calculated as 4.3681 millimolar.

Then calculate the required volume for a stock solution:

2000 nM stock solution was prepared by adding 2.28ul of AP1903 to 5ml of cell media; tube 1.

Add serial dilutions of AP1903 to media:

500 ul was withdrawn from 2000 nM stock concentration after pipetting up and down and was added to 4.5mls of media □ tube 2; 200 nM.

500 ul was withdrawn from 200 nM solution (tube 2) after pipetting up and down and was added to 4.5mls of media □ tube 3; 200 nM.

Add the serial dilutions to JK 6.1 and primary cells; UT and transduced with Lenti-icas9 as follows:

For CCK8 experiment:

100 ul of 2000 nM concentrate was added to 100 ul of JK 6.1 in 10% RPMI (25,000cells/well) □ 1000 nM solution. Further dilutions were carried out in the same way.

For apoptosis/necrosis assay:

500 ul of 2000 nM concentrate was added to 500 ul of T cells in 10% RPMI (100,000cells/well) □ 1000 nM solution. Further dilutions were carried out in the same way.

2.12 Test the effect of immune suppressive medications on T cells transduced with Lenti-icas9 on survival and proliferative capacity

Objective

To investigate the effect of commonly used immunosuppressive agents (Methylprednisolone (MP), Mycophenolate mofetil (MMF) and Cyclosporine A(CSA) on the transduced T cells with Lenti-icas9.

Two assays were used including Annexin V/Topro-3 assay for checking survival and radioactive thymidine assay to check proliferation.

Steps:

Sorting of transduced T cells with Lenti-icas9:

10 ml blood on EDTA were collected from a healthy volunteer followed by ficoll plaque separation of mononuclear cells. Mononuclear cells were then suspended in 5% X-vivo+IL2 100 IU/ml and plated in a 48 well plate 1million cell/ml and activated by CD3+/CD28 activation beads 1:1 ratio. On the next day the cells were transduced with Lenti-icas9 at MOI of 5. Transduction efficacy was checked after 72 hours of transduction as been described above. Four days after transduction cells were stained for both CD3+ FITC and CD19+ APC. Untransduced T cells were used as a control. Using Aria cell sorter, transduced T cells were then sorted for CD3+CD19+ population (reflecting genetically modified T cells).

24 hr. incubation of sorted cells:

Sorted cells were then dispensed in a 48 well plate; 1 million cell /well were cultured in 1ml of 5% X-Vivo media and 100 IU of IL2. Wells with untransduced T cells were used as a control. Cells were then incubated for 24 hours in a humidified incubator (5% CO₂) at 37⁰C to allow cells to rest after sorting.

2.12.1 Test the effect of immune suppressive medications on survival of T cells transduced with Lenti-icas9 using Annexin V/Topro-3 apoptosis /necrosis assay

500 ul of T cell suspension (100,000 cells/well) were dispensed in a 48 well plate including untransduced T cells, T cells with Lenti-icas9. MP 20ug/ml, MMF (0,1,3,5, 7 ug/ml) and CSA (0-800ng/ml) were added to the correspondingly labelled wells.

Platted cells were left in a humidified incubator (5% CO₂) at 37 °C for 96 hours. Cells were collected after 96 hrs. in labelled tubes. 1ml of 1X Binding buffer was added to each 10*5 cells followed by centrifugation at 12,000 rpm for 5min. Supernatants were discarded, and the cell pellets were re-suspended in 100 ul of 1x Binding Buffer per 10*5 cells. 5 ul of Annexin V-FITC was then added per 10*5 cells and cells were incubated for 15 minutes in the dark at room temperature. Cells were then washed by adding 1 mL of 1x Binding Buffer per 10*5 cells and centrifuged at 12,000 rpm for 10 minutes. Supernatants were completely aspirated, and cell pellets were resuspended in 200 ul of 1x Binding Buffer per 10*5 cells. 10ul of Topro -3 stain solution was added immediately prior to analysis by flow cytometry. Drug preparation was explained below.

2.12.2 Test the effect of immune suppressive medications on proliferative capacity of T cells transduced with Lenti-icas9 using Thymidine proliferation assay

Description:

Thymidine incorporation assay utilises a strategy wherein a radioactive nucleoside 3H-thymidine, is incorporated into new strands of chromosomal DNA during cell mitosis. A scintillation beta counter is used to measure the radioactivity of dividing cells denoting percentage of cell proliferation.

Materials:

3H Thymidine produced by Perkin Elmer, Waltham, MA, USA (catalog number NET027W001MC).

Fiberglass filter; Filtermat A, Perkin Elmer Waltham, MA, USA (catalog number 1450-421).

Meltilux for Microbeta filters, Perkin Elmer Waltham, MA, USA (catalog number 1450-441).

Procedure:

100 ul of cell suspension (25,000/well) were dispensed in triplicates a 96 well plate including untransduced and transduced T cells with Lenti-icas9. Serial dilutions of MMF (0-7ug/ml), CSA (0-800 ng/ml) and MP 0.2 ug/ml were added to correspondingly labelled wells. 96 well plate was then left in a humidified incubator (5% CO₂) at 37 °C for 96 hrs. After 4 days, the cells were pulsed by adding 10 uCi of 3H thymidine per

well (1ul of 3H thymidine stock (1mC/ml) + 19 ul of 5% X-vivo) to each well. After pulsing for 18 hours, cells were harvested onto fiberglass filters using automated cell harvester 96 (Tomtec, Hamden, CT, USA). Meltlux solid scintillant was melted onto the dried filtermats and sealed between 2 pieces of plastic film and placed into the cassette to be read into the tr Microbeta counter (Perkin Elmer, Waltham, MA, USA). Incorporated radioactivity was measured as counts per minute (cpm).

Thanks to Dr. Jenny Yeung; institute of child health for assistance in setting in 3H thymidine pulsing and harvesting procedures.

Methods of preparation of immune suppressive drugs:

Materials:

Methylprednisolone (MP) 40mg/ml.

Mycophenolate mofetil (MMF) 500 mg powder.

Cyclosporine A (CSA) 50mg/ml.

Preparation of drugs

Methylprednisolone 40 mg for IV injection

40 mg of MP was added to 39 ml of RBMI solution \square 1 mg/ml. 10 ul of the diluted solution (10 ug) was then to 4 ml of 5% X-Vivo+ IL2 100 IU/ml \square 0.4 ug/ml solution is thus prepared.

Preparation of serial dilution of MMF (0.3- 7 ug/ml)

MMF vial (500 mg) was diluted in 500 ml of RPMI \square 1mg/ml. 3ul (3ug) of diluted solution was added to 5mls of 5% X-Vivo +IL2 100 IU/ml \square 0.6ug/ml concentration. 10 ul (10ug) of diluted solution was added to 5mls of 5% X-Vivo +IL2 100 IU/ml \square 2ug/ml concentration. 30 ul (30ug) of diluted solution was added to 5mls of 5% X-Vivo +IL2 100 IU/ml \square 6 ug/ml concentration. A 50 ul (50ug) of diluted solution was added to 5mls of 5% X-Vivo +IL2 100 IU/ml \square 10 ug/ml concentration. A 70 ul (70ug) of diluted solution was added to 5ml of 55 X-Vivo +IL2 100 IU/ml \square 14 ug/ml concentration.

Preparation of serial dilution of CSA (100-800 ng/ml)

Cyclosporine A vial (50mg/ml) was diluted in 49 ml of RPMI \square ; 1ug/ul. 1 ul (1ug) of diluted solution was added to 5mls of 5% X-Vivo +IL2 100 IU/ml \square 0.2ug/ml (ie

200ng/ml) concentration. 1 ul (1ug) of diluted solution was added to 2.5mls of 5% X-Vivo +IL2 100 IU/ml □ 0.4 ug/ml concentration. Another 1 ul (1ug) of diluted solution was added to 1.25mls of 5% X-Vivo +IL2 100 IU/ml □ 0.8 ug/ml concentration.

2.13 Investigating resistance to AP1903

Post the infusion of AP1903 to the patients in our centre, it was clearly demonstrated that some of the genetically modified T cells had escaped the effect of AP1903 as shown in sector 4.9. Thus, a number of experiments were set to investigate the mechanism of resistance.

2.13.1 Assessing the escape mechanism of genetically modified T cells (retroviral-icas9) for P002, P006 and P007 to the effect of AP1903

2.13.1.1 Testing the effect of AP1903 *in vitro* on sorted CD3+CD19+ cells collected from P002 and P007

10mls of blood on EDTA were collected from Bellicum P002, P004 and P006 after patients/legal guardians' consents. Mononuclear cells were isolated by Ficoll separation as been discussed above. Patients' cells were sorted for CD3+CD19+ cells using Aria sorter as been discussed above. Collected sorted cells were then spun at 12,000 rpm for 5 minutes. Supernatant was discarded, and cell pellets were re-suspended in 5% X-vivo+ IL2 100 IU/ml. 100,000 cells were withdrawn into another tube and centrifuged at 12,000 rpm for 5 minutes, supernatant was discarded, and cells were re-suspended in 200 ul PBS and then stored at -20 for future DNA extraction. At the same time, 2 ml of blood was collected in EDTA from healthy donor, mononuclear cells were isolated by Ficoll separation. These mononuclear cells were used as a negative control.

CCK8 killing assay was used for P002:

Moreover, untransduced and transduced JK 6.1 with Lenti-icas9 (produced from earlier experiments) were also used as negative and positive controls. 100ul of cells (25,000 cells/well) were dispensed in triplicates into a 96 well plate as showed in figure 4.14. Serial dilutions of the drug AP1903 was added to the labelled well (0nM-3000nM) as described above. The plate was then left for 24 hours in a humidified incubator (5% CO₂) at 37°C. 10ul of CCK8 was added to each well and the plate was

left for another 4 hours in a humidified incubator (5% CO₂) at 37°C. Absorbance at 450nm was measured using a microplate reader (FLUOstar OPTIMA) to assess cell viability upon exposure to AP1903.

Annexin V/Topro-3 apoptosis assay was used for P007

500 cells in 5% X-vivo (+ IL2 100 IU/ml) were dispensed in a 48 well plate (50,000 cells/well). Two concentrations of AP1903 were added (10nM and 3000nM) to labelled wells with untransduced and transduced T cells with lenti-icas9. The plate was then incubated at a humidified incubator (5% Co₂) at 37°C for 24 hours. the cells were harvested from the labelled plate next day and apoptosis was assessed using flowcytometry after staining for Annexin V FITC and Topro-3 APC stains as been described above.

Due to limited number of cells collected after sorting in P006 (20,000 cells in total – killing assay was not applicable. P006 was on immunosuppressive drugs- Prednisolone/CSA to control aGvHD with persistently low CD3 cells (100 cells/ul).

2.13.1.2 Assessment for the presence of a possible mutation in icas9 transgene leading to the escape mechanism (lack of effect to AP1903) DNA extraction procedure

Materials:

DNeasy Blood & Tissue Kit; QIAGEN (catalog number 69504) including buffer ALT, buffer AW1, buffer AW2, proteinase K, Mini spin column and collection tubes.

Procedure:

Collected CD3+CD19+ sorted cells from P002, P006, P007 in PBS were left to thaw at room temperature and solution was then withdrawn into a microcentrifuge tube. 20 µl of proteinase K was added to the solution and properly mixed by vortexing for 15 seconds. 200 µl Buffer AL was then added to the mixture and mixed thoroughly by vortexing followed by incubation at 56°C for 10 min. 200 µl ethanol (100%) was then added to the sample and mixed thoroughly by vortexing. The mixture was then pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube. This was followed by centrifugation at 8000 rpm for 1 minute. The DNeasy Mini spin column was then placed into a new 2 ml collection tube and 500 µl Buffer AW1 was added followed by centrifugation for 1 min at 8000 rpm. Again, the DNeasy Mini spin column

was placed into a new 2 ml collection tube and 500 µl Buffer AW2 was added followed by centrifugation for 3 min at 14,000 rpm to dry the DNeasy membrane. Following the centrifugation step, the DNeasy mini spin column was removed and placed in a clean 1.5 ml microcentrifuge tube. 150 µl of deionised water was pipetted directly onto the DNeasy membrane. The column was incubated at room temperature for 1 min, and then centrifuged for 1 min at 8000 rpm. The DNA yield was measured using a Nanodrop (ND 1000 version 3.7.1)

Sequencing of extracted DNA

To check for the presence of a mutation in icas9 transgene, 2 methods were used. For all patients next generation sequencing was used to clarify the presence of any mutation. In P002, sanger sequencing of a cloned icas9 transgene in Pjet vector was used as well.

Two methods were used:

Cloning of icas9 transgene to PJet 1.2 vector followed by sequencing of multiple individual clones derived from sub-cloned amplified PCR product (transgene icas9) – only carried out for P002.

Principal:

PJET1.2/blunt is a linearised cloning vector, which accepts inserts from 6 bp to 10 kb. Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated with the vector in just 5 min. Ligated product can then be efficiently transformed in any of the common laboratory E-Coli bacteria.

Steps:

icas9 transgene from the extracted DNA was amplified using Q5® High-Fidelity DNA Polymerase (M0491) as been described above. PCR product was then purified using QIAquick PCR Purification Kit (catalog number 28106) as described above. A ligation reaction was set using amplified icas9 PCR product and Pjet 1.2 vector [produced by ThermoFisher Scientific (catalog number: K1231)] using T4 DNA ligase (NEB, England; M0202S) as described above. This was followed by transformation of the ligation reaction into One Shot™ Stbl3™ (produced from Invitrogen, ThermoFisher scientific, England; Catalog number:C737303) as described above. 10 colonies were picked up from the transformation plate on next day as described above. Minipreps were prepared using Qiagen miniprep as described above. Sanger sequencing was

carried out using primers that span the icas9 transgene. Primers used were designed using Snapgene software version 3 GSL Biotech LLC. Forward primers used were atcccaccacatgccactct,cccaggacatcggtgtctac, aacaggctctcagggcca. Reverse primers used were cactcttgggtccctccag and ccgccagattccagtttagaagc.

Next generation sequencing of the extracted DNA product from sorted T cells – patients P002, P004, P006.

In preparation for NGS of icas9 transgene, icas9 transgene was first amplified from the extracted DNA using Q5 PCR as been described above. A library preparation was carried out using Nextra XT protocol (illumina). The product was then sequenced in 500 cycles V2 Miseq sequencer (illumina). Sequencing data were illustrated in csv files (comma separated value) which is a bioinformatic friendly excel sheet. Thanks to Dr. Athina Gkazi for performing NGS.

2.13.2 Production of virus vector with icas9 wild and icas9 mutant Objectives

To confirm that the detected mutation in icas9 transgene was responsible for low expression of CD3+CD19+ in primary T cells and induced resistance to the effect of AP1903

Methods:

Generation of a vector with mutant icas9 by site directed mutagenesis

Description:

Site directed mutagenesis (also called oligonucleotide directed mutagenesis) is a molecular biology method that is used to make specific and intentional mutation into a DNA sequence.

Steps:

Two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence were synthesised using QuikChange primer design tool by Agilent: <https://www.genomics.agilent.com/primerDesignProgram.jsp> . in these steps:

- Change section 2 to QuikChange lightning.

- Copy our unmodified vector sequence (retro-icas9) into section 3, and upload sequence in section 4.
- Insert an A at the correct position of the vector.
- Design sense and anti-sense primers (primer 1 and 2).

Primers used for this experiment were as follows: 5'-ctggtgctttaattcctccggaaaaaaacttttcttaaacatca-3' as a sense primer and 5'-tgatgttttaagaaaagtttttccggaggaaattaaagcaaccag-3' as an anti-sense primer.

Materials:

QuikChange lightning site directed mutagenesis kit was produced by Agilent Technologies (Catalog no 210518) including 10X Reaction buffer, QuikSolution, QuikChange lightning enzyme, Dpn 1 enzyme, dNTP

Reaction components:

Component	50 ul reaction
10 X Reaction Buffer	5ul
dsDNA template	10-100ng
Oligonucleotide primer 1	125ng
Oligonucleotide primer 2	125ng
10nM dNTP	1ul
QuikSolution reagent	1.5ul
QuikChange Lightning Enzyme	0.25ul
Nuclease free water	to 50ul

All reaction components were set on ice and mixed properly by pipetting up and down followed by thermocycling using the following cycling parameters outlined as below:

Thermocycling steps were:

Step	Temperature	Time
Initial denaturation	95°C	2minutes
18 Cycles	95 °C	20 seconds
	60 °C	10seconds
	68 °C	30seconds/kb (1minute and 30 seconds) £
Final destination	68°C	5 minutes
Hold	4 °C	

£: Icas9 transgene is 2.2 Kb so the step was carried out for 1 minute and 30 seconds.

Digestion of the Amplification Products

2 µl of the provided Dpn I restriction enzyme was then directly added to each amplification reaction and thoroughly mixed by pipetting the solution up and down several times and then immediately incubated at 37°C for 5 minutes to digest the parental (i.e., the non-mutated) super-coiled dsDNA.

Setting a transformation reaction

The ligation reaction was then transformed into One Shot™ Stbl3™ (produced from Invitrogen, Thermo fisher scientific, England; Catalog number: C737303) as been described above. Next day, success of transformation was confirmed through presence of colonies.

Miniprep of picked up colonies from previous step

Colonies from the transformation plate (4 colonies) were picked up and a Miniprep was prepared using QIAprep Spin Miniprep Kit (catalog number:12123 and 12125) as been described above.

Sequencing of lentiviral plasmid with mutant icas9:

Minipreps with the suspected correct construct were sent for sequencing using primers that span the icas9 transgene. Primers used were designed using Snapgene software version 3 GSL Biotech LLC. Forward primers used were *atcccaccacatgccactct,cccaggacatcgtgtcctac*. Reverse primers used were *cactcttgggtccctccag* and *ccgccagattccagtttagaagc*. Checking the sequence for the insertion of A in icas9 transgene (c1,264insA).

Production of a Lentivirus with icas9 mutant and wild type

Following the institutional protocol, two 3rd generation lentivirus production was set using the plasmid with icas9 mutant (called Lenti-icas9 mutant) and icas9 wild (called Lenti-icas9 wild). Both viruses were produced in parallel using the same settings. For details on methodology, please review earlier sections.

2.13.3 Transduction efficacy of 293T cells transduced with Lenti-icas9 wild type and mutant

2.13.3.1 Assessment of the transduction efficiency using flowcytometry for CD19 expression:

1ml of 293 T was dispensed in 24 well plate at 1×10^5 of 293 T cells/well. The plate was left in a humidified incubator (5% CO₂) at 37°C overnight to allow 293 T to attach to plate walls and start to grow. 12 wells were prepared; 5 for each virus and another for untransduced 293 T cells. Next day, a viral stock from each virus (lenti-icas9 wild and lenti-icas9 mutant) was diluted as been described before. Serial volumes 10, 2, 0.4, 0.08, 0.016 were added to labelled wells. After 72 hours of adding the viruses, transduced 293 T were harvested as previously described. Half of the harvested cells was used to check transduction efficacy by staining for CD19+. The other half was plated in a labeled 24 well plate and the cells were passaged for another 4 days. After 1-week post-transduction, 293 T cells were harvested from the plate and genomic DNA was extracted in a mycoplasma free hood using DNeasy Blood & Tissue Kit; QIAGEN (catalog number 69504) as been delineated above. DNA yield was checked using Nanodrop. For each sample, DNA was diluted to 10ng/ul in DNase free water in preparation for vector copy number assessment using qPCR.

2.13.3.2 Assessment of the transduction efficiency using qPCR

Material

TaqMan DNA MasterMix produced by life Technologies; catalog number 4324018

Procedure:

A MasterMix for qPCR was prepared as shown:

Reagents	Initial concentration	Final concentration	Volume for 1 sample
TaqMan DNA MasterMix	2x	1x	12.5
HIV Fwd primer (psi)	10 mM	0.1 mM	0.25
HIV Rev primer	10 mM	0.1 mM	0.25
Albumin Fwd primer	10 mM	0.1 mM	0.25
Albumin Rev primer	10 mM	0.1 mM	0.25
Sterile H2O	NA	NA	1
DNA	10 ng/ul		10

Every sample was tested in triplicate to have a significative measurement. 15 ul of the mastermix was added to each well in addition to 10 ul of DNA. Standards were added from lowest to the highest to calibrate the expression in addition to cells with already measured 1 copy number (these were considered positive control). Sterile water was added into 3 wells (negative control). Extracted DNA from Lenti-icas9 wild and mutant (serial dilutions 10-0.016) were added to labelled wells. qPCR reaction was run for an hour 30 minutes in a BIO-RAD CFX96 real time machine. Data were imported into Excel sheet and vector copy number was measured as Starting Quantity Psi/ Starting quantity Albumin. Viral titre was calculated using the following equation:

Viral Titre: $(VCN * \text{Number of seeded cells}) / (\text{dilution}/1000)$

2.14 Test the effect of AP1903 on 293T transduced with Lenti-icas9 wild and Lenti-icas9 mutant

Based on results from VCN, 293T cells with high VCN were selected for the experiment- without sorting for CD19+ expression. 100 ul of cell suspension (50,000/well) was dispensed in triplets in a 96 well plate; these include untransduced cells; UT 293T (as a control), transduced 293T cells with Lenti- iCas9 wild 10, transduced 293T cells with Lenti-cas9 wild 2, transduced 293 T cells with Lenti-icas9 mutant 10 and transduced 293T cells with Lenti-icas9 mutant 2.

AP1903 was added in increasing concentrations (from 0-100 nM). The plate was then incubated for 24 hours in a humidified incubator (at 37°C and 5% CO₂). 10ul of CCK8 was added to the wells in the tested plate. The plate was then left for 4 hours in a humidified incubator (at 37°C and 5% CO₂). Absorbance at 450nm was measured using a microplate reader (FLUOstar OPTIMA) to assess cell viability upon exposure to AP1903.

**Chapter 3:
Results from clinical trial**

3.1 Summary of clinical results:

Seven patients were enrolled to the clinical trial. Two patients (P004, P008) have been excluded due to 1) the production of HLA antibodies against the donor (P004) and 2) poor production of BPX-501 (P008). P007 was recruited under a compassionate patient program due to a change in eligibility criteria (not to include JMML cases). Thus, in total, six patients received the genetically modified T cells (BPX-501) between D+21-±14 post-HSCT.

All patients had 100% engraftment at a median follow-up of 19 months of (range:5-36m). Two patients passed away. P003 passed away at 35 months post-transplant due to severe inflammatory reaction in response to disseminated atypical mycobacteria. P006 passed away at 5 months post-transplant due to gut bleeding on top of active aGvHD. Significant viral reactivation requiring prolonged courses of anti-virals was encountered in only 1 patient (P006). All patients developed Grade I-III aGvHD. However, Grade III was only recorded in one of them (P006). Three patients received one or two doses AP 1903 to switch off aGvHD; P002, P003, P006. P002 had achieved complete response, P003 had a partial response and P006 died out of uncontrolled aGvHD. P007 developed encephalopathy 19 days post- BPX-501. The condition was considered possibly related to BPX-501 infusion. He was treated with Dexamethasone and received 2 doses of AP1903 with complete resolution of the neurological symptoms with no sequel. TCR αβ cells appeared early post-transplant and by 2 months post-transplant, they predominated TCR γδ T cells. Median CD4 at 1- and 3-months post- transplant was 65 (0-240) and 375 (140-1340) cells/ul respectively. At 6 months post-HSCT, the median CD4 counts and CD8 counts were 290 (10-650) cells/ul and 520 (range: 0-740) cells/ul; respectively.

Three patients (out of 4 who were on immunoglobulin replacement at transplant) discontinued immunoglobulin replacement therapy; P001, P002 and P005. P001 and P002 had completed their vaccination schedule and had adequate response to Tetanus and Pneumococcal vaccine. Patients' characteristics and detailed description on immune recovery of individual cases were discussed in text and shown in table 3.1, figure 3.1, figure 3.2, and figure 3.3.

P002 and P007 had difficult to treat malignancies; relapsed non-Hodgkin lymphoma (NHL) of bone in the context of a combined immune defect) and juvenile myelomonocytic leukemia (JMML); respectively. P002 had completed 3 years post-

transplant with no evidence of recurrence. However, he developed secondary osteosarcoma of the femur –at the site of previous radiotherapy. He is now in complete remission after surgical above-knee amputation. At last follow-up of 8 months in our centre, P007 was disease free. Long-term follow-up assessments, in particular assessment of puberty have shown encouraging outcomes in 3 evaluable cases (above 10 years of age and >1-year post-HSCT). P001 had evidence of secondary sexual characters appearing at 3 months post-HSCT followed by her first menses at 6 months post-HSCT. P005 had pre-transplant ovarian dysfunction -based on laboratory testing, however Luteinizing hormone (LH) and estradiol normalised post-transplant and P005 had her first menses at 3 months post-HSCT. P002- a boy- had entered puberty 6 months post-transplant with normal assessment of puberty at 1-year post-HSCT.

3.2 Detailed description of patients who had a TCR $\alpha\beta$ /CD19 depleted transplant followed by (BPX-501) in the same center

P001

Background

P001 is currently 18 years old and completed 3 years post-transplant for IL10R B defect. She presented at the age of 2 years with Crohn's like disease (persistent diarrhea, rectal bleeding and perianal abscesses) that was refractory to medical treatment including high dose Prednisolone therapy. P001 went through multiple gut surgeries ending in pan-colectomy and ileostomy at the age of 9 years. However, she continued to have complicated courses with multiple attacks of gut bleeding, perianal abscesses and multiple fistulas. At the age of 15 years, she developed tonic clonic convulsions with MRI scans consistent with PRES syndrome. Extended donor search failed to find a matched donor. Thus, she was recruited to BP004 clinical trial and had a conditioned Treosulphan 42mg/m² (Treo)/Fludarabine 150mg/m² (Flu)/Thiotepa 10mg/Kg (TT)/rATG 15mg/Kg TCR $\alpha\beta$ /CD19haploidentical (father) transplant on 3/09/2015. BPX-501 was infused on 17/09/2015.

Post-transplant complications:

Her transplant was complicated with CMV viraemia at D+32 (maximum viral load: 3134 copies/ml) that spontaneously resolved without medical intervention at D+119. On D+40, P001 developed skin aGvHD stage 1-2 D+40 that completely resolved by D+80 on topical Steroids and Tacrolimus therapy. P001 had severe osteopenia

reported pre-HSCT with a z score of -4.5 on dual energy X-ray absorptiometry (DEXA) scan. Zolindronic acid was delayed until 6 m post-HSCT so not to interfere with the graft. She then received a monthly dose of Zolindronic acid (in total 2 doses) and follow-up DEXA scan showed a normalised z score for body weight.

Engraftment and immune reconstitution:

P001 had achieved neutrophil and platelet engraftments by at D+16 and D+8, respectively. At 3-year post-transplant, P001 remained 100% donor engrafted. CD3+CD19+ cells started to increase steadily from 1m post-HSCT; rising from 0.65 cells/ μ l to 59 cells/ μ l at 2m and remained 50 cells/ μ l at 30m post-HSCT (Figure). Coinciding with adoptive T cell expansion, endogenous TCR $\alpha\beta$ +CD19- T cells started to rise from 100 cells/ μ l at 1m post-transplant reaching 310 cells/ μ l and 1000 cells/ μ l at 2m and 6 m post-transplant respectively. CD4 cells >300 cells/ μ l and B cells > 200 cells/ μ l were achieved at 5- and 4-months post-transplant; respectively (Figure 3.1, Figure 3.2).

Naïve T cells appeared at 3 months post-HSCT and by 6m, naïve CD4 reached 400 cells/ μ l and naïve CD8 cells were 150 cells/ μ l (Figure 3.1). T-cell receptor excision circles (TRECS) appeared at 3m post-HSCT (1690) and reached 12673 at 9m post-transplant. P001 was immunoglobulin independent at 9m post-transplant with adequate vaccine response to tetanus (>0.4 IU/ml) and pneumococcal vaccine (12/13).

Long term follow-up:

P001 had her first menses at 6 m post-transplant and since then her menses were regular. Thyroid function tests and LH, FSH and estradiol were normal at 1-year follow-up. Pulmonary function tests and Echo were recorded as normal for age at 6 m post-transplant. Repeated follow-up endoscopies were normal apart from the persistence of a large recto-vaginal fistula and some diversionary type colitis (sequel of her original disease). No surgical intervention was taken due to 1) lack of clinical symptoms 2) high risk operation.

P002

Background

P002 is currently 18 years old and 3 years post-transplant for an unidentified combined immune defect and refractory non-Hodgkin lymphoma (NHL) of the femur.

P002 presented at the age of 5m with repeated chest, eye, and urinary tract infections requiring IV antibiotic therapy. At the age of 5 years, he had widespread Molluscum contagiosum and was failing to thrive. His investigations revealed normal CD3 and normal PHA but slightly low CD4 and low naïve CD4 cells for age. He had evidence of active inflammation with high IgG up to 35 mg/gl and high $\gamma\delta$ T cells constituting 26% of all CD3. He was thus considered as a case of combined immune deficiency. At the age of 7 years, he developed low grade EBV viraemia (500-34,000 copies/ml) together with severe gastritis (biopsy showing EBV driven lymphoproliferation in the upper gut) for which he received 4 doses of Rituximab with improvement in GI symptoms and resolution of viraemia. At the age of 13 years, he developed Enterobacteriaceae driven right humerus osteomyelitis that responded to IV antibiotics and was discharged from hospital. He was readmitted 2 month later with a significant amount of weight and severe leg pain. Femoral biopsy confirmed a diffuse large B cell lymphoma (strongly CD20 and EBER weakly positive). A technetium bone scan showed abnormalities corresponding to the femoral lesion but no other bone lesions. P002 received GRAB chemotherapy with Rituximab. He tolerated chemotherapy well with no significant infections. One week after completion of chemotherapy, his symptoms recurred with MRI showing tumor recurrence. He thus completed a course of Rituximab / Cyclophosphamide/Doxorubicin Hydrochloride / Vincristine (other name: Oncovin) /Prednisolone (R-CHOP) and 3-week course of radiotherapy on his femur. A repeat MRI/bone biopsy showed fibrotic changes with no evidence of malignancy. With a need to proceed to an urgent transplant, this patient was given a conditioned Treosulphan 42mg/m² (Treo)/Fludarabine 150mg/m² (Flu)/Thiotepa 10mg/Kg (TT)/ rATG 15mg/kg TCR $\alpha\beta$ /CD19 haploidentical (father) transplant on 09/02/2016. BPX-501 was infused on 28/02/2016.

Post-HSCT complications:

The transplant course was complicated with low-grade CMV viraemia since D+20 (500 copies/ml) reaching to a maximum of 42,586 copies/ml at D+40. Due to rapid immune recovery, P002 was only given 2 doses of Ganciclovir and 2 doses of Valganciclovir. CMV viraemia resolved at D+76. He had another CMV reactivation at D+120 reaching 17,247 copies/ml that resolved spontaneously in 1-week time. No further viral reactivations were recorded (Figure 3.6).

P002 developed acute skin and upper gut GvHD that required the use of switch off medicine; AP1903. Details on aGvHD and use of AP1903 are discussed in section 3.3.

At 11 m post-transplant, P002 developed a fracture in left femur at the site of previous tumor. Bone biopsy confirmed absence of malignant tissue. DEXA scan and Vitamin D levels were normal. Orthopedic team recorded fragile bone during operative correction. He had a metallic nail inserted. A couple of months later, he developed a fracture in his left humerus upon minor trauma requiring casting. To date, both fractures are slowly healing as been recorded in follow-up X-rays. PID BRIDGE research study showed that P001 was homozygous for COL1A1 c.2167G>A p. (Ala723Thr). Segregation analysis showed that his father, mother and elder sister are heterozygous for the same mutation. This is a novel pathogenic mutation in type I collagen –based on clinical picture and segregation analysis- responsible for osteogenesis imperfecta.

Engraftment and immune reconstitution:

P002 had achieved neutrophil and platelet engraftments at D+18 and D+8; respectively. At 3 years post-transplant, P002 remained 100% donor engrafted. Endogenous TCR $\alpha\beta$ T cells started to rise from 330 cells/ul at D+30 reaching a stable level ≥ 1000 cells/ μ l at 6 months post-HSCT. P002 achieved CD4 cells >300 cells/ μ l and B cells >200 cells/ μ l at 3 m post-transplant. Again, naïve T cells were detected at 3m post-HSCT and by 6 m naïve CD4 were 260 cells/ μ l and naïve CD8 cells were 120 cells/ μ l (Figure 3.1 and Figure 3.2). TRECS were 1593 and 8780 at 3 and 12-month post-transplant.

At 6-month post-transplant, P002 was immunoglobulin independent and had adequate vaccine response to tetanus (0.32 IU/ml) and pneumococcal vaccine (10/13). Molecular spectratyping showed all V beta families but only few having a Gaussian distribution at 3-month, 6-month, 1-year and 2-year post-transplant.

Long term follow-up:

At the age of 16 years, his endocrine assessment showed normal thyroid function and normal LH and testosterone hormone. His puberty was staged at P4/5, G4/5 with 10mls testis and apparent facial hair. Pulmonary function tests and echocardiogram (ECHO) were recorded as normal for age at 6 m post-transplant.

Unfortunately, at the age of 18 years, P002 developed high grade osteosarcoma of the left knee- site of previous radiotherapy and previous fracture. PET scan of the whole body and CT chest and abdomen revealed no evidence of metastasis. His condition was treated with above knee amputation with a safety margin. He is

currently alive and well. There are 10 cases reported in the literature of patients with osteogenesis imperfecta developing osteosarcoma (Jewell 1940, Klenerman et al, 1967, Lasson et al, 1978, Reid et al, 1979, Rutkowski et al, 1979, Gagliardi et al, 1995, Bedi et al, 1999, Maiya et al, 2002, Takahashi et al, 2004). The combination of the underlying genetic diagnosis-osteogenesis imperfecta and previous radiotherapy to the knee has potentially contributed to the development of bone tumor.

P003:

Background and post-HSCT complications:

P003 was 4 years old, 2 years and 11 months post-transplant at the time of his death. He presented at the age of 10 months with a picture consistent with Hemophagocytic lymphohistiocytosis (HLH) including fever, pancytopenia, and hepatosplenomegaly with low fibrinogen, raised ferritin and high triglycerides. Viral PCRs for EBV, CMV and adenovirus were negative. Leishmania was negative by PCR. Perforin expression was abnormal on NK cells and subsequently, he was found to have a compound heterozygous mutation in c.50del and c.46C>T. MRI brain was normal. He was started on HLH-94 with good response and normalisation of hepatosplenomegaly and blood abnormalities. Pre-transplant abdominal ultrasound and CT showed 2 hypoechoic cystic lesions in the liver of uncertain significance. P003 had pre-transplant enteropathy with growth flattening. This enteropathy was sought to be Noro II related. Extended donor search failed to find a matched donor. Thus, he was recruited to BP004 clinical trial and had a conditioned Treosulphan 42mg/m² (Treo)/Fludarabine 150mg/m² (Flu)/Thiotepa 10mg/Kg (TT)/rATG 15mg/Kg TCR αβ/CD19 haploidentical (father) transplant on 16/03/2016. BPX-501 was infused on 30/03/2016.

Post-HSCT complications

P003 had a quite complicated post-HSCT course with difficult to treat aGvHD, persistent Noro II enteropathy, steroid induced fractures, thrombotic microangiopathy (TMA), multiple liver nodules, acute pancreatitis (prolonged TPN therapy) and late onset infections. P003 developed aGvHD grade III; skin and gut that was steroid dependent. On D+156, he was administered a dose of AP1903 in an attempt to control aGvHD and allow steroid tapering. However, his enteropathy was an ongoing issue and he remained on steroid therapy till his death at 35 months post-transplant. Details on aGvHD and the use of AP1903 are discussed in section 3.3.

Due to prolonged steroid therapy and subsequently poor immune system post-transplant, P003 had persistent Noro II virus in stool at a CT of 16. Due to prolonged steroid therapy, P003 developed severe osteopenia with T7 vertebral body fracture. Thus, was started on Zolindronic acid therapy at 14 months post-transplant.

At 9 months post-transplant, P003 developed persistent uncontrolled refractory hypertension. Laboratory assessment showed albuminuria with an albumin creatinine ratio of 184 and thrombocytopenia. Blood film showed presence of fragment cells. Direct antiglobulin was negative and ADAMT13 was normal together with raised terminal complement complex of 271 ng/ml. Thus, the diagnosis of TMA was confirmed. Eculizumab was started immediately on weekly basis for 1 month then every 2 weeks for another month. P003 showed good response with resolution of thrombocytopenia, albuminuria and controlled blood pressure on anti-hypertensive medications. Eculizumab was spaced and discontinued at 4 months from the start of TMA. TMA was considered unrelated to BPX-501 and AP1903; first as it occurred more than 8 months after both investigational therapies and second because it was considered to be a vascular instability reflecting ongoing inflammation from difficult to treat aGvHD or disease related enteropathy.

Due to prolonged TPN and possible steroid therapy, P003 developed acute pancreatitis at 20 months post-transplant with severe abdominal distension leading to a respiratory compromise and thus the need for PICU admission mechanical ventilation for 4 days. Lipids were dropped from TPN. In 2 months' time, he recovered with no sequel.

Since early post-transplant (D+35), P003 developed mild transaminitis; ALT: 50-100 IU/L with normal bilirubin and normal GGT. At 6 m post-transplant, P003 started to show evidence of moderate transaminitis with ALT reaching 143 IU/L with raised GGT reaching 701 IU/L but still normal bilirubin. Liver US showed multiple hepatic nodules. Liver biopsy showed extramedullary hematopoiesis and foamy macrophage. Changes were thought to present regenerative nodules due to prolonged steroid therapy. After a period of resolution of raised GGT, it crept up again at 1-year post-transplant reaching 9388 IU/ml again with normal bilirubin, moderately raised ALT of 367 IU/ml, normal bilirubin, normal Prothrombin (PT), normal Partial thromboplastin time (PTT) with slightly low fibrinogen. Magnetic resonance imaging (MRI) and US abdomen showed no changes in the number or appearance of hepatic nodules. α -feto proteins were repeatedly negative. Low fibrinogen was a sign of ongoing inflammation rather than a sign of liver cell impairment. Repeat liver biopsy showed

no evidence of cGvHD, however possible drug related changes. At 2 years post-transplant, ALT was 61 IU/L, GGT: 304 IU/L with normal bilirubin and albumin of 31 mg/dl.

At 2 years post-transplant, P003 developed nodular skin lesions attached to the skin surface and showing signs of inflamed skin. P003 was not given BCG vaccine as a neonate. Viral PCRs for EBV, CMV and Adeno were negative. Fungal scrapping was negative. Histopathology and culture confirmed the presences of aggregated histiocytes with positive Ziel-Neelsen stain for Acid fast bacilli. PCR confirmed the presence of Mycobacteria Chelonae for which he was treated with Meropenem/Amikacin/Clarithromycin therapy. Prednisolone therapy was gradually tapered to 0.1mg/kg. Unfortunately, P003 had a severe inflammatory response syndrome (IRIS) in response to disseminated atypical mycobacterial disease. Blood cultures and bronchoalveolar lavage confirmed the presence of Mycobacteria Chelonae. CT scans of chest and abdomen recorded diffuse pulmonary infiltrates and multiple liver lesions. During this time, CD3+ T cells had significant expansion reaching 440 cells/ul; only 0.8% of the T cells were CD3+CD19+ T cells. P003 was admitted to PICU on mechanical ventilation. He initially responded to pulsed methyl prednisolone therapy, however, his condition deteriorated quickly, and he was put into high frequency oscillation ventilation. An attempt of anakinra was tried for a week with no response. He was on high frequency oscillation ventilation for 10 days and continuous veno-venous hemofiltration (CVVH) for renal shut down. He peacefully passed away at 2 years and 11 months post-transplant. Parents declined post-mortem analysis.

Another reported complication was Fanconi-renal tubular defect that was noted since 1-year post-transplant and possibly related to Acyclovir and Cyclosporine A therapies.

Engraftment and immune reconstitution:

P003 had achieved neutrophil engraftment and platelet engraftment by at D+19 and D+8, respectively. Patient remained 100% engraftment at 2 years post-transplant. Due to early and prolonged steroid therapy in addition to other immunosuppressive medications, P003 remained immune compromised. His total CD3 went from 250 cells/ μ l at D+30 to levels between 30-50 cells/ μ l at 1 and 2-year post-transplant (Figure 3.3).

P005

Background

P005 is currently 14 years old and 8 months post-transplant for PI3K R1 defect. She presented at the age of 18m with repeated chest infections and failure to thrive. Computerized Tomography (CT) scan of the chest showed widespread bronchiectasis. She had previous BAL positive for adenovirus and H1N1. At the age of 13 years, she had EBV driven Lymphoproliferative disease with huge lymphadenopathy and hepatosplenomegaly treated with Rituximab in conjunction with a short course of steroid therapy. She also developed CMV parotitis. She was diagnosed to have an activating PI3KR1 mutation; c.1425+1G>T (APDS 2). She was maintained on Sirolimus as a bridge therapy to transplant. Pre-transplant pulmonary function test showed FEV1 to be 66.7% predicted of normal and FVC of 64% predicted of normal. She was also known to have a β thalassemia trait. Pre-transplant, P005 had an endocrine review as mother was anxious about a possible delay in puberty. Her endocrine bloods showed very high LH. Extended donor search failed to find a matched donor. Thus, she was recruited to BP004 clinical trial and had a conditioned Treosulphan 42mg/m² (Treo)/Fludarabine 150mg/m² (Flu)/Thiotepa 10mg/Kg (TT)/rATG 15mg/Kg TCR $\alpha\beta$ /CD19 haploidentical (father) transplant on 25/08/2017. BPX-501 was infused on 07/09/2017.

Post-HSCT complications

Transplant course was complicated with CMV reactivation and late onset aGvHD. P005 had low level CMV viraemia (maximum: 7190 copies/ml) at D+20 that was treated with IV Ganciclovir due to concerns of disease development based on pre-transplant history. Viraemia resolved with no sequel by D+40. Interestingly, she did not develop EBV reactivation post-transplant (Figure 3.6).

P005 developed late onset stage1 aGvHD at D+111 post-HSCT. Appearance of aGvHD coincided with a noted rise in CD3+CD19+ cells at 3 months from 98 cells/ul to 130 cells/ul. aGvHD completely responded to topical therapy with no need for AP1903 infusion.

Engraftment and immune reconstitution:

P005 had achieved neutrophil and platelet engraftments by at D+16 and D+7, respectively. Patient remained 100% engraftment at last follow-up; 8 months post-transplant. CD3+CD19+ cells started to increase steadily from 6 cells/ μ l at D+30 to 98 cells/ μ l at D+60. Between 3-6 months post-HSCT, CD3+CD19+ cells were ranging between 130-140 cells/ μ l. Coinciding with adoptive T cell expansion, endogenous TCR $\alpha\beta$ T cells started to rise from 760cells/ μ l at D+30 reaching 1000 cells/ μ l at 7 months post-transplant. P005 had stable CD4 cells of 260 cells/ μ l for 4 months post-transplant. B cells were stable at 110-130 cells/ μ l. Naïve CD4 appeared at 2 months post-transplant –recorded as 30 cells/ μ l and rose reaching 110 cells/ μ l at 6 months post-transplant (Figure 3.1, Figure 3.2). P005 discontinued immunoglobulin replacement therapy at 7 m post-transplant and returned to her home country with a plan to start vaccination in 3 months' time.

Long term follow-up:

By 3 months, LH was normalised from being 24.8 International Unit (IU)/L (0.4-4.6) pre-transplant to 2.7 IU/L. P005 had her menses at 3 months post-transplant. A repeat PFT was done at 3- and 7-months post-transplant with a stable FEV1 of 67% and FVC of 67.7% predicted of normal. Echo was normal at 6 months post-transplant.

P006

Background

P006 was 4 and half years old and 5 months post-transplant at time of his death. He presented at the age of 1 year with fever, easy bruising and pancytopenia when he was subsequently diagnosed as a case of standard risk acute lymphoblastic leukemia (ALL). He was treated with UKALL 2011. Whilst on maintenance 2 years later, he developed headaches and photophobia with investigations confirming isolated CNS relapse. Thus, he was treated with R3 protocol. At time of transplant he was in the second complete remission (CR2). P006 had previous supraventricular tachycardia and was known to have WPW syndrome to which he received 6m of Atenolol therapy. He had intermittent jaundice and was known to have Gilbert disease. P006 was noted to have developmental and speech delay as part of autistic spectrum disorder. Extended donor search failed to find a matched donor. Thus, she was recruited to BP004 clinical trial and had a conditioned TBI (14.4Gy)/Cyclophosphamide

120mg/kg/rATG 15mg/Kg TCR $\alpha\beta$ /CD19 haploidentical (father) transplant on 31/08/2017. BPX-501 was infused on 07/09/2017.

Post-HSCT complications:

Transplant course was complicated with CMV and AdV reactivations, aGvHD and death (from uncontrolled GvHD).

P006 had CMV viraemia on D+32 (44,493 copies/ml) that peaked reaching 90,024 on D+39. He was treated with Ganciclovir and Foscarnet therapy followed by Valganciclovir therapy. P006 had chronic adenoviral excretion in stool from D+3 post-HSCT till death. He had also intermittent Noro II in stool. He developed AdV viral reactivation on D+48 (13 days after BPX-501 infusion) at a titre <10, 000 cells/ul. He responded to 2 doses of Cidofovir and was subsequently shifted to oral Brincidofovir. AdV viraemia was cleared at D+90. P006 had multiple interruption of Brincidofovir therapy for many reasons- in particular when he developed severe diarrhea and there was concern that it was Brincidofovir related. While on steroid therapy for aGvHD, he had adV and CMV reactivation at D+105 and D+115 respectively. He thus received both Ganciclovir and Brincidofovir therapy. At time of death, P006 had no CMV viraemia but low AdV in blood (560 copies/ml).

P006 developed Grade III aGvHD; skin, upper gut and lower gut starting 10 days after BPX-501. P006 received 2 doses of AP1903 in an attempt to control aGvHD. However, despite initial response, his gut GvHD did not settle. Prednisolone therapy was commenced with complete remission. With the presence of CMV and AdV viraemia, multiple attempts were sought to taper steroid therapy. However, this was associated with a flare-up of GvHD of gut leading to his death from significant uncontrolled gut bleeding.

Details on GvHD and the use of AP1903 are discussed in section 3.3

Engraftment and immune reconstitution:

P006 had achieved neutrophil engraftment and platelet engraftment by at D+21 and D+8, respectively. Patient remained 100% engraftment at last follow-up; 5 months post-transplant. Endogenous TCR $\alpha\beta$ T cells started to rise from 10 cells/ μ l at D+60 reaching 310 cells/ μ l and 210 cells/ μ l at 3 months and 5 months (pre-death) post-HSCT; respectively. P006 had CD4 cells and CD8 cells of 190 cells/ μ l and 170 cells/ μ l at 3 months post-transplant. Naïve CD4 and naïve CD8 appeared at 3 months

post-transplant –recorded as 10 cells/ μ l and 40 cells/ μ l; respectively. B cells were not detected until death; figures 3.1 and 3.2.

P007

Background

P007 is currently 7 years old and has completed 1-year follow-up post-HSCT for juvenile myelomonocytic leukemia (JMML). At the age of 5 years, P007 developed generalised lymphadenopathy and hepatosplenomegaly together with a leukoerythroblastic blood film with nucleated red cells and monocytosis. HbF was noted to be high; 54%. A bone marrow confirmed the diagnosis of JML. Molecular genetics showed a c35G>A missense somatic mutation u K-ras. Meanwhile, P007 had EBV viraemia (64,000 copies/ml) coinciding with high fever and mouth ulcers. P007 was thus treated with 4 cycles of 5-Azacytidine together with four doses of Rituximab for his EBV viraemia. Subsequently, he underwent a TCR $\alpha\beta$ /CD19 depleted maternal haploidentical transplant with Treosulphan/Cyclophosphamide/Melphalan/ATG on the 03 August 2017. After achieving 60% donor engraftment, P007 subsequently developed immune mediated rejection of the graft on D+18 with 0% donor engraftment. He has persistent weak anti-HLA against the donor for which he received Bortezomib and plasmapheresis. P007 proceeded to a second haploidentical transplant (using father as the donor) with (Teltschik et al, 2016); Flu 120mg/m² /Cyc 120 mg/kg/TT 5mg/Kg / TBI 4Gy/ rATG 4.5mg/Kg on 13/09/2017. BPX-501 on a compassionate basis was administered on D+21.

Post-HSCT complications

Transplant course was complicated with CMV viraemia, engraftment syndrome, T cell mediated neurotoxicity, aGvHD and cGvHD.

P007 had CMV reactivation post-1st transplant for which he received 2 weeks of Ganciclovir therapy. At D0 he was noted to have low CMV viral load of 5738 copies/ml thus received 2 weeks of Foscarnet therapy. CMV viraemia was intermittent between D+30 and D+70; highest viral load was 1963 copies/ml.

P007 had evidence of T cell mediated neurotoxicity post-BPX-501 infusion. His condition completed resolved with no sequel with a combination therapy of steroids and 2 doses of AP1903. The details of this event are discussed in section 3.3.

P007 also developed Grade III skin, gut aGvHD that responded to steroid therapy. While on low dose steroid therapy, he developed skin -limited cGvHD. Details on aGvHD and cGvHD are discussed in section 3.3.

P007 had mild renal impairment with raised creatinine 53 umol/litre (23-37) and raised urea – this is partially related to poor oral intake of fluids and partly related to drug toxicity (Acyclovir prophylaxis).

Engraftment and immune reconstitution:

P007 had achieved neutrophil and platelet engraftments by at D+20 and D+11; respectively. Patient remained 100% engraftment at last follow-up; 8 months post-transplant. TCR $\alpha\beta$ T cells started to appear from 2 months post-HSCT 260 cells/ μ l and remained above 1000 cells/ μ l from 6 months post-HSCT. CD4 cells rose from 50 cells/ μ l at 2 months post-HSCT to 130 cells/ μ l and 290 cells/ μ l at 4 and 6 months post-HSCT; respectively. CD8 cells were stable at 210 cells/ μ l from 2nd to 5th month post-HSCT followed by a rise to 740 cells/ μ l at 6 months post-HSCT. B cells were stable at 110 cells/ μ l at 7 months post-HSCT. Naïve CD4 and naïve CD8 cells appeared at 6 months post-transplant –recorded as 10 cells/ μ l and 20 cells/ μ l; respectively (Figure 3.1, Figure 3.2). P007 had discontinued immunoglobulin replacement therapy at 4 m post-transplant. P007 has returned to his home country at 8 months post-HSCT with a plan to start vaccination at one-year post-HSCT.

Table 3.1. Characteristics of patients who were enrolled to receive BPX-501 post-TCR $\alpha\beta$ /CD19 depleted grafts

No	Diagnosis / age at presentation (m)	Gender/ Age at transplant in years	Significant Pre-transplant morbidities	Conditioning Donor source CD34+ dose (X10 ⁶ /Kg) TCR $\alpha\beta$ dose (10 ⁴ /Kg)	Time to BPX-501 infusion	Days to NT and PLT engraftment	GvHD (Grade)	Post-transplant complications	Outcome/ Last Follow Up (months)
P001	IL10RB defect/ 2m	F/ 15y	Pancolectomy and ileostomy Multiple fistulas Significant hearing loss	Treo/Flu/TT/rATG 15 Paternal 13.22 0.25	D+14	16/8	aGvHD skin1 None	Low grade CMV viraemia (spontaneous recovery) Persistent rectovaginal fistula	A/W Off Ig 36m

P002	CID/NHL/ 5m	M/15y	Repeated infections Upper gut ulcerations (? EBV PTLD) EBV driven NHL (chemotherapy and radiotherapy to Lt femur) Autistic spectrum disorder	Treo/Flu/TT/rATG 15 Paternal 13.95 0.22	D+14	18/8	aGvHD skin1, UG 1 None	Low grade CMV viraemia Repeated fractures (COL1A1 c.2167G>A p.(Ala723Thr) .	A/W Off Ig 36 m
P003	Perforin HLH/10m	M/3y and a half	HLH- (HLH 94) Flattered growth Chronic Noro II enteropathy	Treo/Flu/TT/rATG 15 Paternal 13.52 0.25	D+14	19/8	aGvHD skin3, possible LG1	Chronic enteropathy Liver nodules and elevated GGT	Deceased Severe inflammatory response due to disseminat

P005	PI3KR1 mutation; c.1425+1 G>T (APDS 2)	F/1.5y	Flattered growth Repeated chest infections Bronchiectasis EBV LPD CMV parotitis	Treo/Flu/TT/rATG 15 Maternal 13.3 1.11	D+18	16/7	Late onset aGvHD skin 1 None	Low grade CMV viraemia	A/W Off Ig 8 m.
P006	ALL CR2	M/1y	Gilbert disease WPW syndrome (Atenolol for 6m) Autistic spectrum disorder	TBI (14.4Gy)/Cyclophosphamide 120mg/kg/rATG 15 Paternal 24.06 0.14	D+35	21/8	aGvHD skin3/U G1 aGvHD LG2 None	CMV and adeno viraemia Pneumatosis intestinalis	Deceased GI bleeding; uncontrolled gut aGvHD

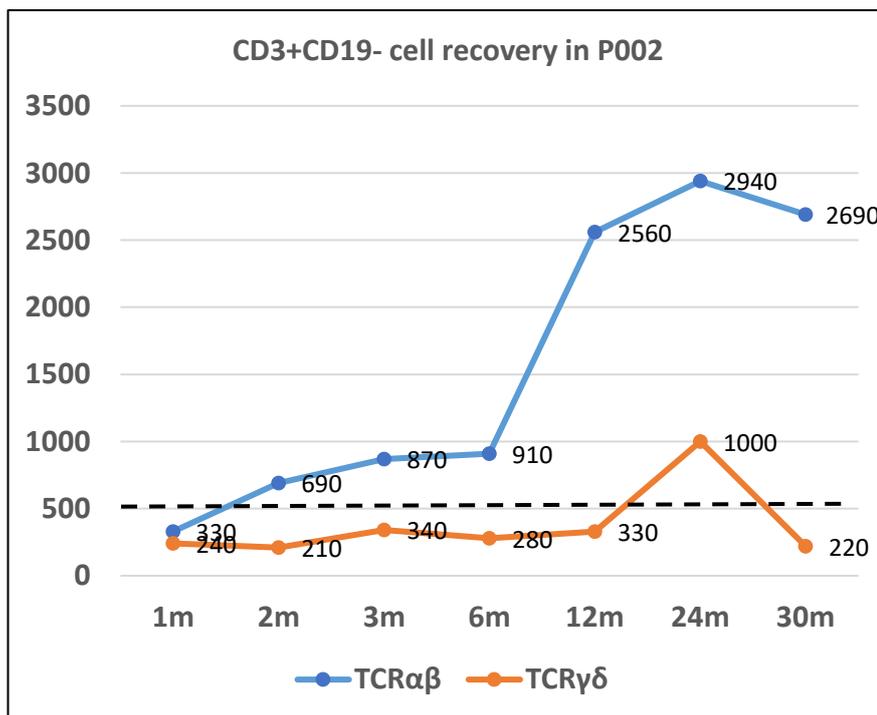
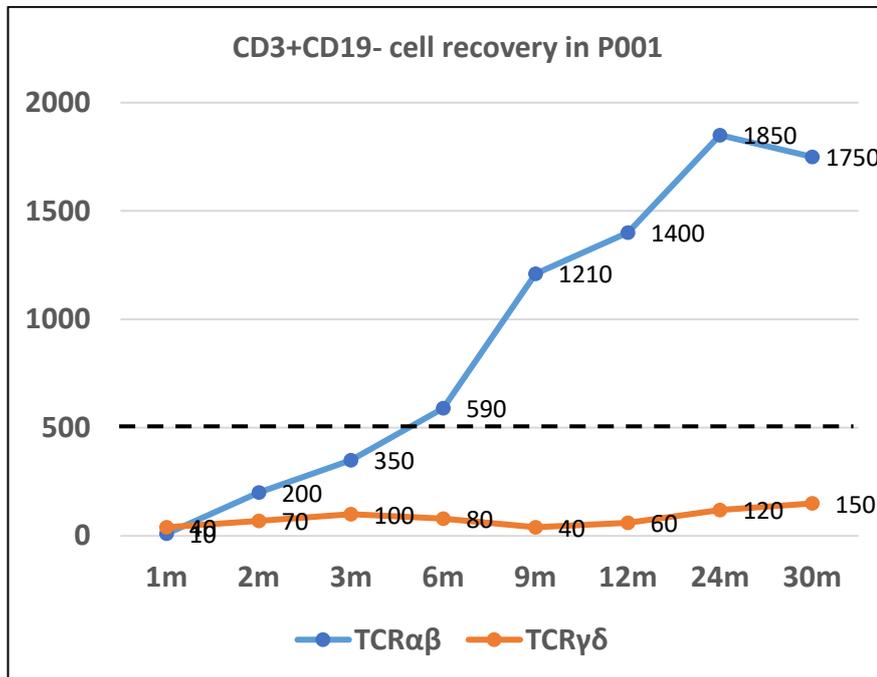
									5m
P007	JMML	M/5y	EBV viraemia (Rituximab) Previous Fungal chest nodules Failed TCR $\alpha\beta$ /CD19 depleted maternal haploidentical transplant (aplastic graft failure) CMV viraemia (Ganciclovir therapy)	Flu 120mg/m ² /Cyc 120mg/kg/4Gy TBI/TT 5mg/Kg/rATG 4.5mg/Kg Paternal 19.6 0.07	D+21	20/11	aGvHD skin 1, UG1, LG2 cGvHD- skin limited	Engraftment like syndrome T cell mediated encephalopat hy Low grade CMV viraemia	A/W 6m
P008	AML M7 CR1	F/6y	Previous PICU admission for	Treo/Flu/TT/rATG15 Paternal	She was later excluded from the study due				

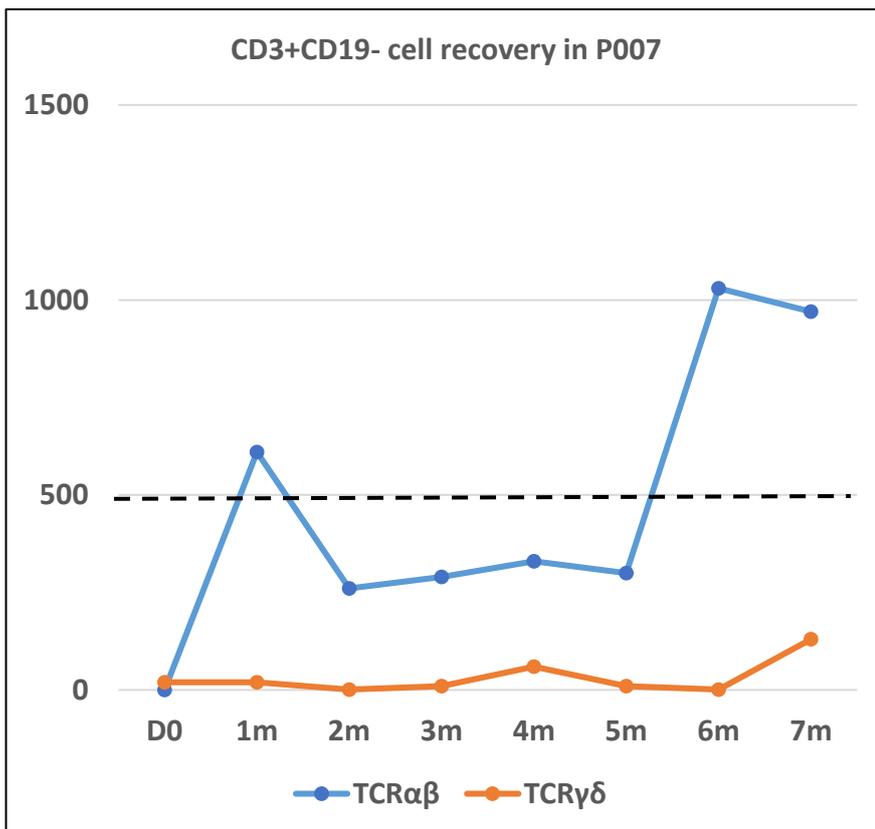
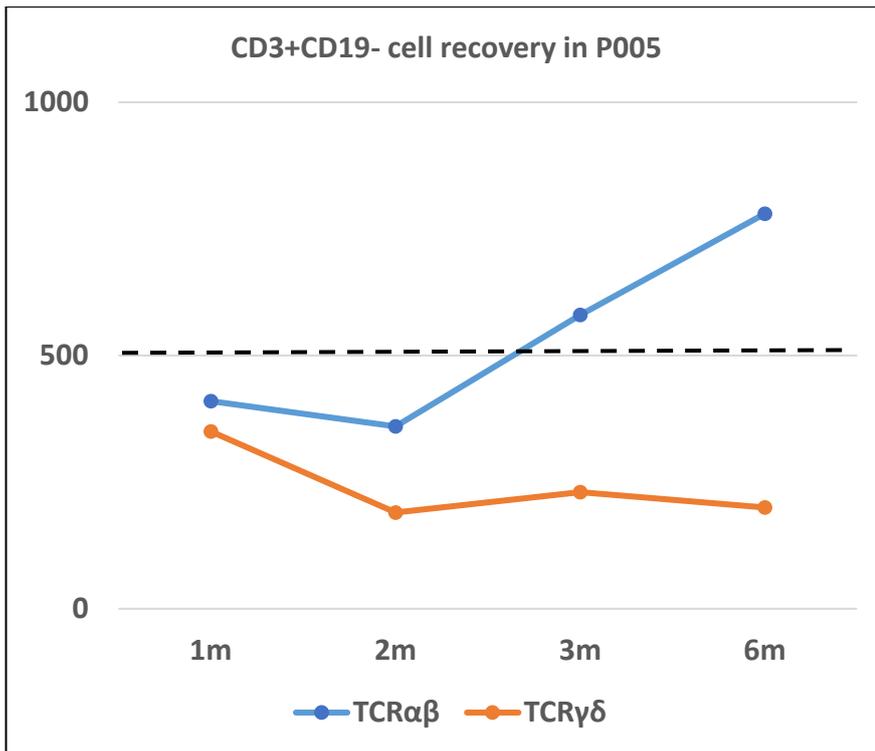
			infection/leukocytosis (MV +inotropes; 10 days) Inter-sphincteric perianal abscesses		Poor BPX-501 production with only 66% CD3+CD19+selection.
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Abbreviations: A/W: alive and well. ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, CR: complete remission, Treo: Treosulphan, Flu: Fludarabine, TT: Thiotepa, rATG: rabbit anti-thymocyte globulin, rATG 15: dose given was 15 mg/kg, TBI: total body irradiation, aGvHD: acute graft versus host disease, cGvHD: chronic versus host disease, HLH: hemophagocytic lymphohistiocytosis, M: male, F: female

Figure 3.1. Pattern of CD3 cell recovery post-HSCT among enrolled patients

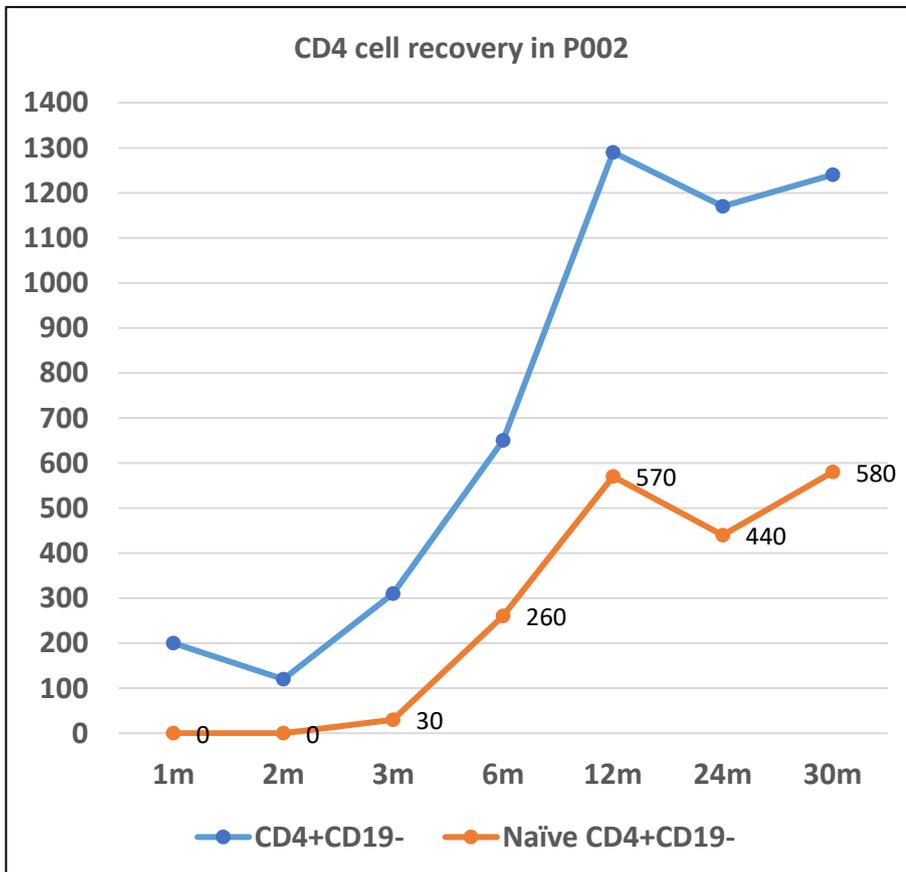
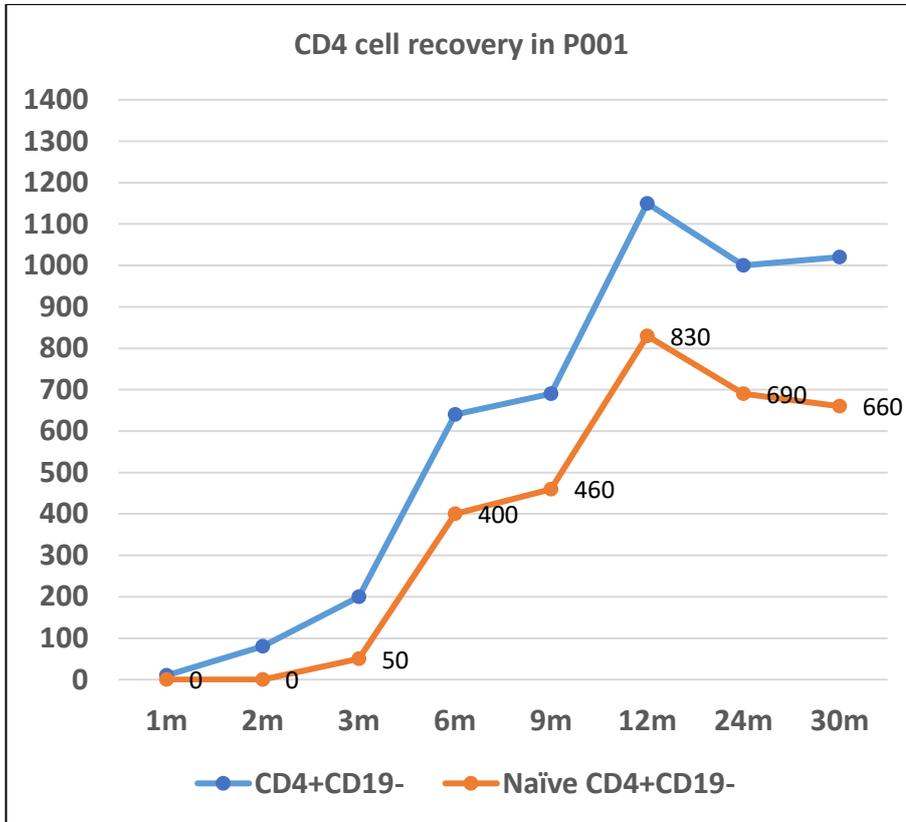
a) Early endogenous T cell recovery with appearance of TCR $\alpha\beta$ T cells

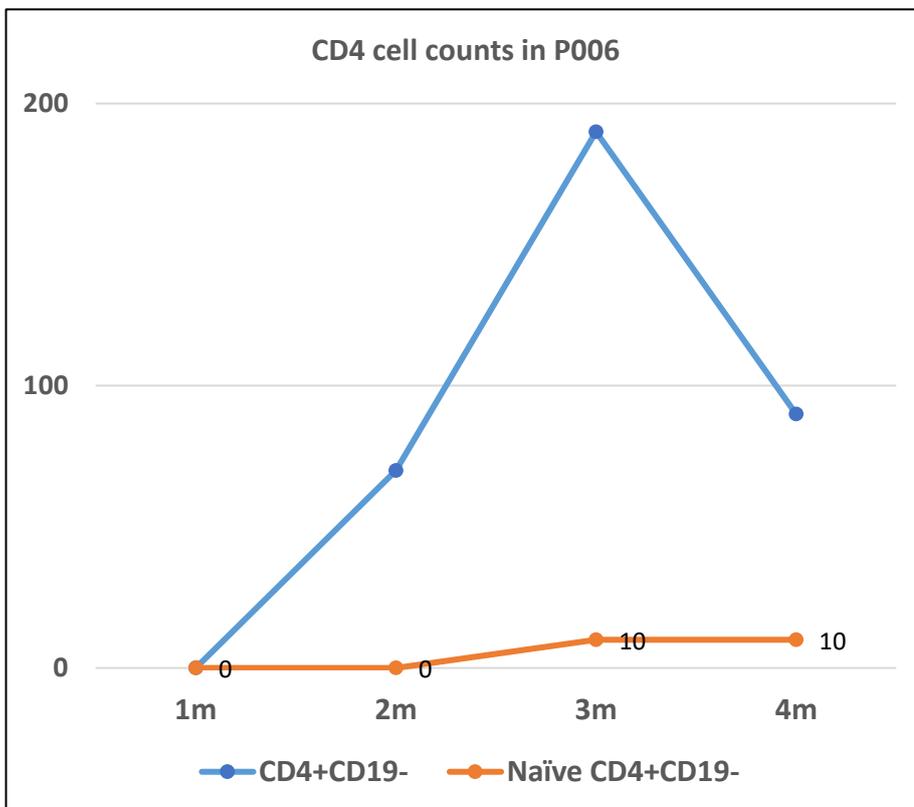
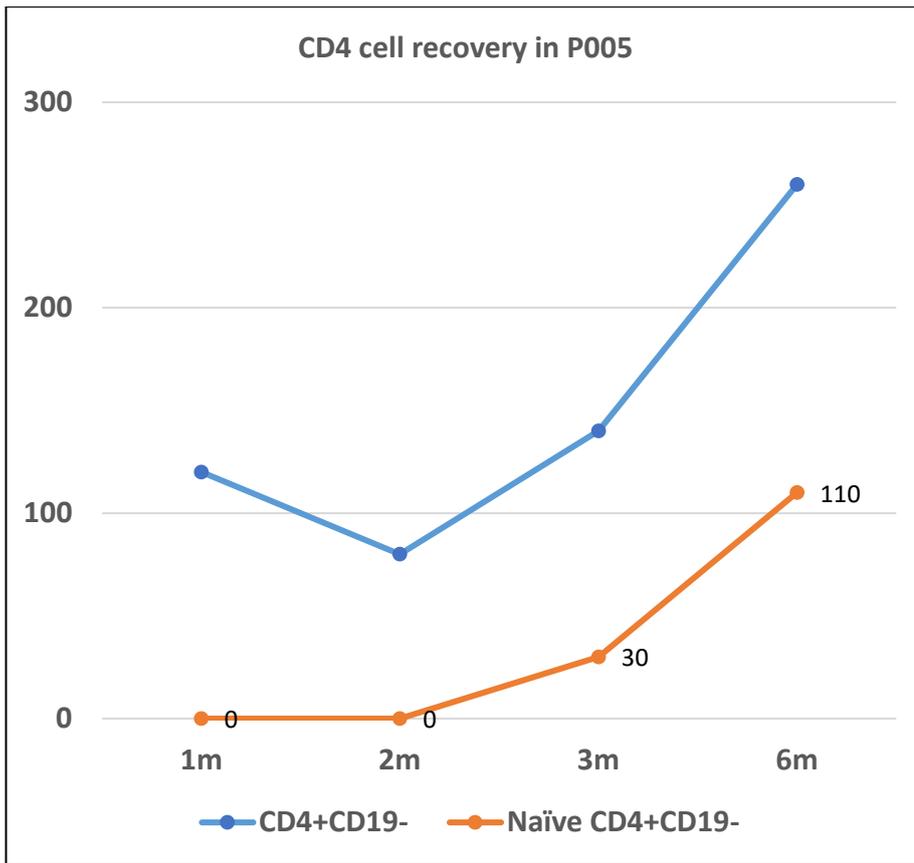


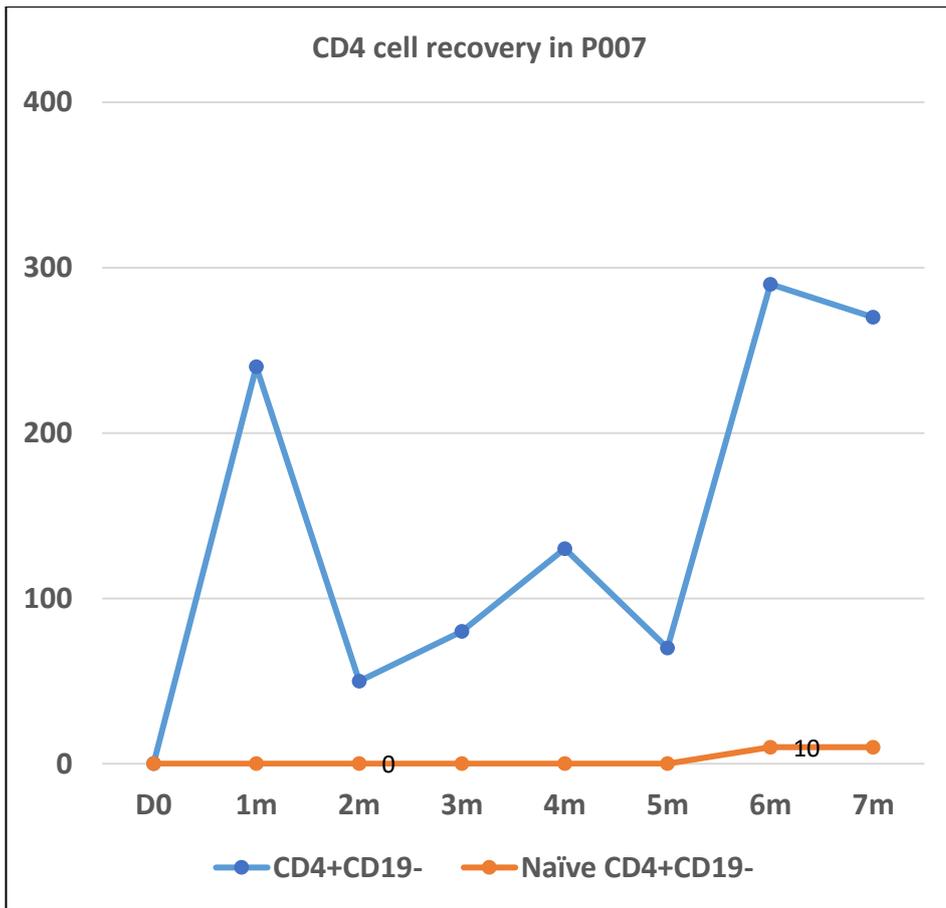


TCR $\alpha\beta$ T cell counts reached 500 cells/ul by 6 months post-HSCT

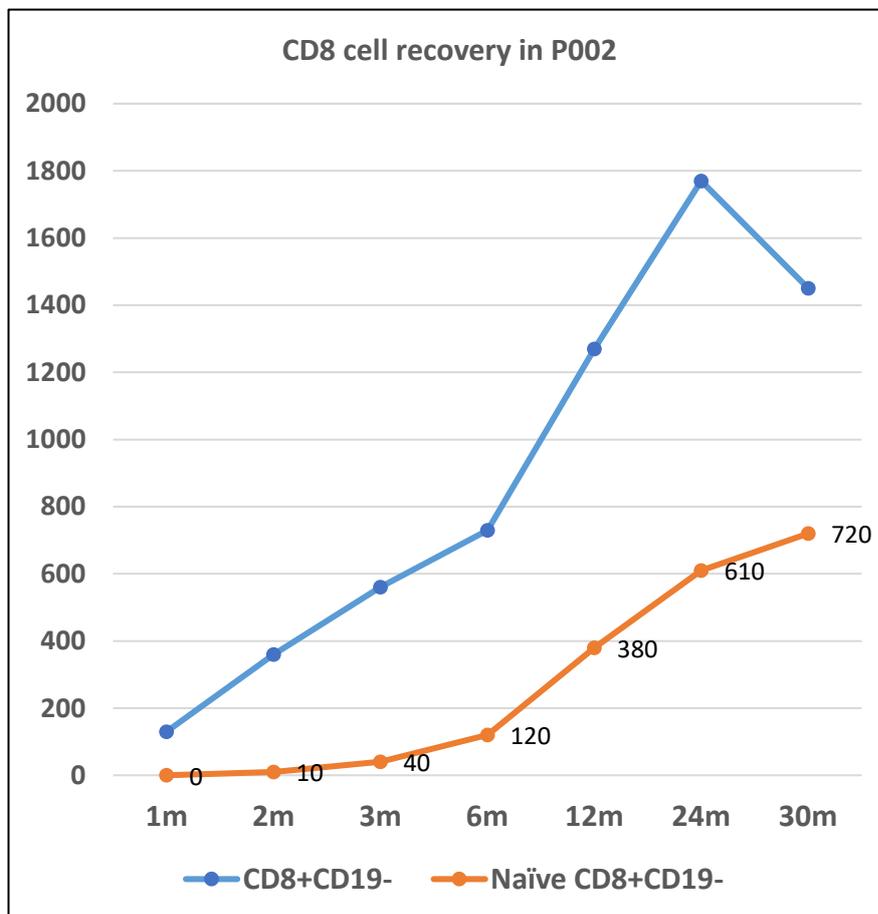
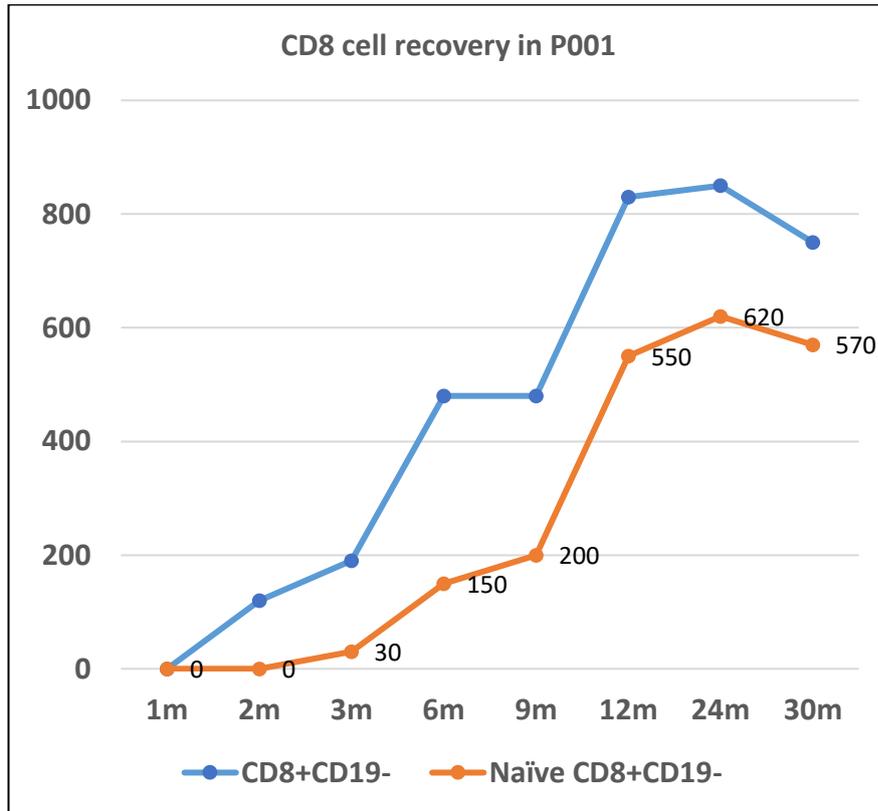
b) Appearance of CD4 naïve T cells from 3 months post-transplant denoting thymic immune recovery

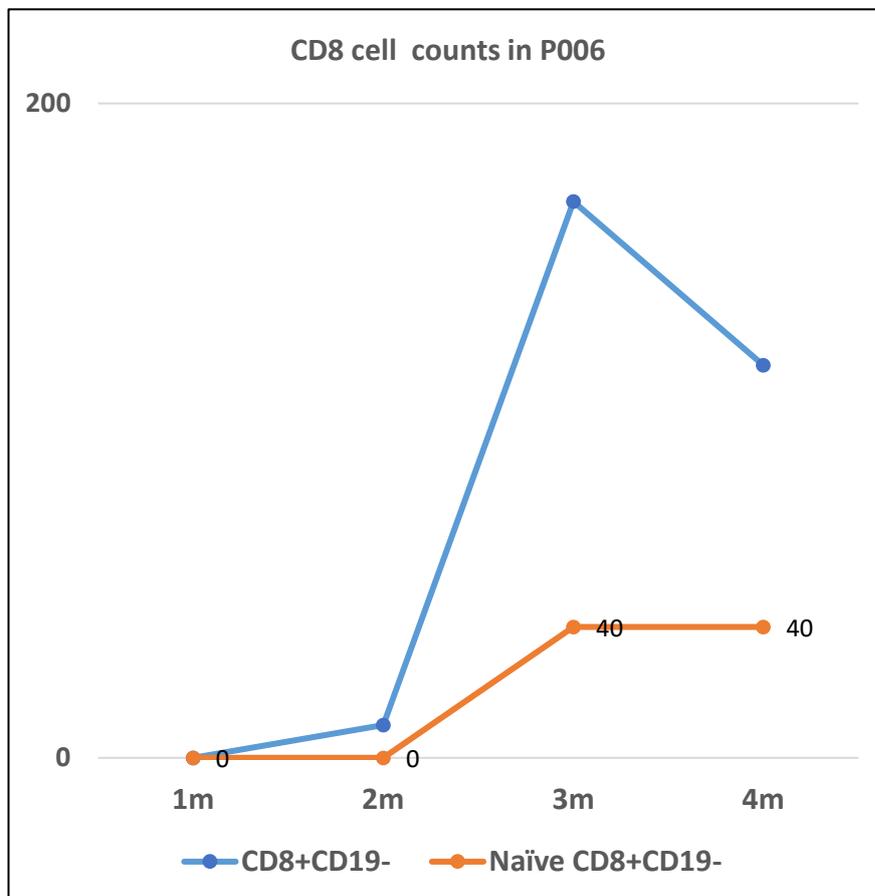
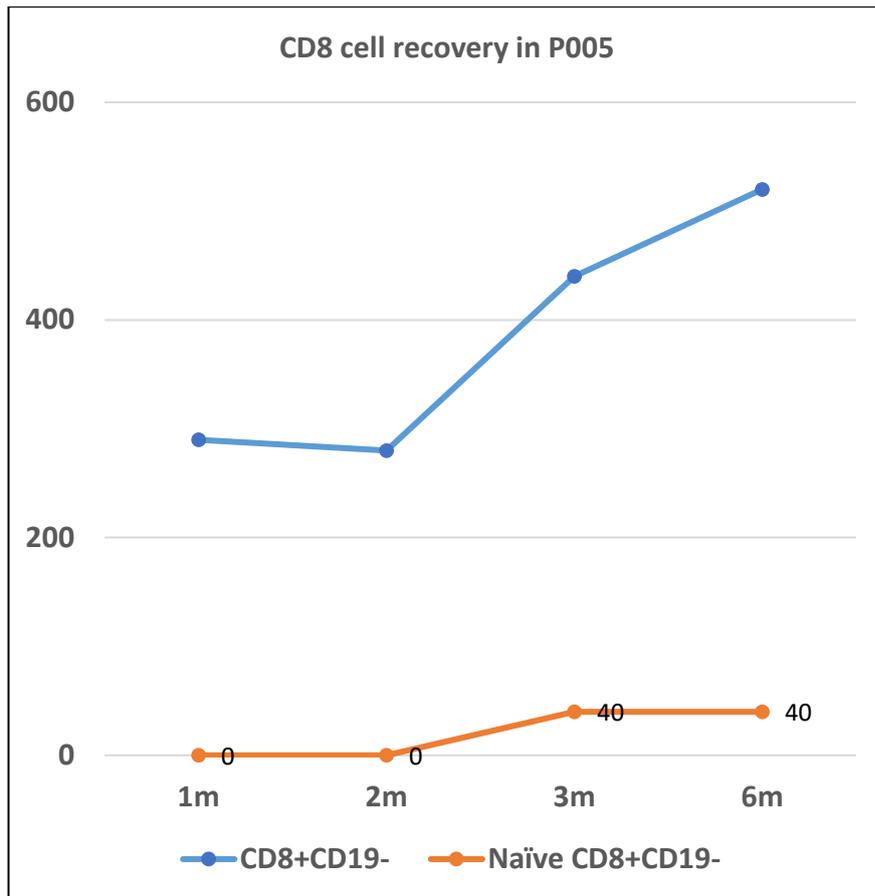


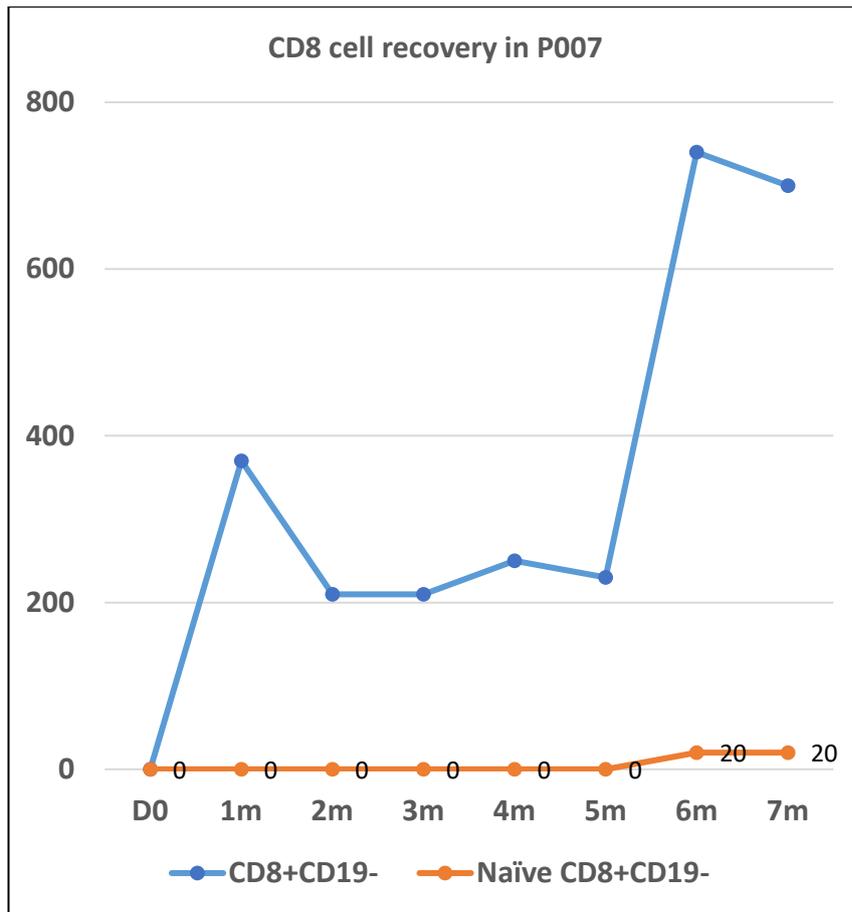




c) CD8 and CD8 naïve T cell counts recovery

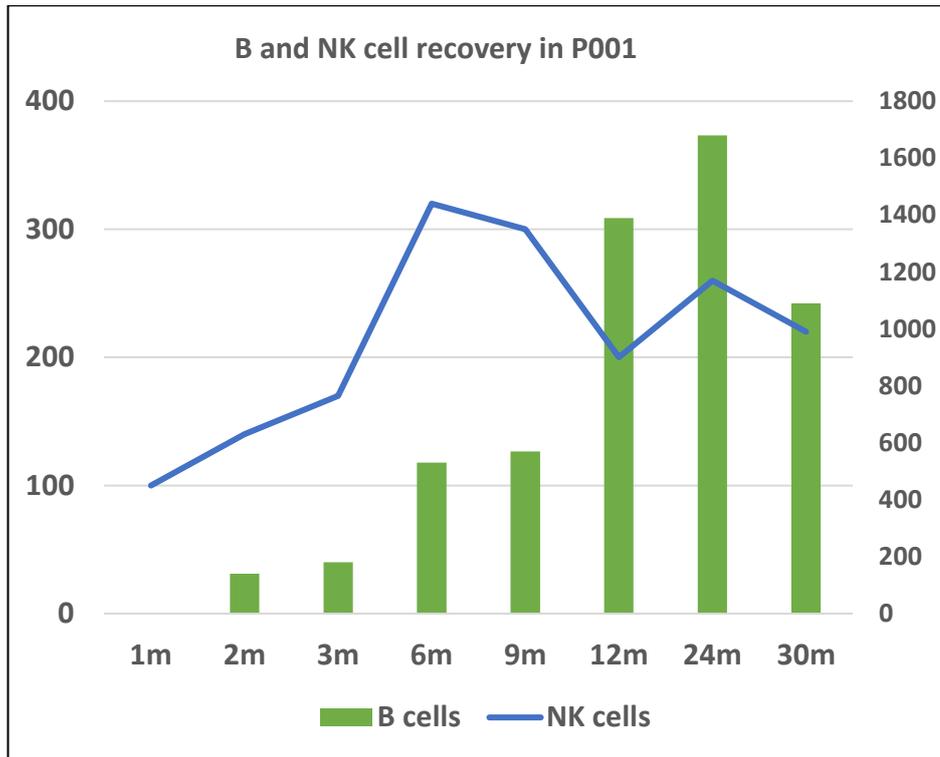


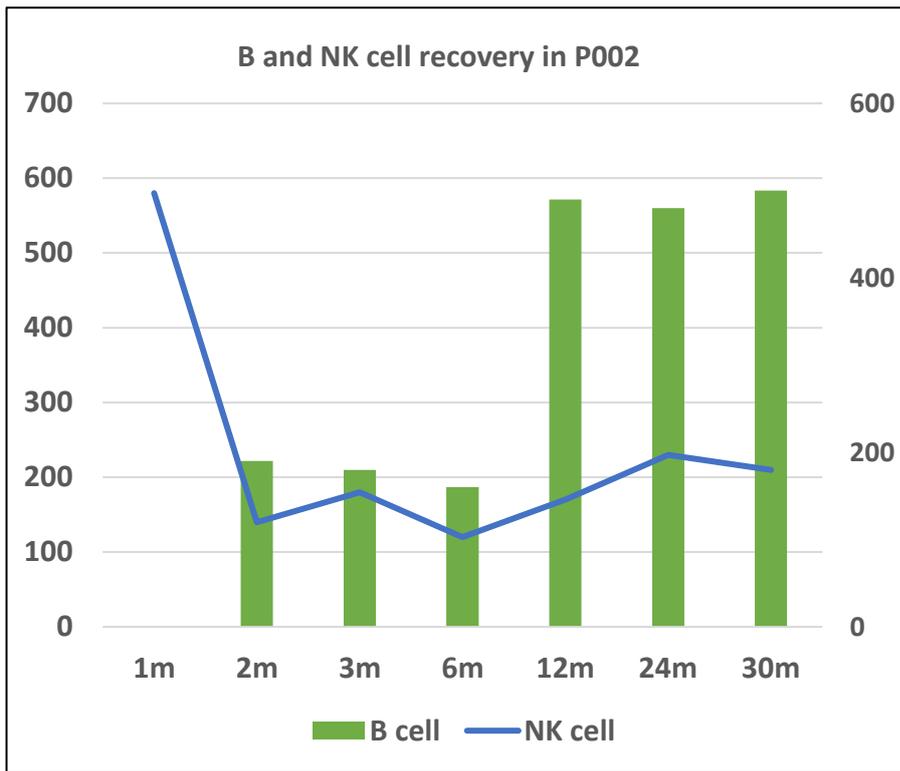
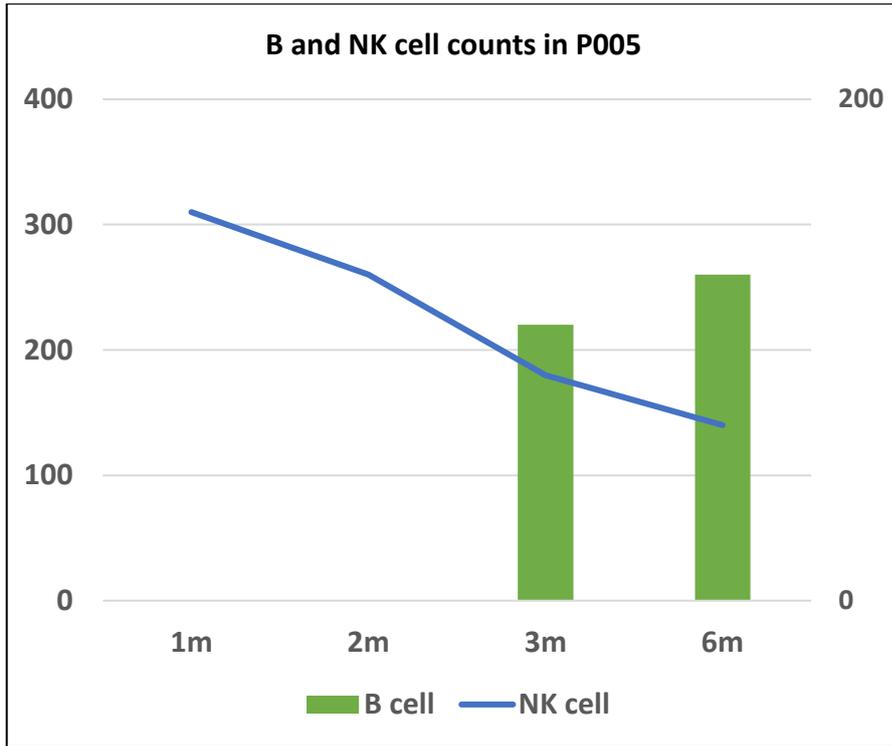


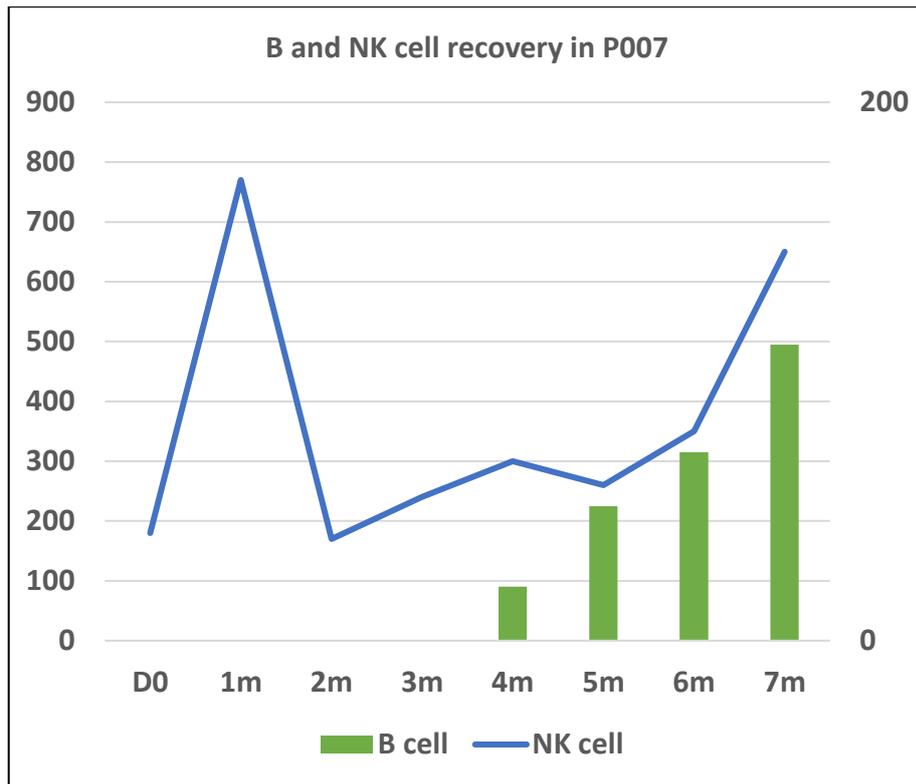


X-axis represents absolute counts (cells/ul) and y-axis represents time post-HSCT. Abbreviations m: months.

Figure 3.2. Pattern of B and NK cell recovery post-HSCT: Expansion of B cells and relative drop in NK cells at 2-3m post-HSCT



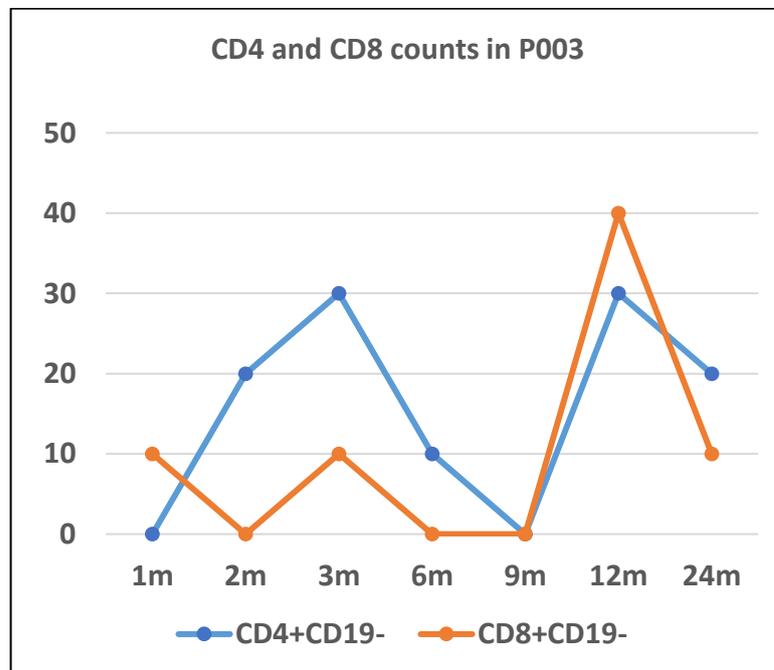
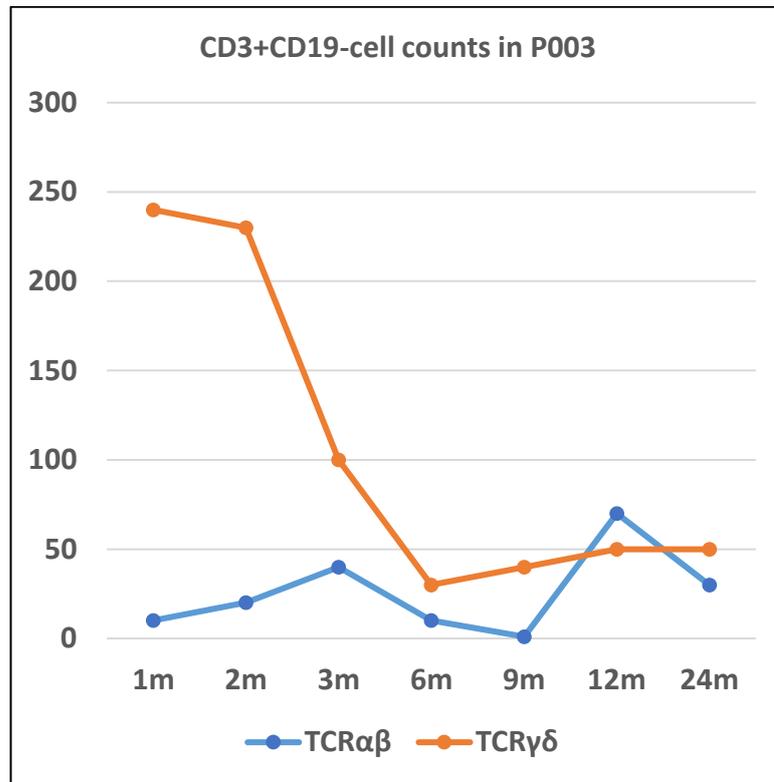




Primary Y-axis (to the left) represents absolute NK counts (cells/μ) and secondary Y-axis (to the right) represents absolute B cell counts (cells/μl). X-axis represents time post-HSCT in months.

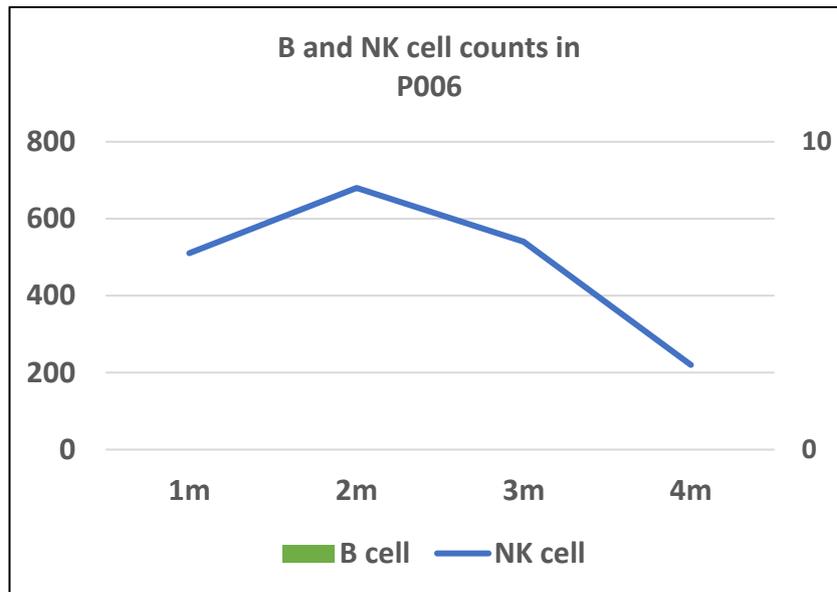
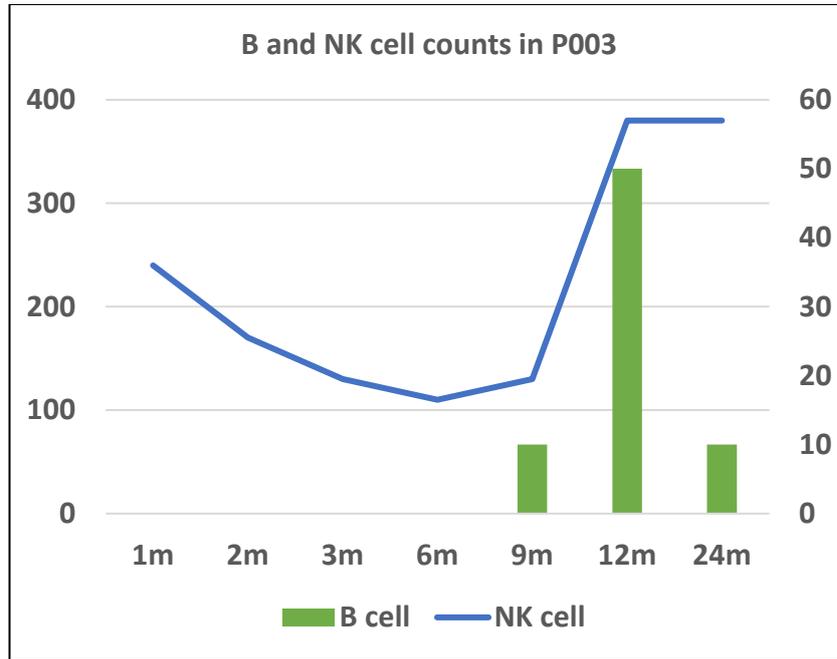
Figure 3.3. Poor immune recovery in P003 and P006

a) T cell counts post-HSCT in P003



X-axis represents absolute counts (cells/ul) and y-axis represents time post-HSCT. Abbreviations m: months.

b) Impaired B cell count recovery in P003 and P006 in the context of steroid therapy



Primary Y-axis (to the left) represents absolute NK counts (cells/μ) and secondary Y-axis (to the right) represents absolute B cell counts (cells/μl). X-axis represents time post-HSCT in months.

3.3 Detailed description of patients who received AP1903 in the same center

All patients in our centre who received BPX-501 developed aGvHD of variable grades as highlighted in the above sector. Four out of 6 patients received AP1903 to manage aGvHD; P002, P003, P006. P007 developed encephalopathy after BPX-501 infusion that was treated with a combination of steroid therapy and AP1903.

Among evaluable TCR $\alpha\beta$ cases (who completed 100 days post-HSCT), 1-year cumulative incidence of aGvHD grade I-IV was 61.5% (8/13); 5 had grade I skin, 2 had grade II skin \pm engraftment syndrome and 1 had grade III upper and lower gut. All TCR $\alpha\beta$ +BPX-501 patients developed aGvHD grade I-IV (100%); 2/6 had grade I skin while 4 had evidence of visceral aGvHD. Median time to aGvHD was 35 days (range: 20-111 days) among TCR $\alpha\beta$ cases+BPX-501; Table (3.2). cGvHD occurred in one TCR $\alpha\beta$ +BPX-501 patient (1/6, 16.6%). However, it was skin limited and resolved completely by topical treatment.

P002:

P002 developed skin aGvHD stage 1-2 at D+20 that responded to topical steroid therapy. By D+40, he developed abdominal pains and vomiting and was TPN dependent. Upper Gut endoscopy showed evidence of upper gut aGvHD. CD3+CD19+ cells showed a significant expansion coinciding with his clinical symptoms; from being barely detectable at D+30 to 59.6 and 150.4 cells/ul at D+40 and D+60; respectively. He was thus given a dose of AP1903 0.4 mg/kg. Coinciding with a significant drop of CD3+CD19+ cells (reaching 4 cells/ul), by D+70, his TPN was dropped and completely discontinued at 3 months post-transplant.

Of note, there was a slight transient drop in CD8 +CD19- counts at time of AP1903 infusion from 800 cells/ul at D+60 to 560 cells/ul at D+67. This pattern was not seen among CD4+CD19- cells; 130 and 150 cells/ul at D+60 and D+67, respectively. This drop did not coincide with CMV reactivation (Figure 3.6).

On D+114, there was a noted re-surge of CD3+CD19+ cells in the peripheral blood; 13.6 cells/ul reaching a peak of 52 cells/ul at D+128. However, this re-surge did not coincide with recurrence of aGvHD. At 24 months post-HSCT, CD3+CD19+ cells were detectable at 24 cells/ul (Figure 3.4).

P003:

P003 developed skin aGvHD stage 2-3 at D+20 post-transplant together with extensive diarrhea and metabolic acidosis. Thus, Feeds were discontinued, and he was started on Prednisolone of 2mg/kg together with resolution of skin rash and improvement in stool output. Upper and lower gut endoscopies were normal and lower gut biopsy showed no evidence suggestive of aGvHD, only finding was foamy macrophage. On each attempt of dropping the steroid dose to 0.5 mg/kg, P003 had a flare of skin± gut symptoms. Thus, he was started on Ciclosporin A therapy and Prednisolone was increased back to 2mg/kg. from D+128 to D+156, a recorded rise in percentage of CD3+CD19+ from being between 0.65 since transplant to 5% (CD3: 110 cells/ul) at D+128 (CD3:140 cells/ul) and 10% at D+156. Thus, the consensus was to administer a dose of AP1903 0.4mg/kg with an attempt that AP1903 can facilitate steroid tapering. Though CD3+CD19+ cells reached 0% in peripheral blood however P003 had difficulty in tapering steroids with recurrence of gut symptoms. A repeat endoscopy and lower gut biopsy were normal apart from foamy macrophage but due to ongoing symptomatology, he was given Infliximab and Basilizumab

infusions. In addition, he was kept nil by mouth and was maintained on TPN. Interestingly after AP1903 infusion and with tapering of steroids, P003 never had a flare of his skin aGvHD as used to occur pre-AP1903 infusion. Of note, P003 had a recurrence of gut symptoms in each attempt of starting the feeds. A third biopsy of the gut showed same picture recorded in the earlier 2 biopsies. Till death, he was maintained on prednisolone and ciclosporin A and TPN to control his enteropathy (Figure 3.4).

P006:

P006 developed stage 1 aGvHD of the skin on D+17 that required 10 days of topical steroids and Tacrolimus cream to fade out. Thus BPX-501 infusion was deferred. Based on the complete resolution of aGvHD and in the presence of viral reactivation, it was agreed to proceed with infused of BPX-501 on D+35. 10 days after the infusion of BPX-501, he started to develop skin aGvHD stage 1-2 coincided with the detection of CD3+CD19+ T cells. His skin failed to respond to topical treatment and evolved into stage 3 skin and stage 1 upper gut aGvHD. Lymphocyte subsets showed an expansion of CD3+CD19+ cells; constituting 7.3% of total lymphocytes (38.6 cells/ μ l). AP1903 was infused on D+56. Both skin and upper gut aGvHD resolved completely within 48 hours of AP1903 infusion with the disappearance of CD3+CD19+ cells from the periphery. However, nine days later, the symptoms of upper gut aGvHD recurred with vomiting, retching and poor oral intake together with a recurrence of skin rash stage 1. Lymphocyte subsets showed a resurgence of the CD3+CD19+ cells; constituting 2.4% of total lymphocytes (35.04 cells/ μ l). A second dose of AP1903 was thus infused on D+72 with no obvious response. His lymphocyte subsets showed a transient brief drop in CD3+CD19+ cells to reach 22 cells/ μ l followed by re-surge of marked cells; constituting 2.6% of total lymphocytes (53.8 cells/ μ l) (Figure 3.4). He was thus started on prednisolone therapy 1mg/kg on D+74 with good response and resolution of both upper gut and skin aGvHD.

On D+92, P006 developed profuse diarrhea 7-8 times/day together with on/off retching symptoms and 2-3 vomitus per day. He had no abdominal pains and no blood in stool or vomit. Despite being on 3 anti-sickness medications (Ondansetron, Prochlorperazine, Hyoscine patch). He lost 1kg of weight during this episode. D+100 Lymphocyte subsets showed; CD4:190 cells/ μ l, CD8: 170 cells/ μ l and CD3+CD19+ cells: 40.09 cells/ μ l. Blood viruses showed low CMV viraemia 880 copies/ml with no detectable adenovirus in blood by PCR. Stools were positive for adenovirus and Noro II virus. Brincidofovir was thought to be responsible for his ongoing diarrhea and thus

was discontinued. He started TPN and remained nil by mouth. His vomiting completely resolved, however, he continued to have ongoing diarrhea with extensive napkin ulcerations. Thus, Esophagogastroduodenoscopy was performed on D+107 and biopsy was consistent with lower gut aGvHD. There was no evidence of upper gut aGvHD. Viral inclusion was not identified for AdV. His FBC showed a drop-in platelet to $99 \times 10^9/L$ after being normal. Since then, Platelets remained between $60-80 \times 10^9/L$ possibly reflecting active marrow aGvHD. P006 was started on Prednisolone 2mg/kg/day and Ciclosporin on D+110. A week later, P006 started to show signs of clinical improvement with stools getting more formed. Prednisolone dose was tapered to 1mg/kg/day and 2 weeks later to 0.5mg/Kg/day. However, he continued to have problems with CMV and adenoviral reactivation. Virology PCRs showed CMV viraemia of 36,238 copies/ml and adenoviraemia 4,364 copies/ml. Four days after steroid tapering, D+141, his diarrhea got worsened with abdominal bloating and pains with evidence of surgical emphysema and X-ray showing pneumatosis intestinalis. Brincidofovir was discontinued on D+150. Gut rest was commenced and Meropenem as well as amikacin and Metronidazole treatment were started. His Methylprednisolone dose was reduced from 0.5 to 0.4 mg/kg day in view of his pneumatosis on D+159.

However, he continued to deteriorate and on the evening of D+159, P006 had a single episode of mild melena that was abruptly followed by a sudden and catastrophic gastro-enteric bleeding episode on next day. At the time of the bleeding, his platelets were $45 \times 10^9/L$ and clotting was deranged. P006 became agitated with metabolic acidosis, tachycardic and hypovolemic shock that required resuscitation with 60 ml/kg fluids, blood products, octreotide infusion as well as inotropes and mechanical ventilation. After a short period of stabilisation, in the morning of D+162, he suddenly deteriorated with multiple episodes of catastrophic gastrointestinal (GI) bleeding requiring multiple red blood cells (RBC) and Platelet transfusion in addition to vasopressors. Unfortunately, he arrested in few hours despite supportive care. The cause of death was deemed catastrophic gastrointestinal bleeding in the context of pneumatosis intestinalis and ongoing active gut aGvHD and adenoviral infection; following a haploidentical alpha beta T cell depleted stem cell transplantation for acute lymphoblastic leukemia.

P007:

Nineteen days after BPX-501 infusion, P007 developed culture -negative high fever with no evidence of GvHD or signs of infection. He remained 100% donor engrafted in all lineages including monocytes with no evidence of disease relapse. Virology/microbiology/Toxoplasma PCR screens were negative. Thus, this fever was treated as a suspected allo-immune engraftment phenomenon and the patient was started on 1mg/kg prednisolone. In twelve hours, his fever settled with no other recorded symptoms. After 7 days, steroids were reduced to 0.5 mg/kg. Five days after reduction of steroid dose, P007 started to show neurological symptoms with confusion and abnormal movements. On D+52, P007 started to show neurological symptoms with confusion and abnormal movements. There was no evidence of any motor or sensory manifestations and there were no recorded seizures. EEG was suggestive of encephalitis/encephalopathy. MRI brain showed the presence of bilateral white matter areas of signal abnormality with a frontal predominance, consistent with a leukoencephalopathy. According to the MRI report this finding would be consistent with treatment related neurotoxicity rather than opportunistic infection. CSF was negative for bacteria, fungi and negative for HSV1, 2, HHV6, VZV, parechovirus, enterovirus, EBV, CMV, AdV, JC, respiratory viruses, measles and mumps. CSF was screened for the presence of lymphocytes by flowcytometry. There were very few lymphocytes in the CSF (<10); majority were CD3+CD19- cells by flow with a few CD3+CD19+ cells were seen in figure 3.5. Bloods taken on D+56 showed evidence of peripheral expansion of CD3+ CD19+ cells constituting 14% of total lymphocytes (68 cells/ul). Bone marrow aspirate carried out on the same day showed normal morphology and 100% donor engraftment. P007 was started on dexamethasone therapy; in a dose equivalent to 2mg/kg of prednisolone on D+58. He remained obtunded with no evidence of neurological improvement 72 hours after steroid therapy. Repeat MRI on showed progression of the diffuse white matter, thalamic and basal ganglia signal changes. Cerebellar involvement was also noted (Figure 3.5).

There was no evidence of pathological enhancement or mass effect. A dose AP-1903 was administered on D+ 64. CD3+CD19+ cells dropped from 52 cells/ul pre-infusion to 10 cells/ul 4 hours post-infusion (Figure 3.4). Patient was also started on weekly Cidofovir and high dose IVIG therapy. Five days post-AP1903 infusion, the patient started to show signs of improvement and his Glasgow coma score increased from 10-11 to 12-13. His dexamethasone dose was tapered to a dose equivalent to 1mg/kg of prednisolone. Though, he started to show clinical improvement, modified CD3+CD19+ cells were again detected in peripheral blood where they constituted

67% of total T cells (53 cells/ μ l). Thus, another dose of AP1903 was infused on D+72. P007 continued to show evidence of neurological improvement being less confused and having appropriate verbal responses. A repeat CSF on D+76 showed no cells and no evidence of viral, bacterial, fungal or Toxoplasma infection by PCR. Bone marrow aspirate carried out on the same day showed normal morphology and 100% donor engraftment. His dexamethasone dose was tapered to a dose equivalent to 0.5 mg/kg of prednisolone since D+78 and completely discontinued on D+90. P007 had complete neurological recovery with no sequel. Encephalopathy was reported to MHRA as a serious unexpected serious adverse event (SUSAR), possibly related to BPX-501 infusion. Of note, despite 2 doses of AP1903 infusion, CD3+CD19+ cells fluctuated between 10-50 cells/ μ l and never disappeared from the periphery. Marked T cells were recorded as 50 cells/ μ l at last follow-up.

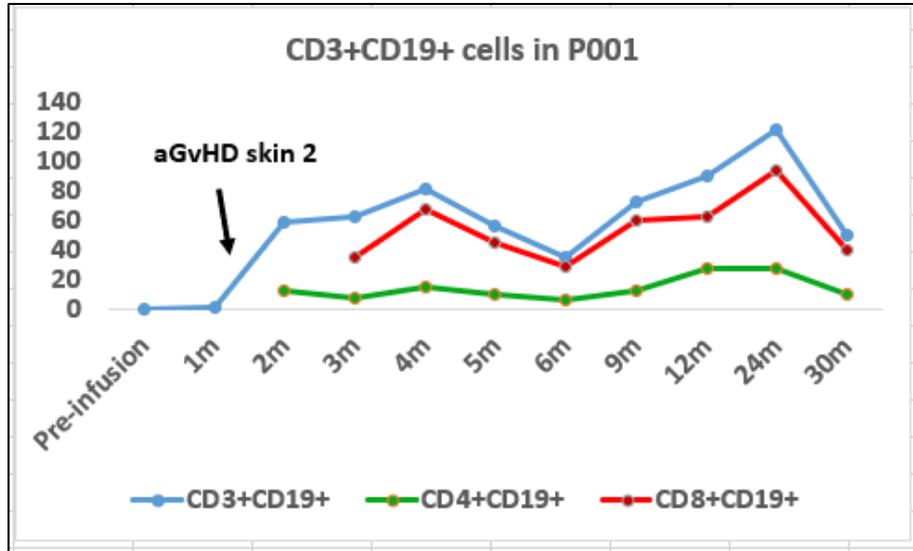
Fifteen days following discontinuation of Prednsiolone therapy for T cell mediated neurotoxicity, P007 had evidence of intermittent stage 1 aGvHD rash that responded to topical treatment. At 4 months post-HSCT, P007 lost his appetite with 1-2 vomitus per day and diarrhea 2-3 times per day. Esophagogastroduodenoscopy and colonoscopy with biopsies showed features consistent with GVHD affecting stomach, duodenum as well as left and right colon. P007 was started on Methylprednisolone for his gut GvHD with improvement in his upper and lower gut GVHD symptoms. Due to previous history of encephalopathy, steroid dose was slowly tapered over 3m period. P007 returned to his home country at 8-month post-HSCT and was maintained at 0.25 mg/kg of Prednisolone therapy. While on low dose steroid therapy, P007 developed cGvHD rash; rash over the knuckles with roughness, and no skin tightness (scleroderma).

P007 had cGvHD screen for organ affection and was noted to have no eye or oral symptoms. Genital area was free and musculoskeletal examination was normal. PFT showed improved in lung function in comparison to pre-transplant data with FEV1=92.1% of predicted and FVC of 90.3% of predicted. Thus, his cGvHD was limited to skin. Topical steroids led to a complete resolution of the rash in 2 weeks' time.

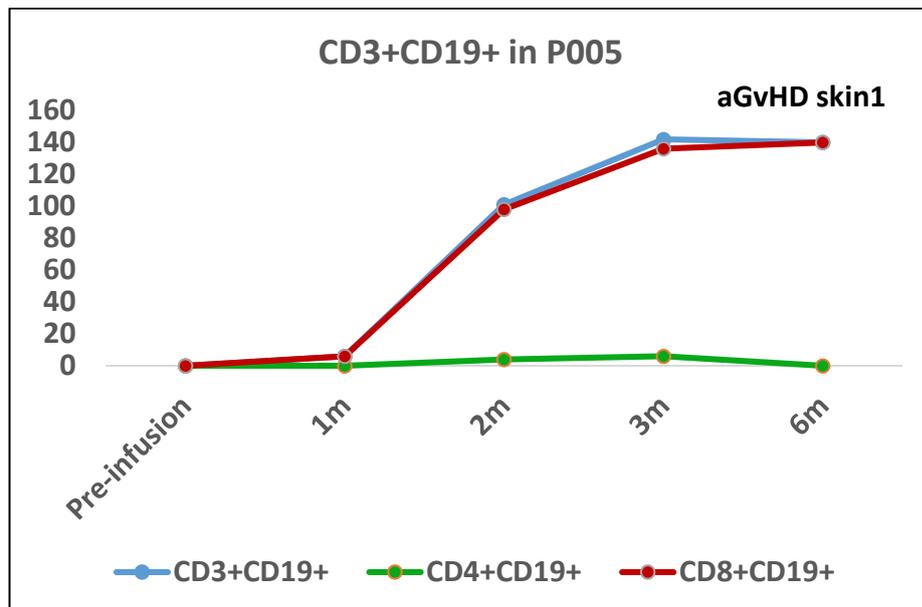
Figure 3.4. CD3+CD19+ cell expansion post-infusion and GvHD

a) Patients who did not require AP1903 infusion

a-1) Persistence of CD3+CD19+ cells for 30 months post-HSCT

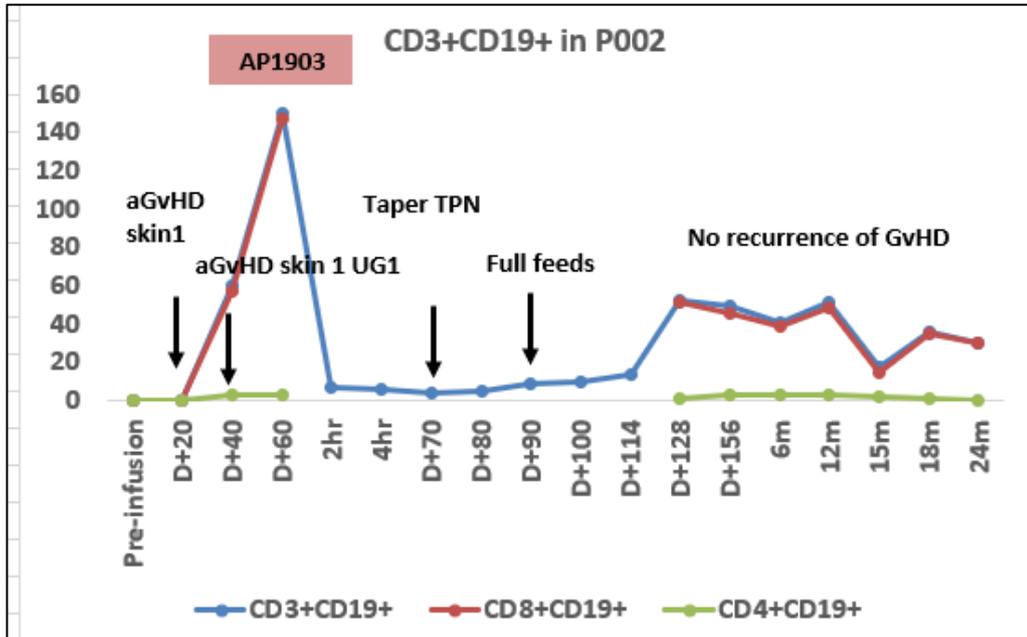


a-2) Expansion of CD3+CD19+ cells in P005

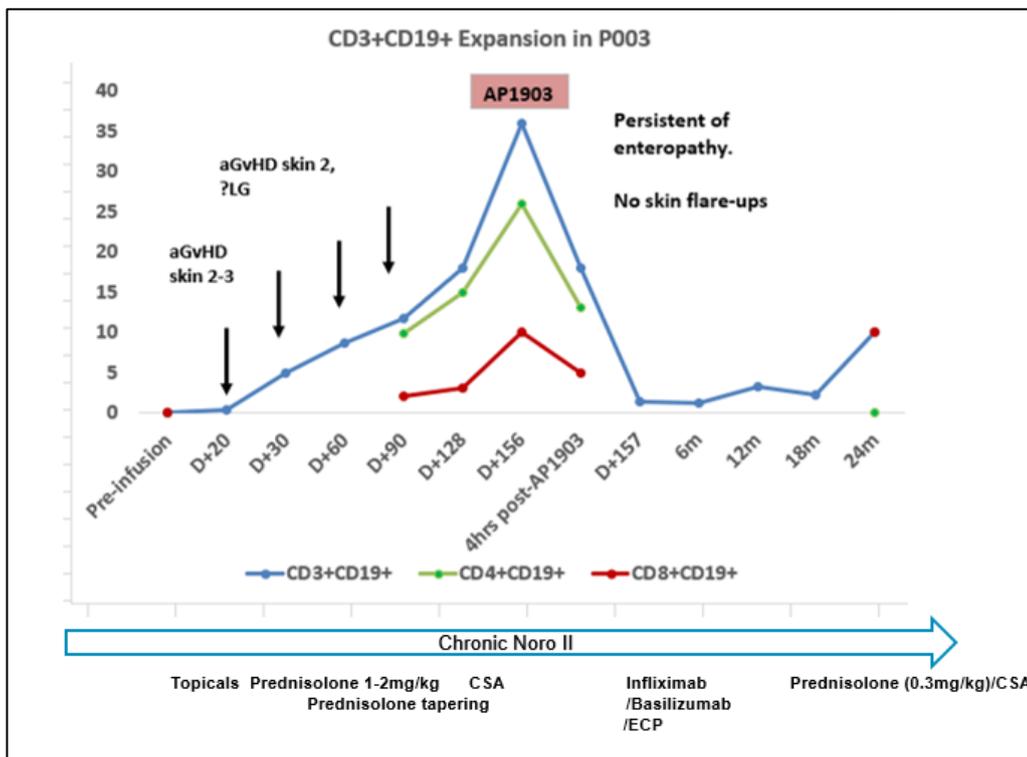


b) Patients who required AP1903 infusion

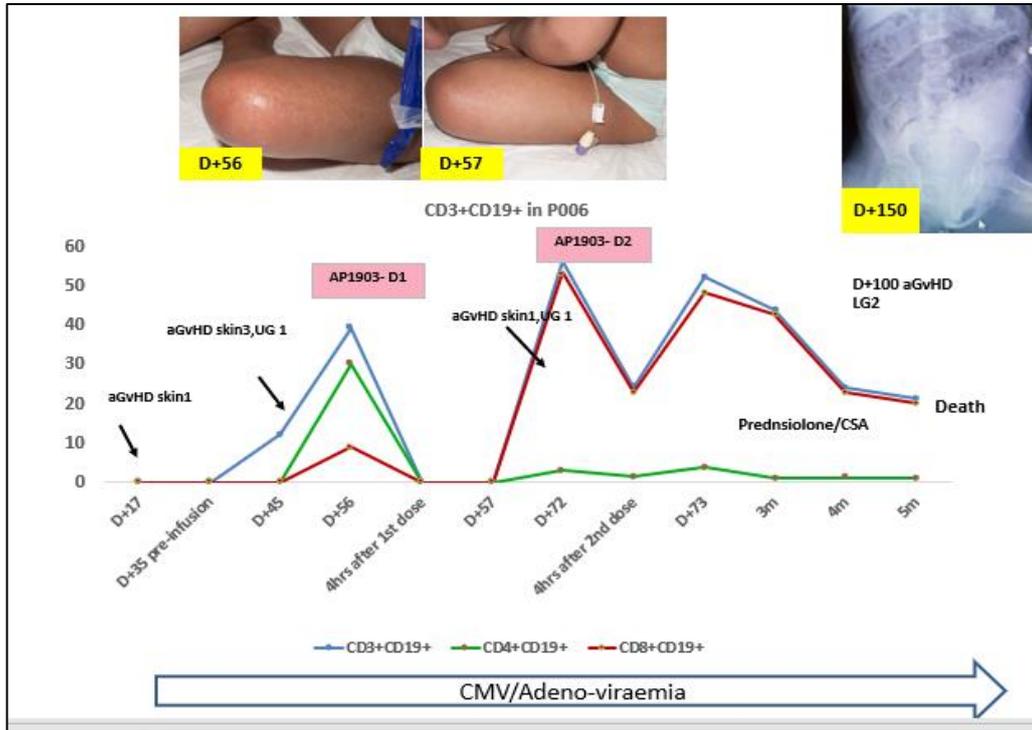
b-1) Pattern of CD3+CD19+ expansion in the context of aGvHD in P002



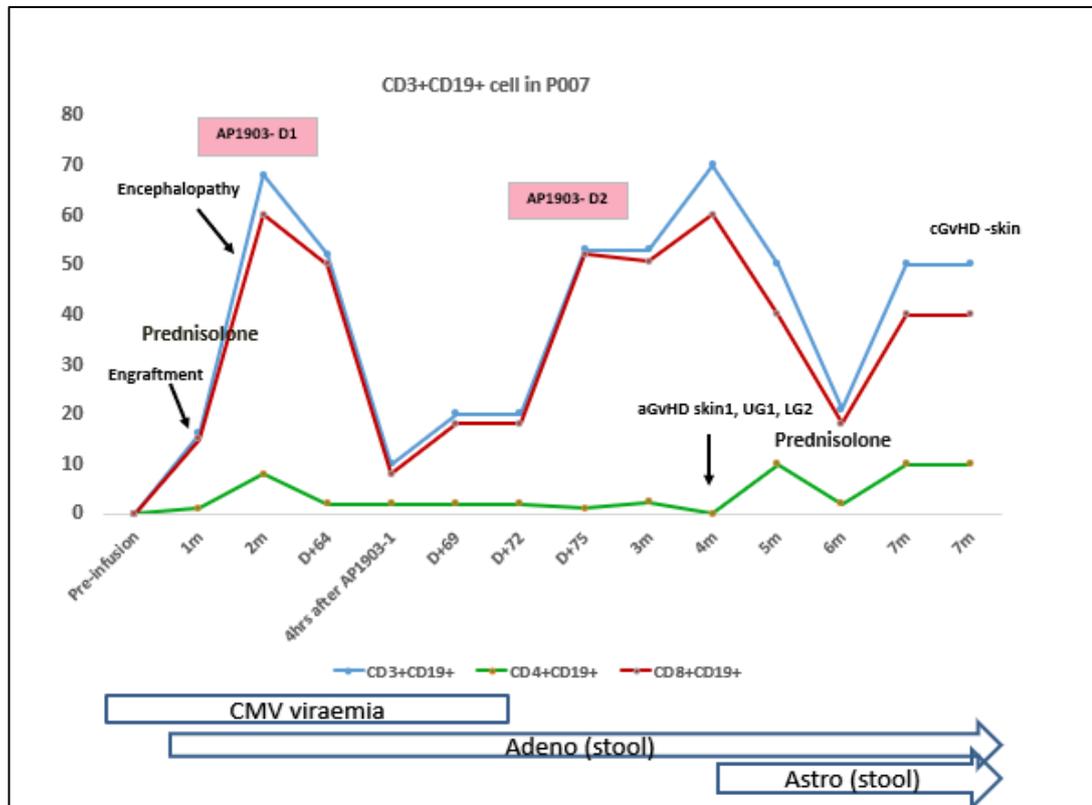
b-2) Pattern of CD3+CD19+ expansion in the context of aGvHD/viral infection in P003



b-3) Pattern of CD3+CD19+ expansion in the context of aGvHD/viral infection in P006



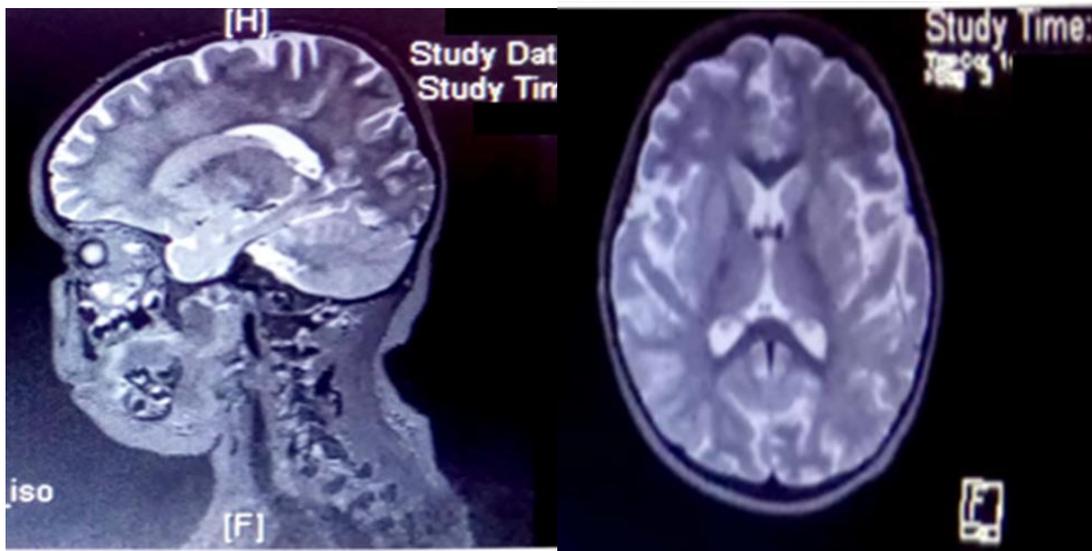
b-4) Pattern of CD3+CD19+ expansion in the context of allo-immunity/viral infection in P007



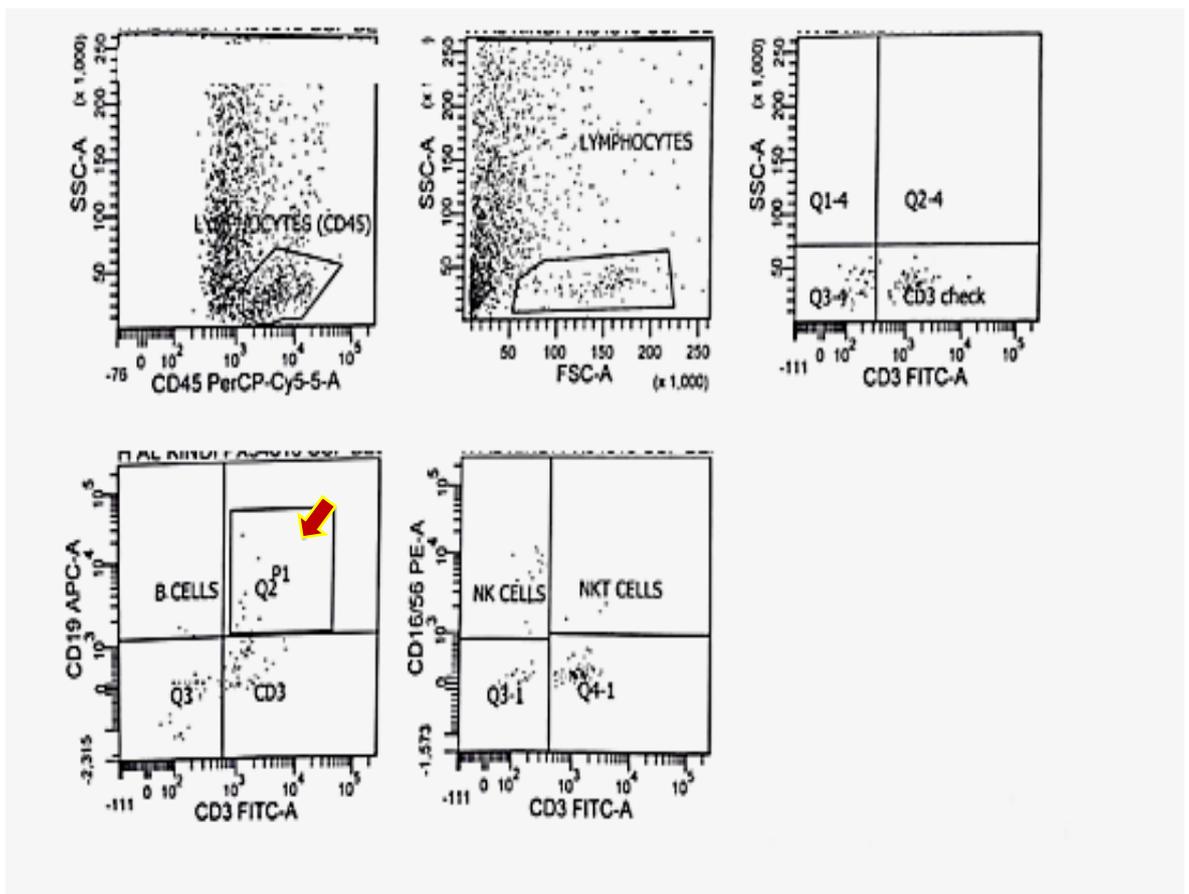
X-axis represents absolute cell counts (cells/μl) and Y-axis represents time post-HSCT. Arrows in figure b-2 represent multiple flare-ups of skin aGvHD stage 2 in addition to persistent gut enteropathy in P003. P006 and P007 received 2 doses of AP1903 (D1: dose 1, and D2: dose 2).

Figure 3.5: Neurotoxicity post-BPX-501 infusion in P007

a) MRI brain imaging showing diffuse white matter changes



b) Presence of CD3+CD19+ cells in CSF as detected by flowcytometry



The red arrow points to the presence of modified T cells (CD3+CD19+ cells) in CSF

Table 3.2: Characteristics of patients who developed GvHD post-TCR $\alpha\beta$ /CD19 depleted graft + BPX-501 infusion

<u>Pt ID</u>	Diagnosis	Conditioning/stem cell source	GvHD prophylaxis	TCR $\alpha\beta$ dose (X10 ⁴ /Kg)	aGvHD grade	Time to aGvHD	Treatment	Outcome
P001	IL10R B defect	Treo/Flu/TT/ATG 15 Paternal	None	0.25	Skin 2	40	Topicals	CR
P002	CID/NHL	Treo/Flu/TT/ATG 15 Paternal	None	0.22	Skin 2, UG1	20	AP1903	CR
P003	Perforin HLH	Treo/Flu/TT/ATG 15 Paternal	None	0.25	Skin3, ? LG1	20	Steroids/CSA AP1903	PR
P005	PI3K R	Treo/Flu/TT/ATG 15 Maternal	None	1.11	Late Skin 1	111	Topicals	CR

P006	ALL in CR2	TBI(14.4Gy)/Cyc/ ATG 15	None	0.14	Skin 1 (pre- PBX-501)	17	Topicals	CR
		Paternal			Skin3, UG1	45	AP1903-dose 1	CR
					Skin1 , UG1	75	AP1903-dose 2 Steroids	NR CR
					LG2	100	Steroids/CSA	PR
P007 2 nd Graft	JMML	Flu /Cyc/4Gy TBI/TT /ATG 4.5mg/Kg	None	0.07	Engraftment Syndrome (fever)	30	Systemic steroids	CR
		Paternal			T cell mediated neurotoxicity	120	Steroids/AP1903 Steroids	CR CR

					Skin 1, UG1, LG2			
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LG: lower gut, UG: upper gut, CSA: ciclosporin A, MP: methylprednisolone, Flu: Fludarabine, Cyc: Cyclophosphamide, TBI: total body irradiation, ALL: acute lymphoblastic leukemia, JMML: Juvenile myelomonocytic leukemia, Treo: Treosulphan, TT: Thiotepa, Flu: Fludarabine, ATG: anti-thymocyte globulin, TBI: total body irradiation CR: complete response, PR: partial response, NR: no response

3.4 Viral reactivation in TCR $\alpha\beta$ /CD19 depleted grafts + BPX-501 infusion

Viral reactivation; CMV or AdV viraemia, was recorded among 83% (5/6) of TCR $\alpha\beta$ +BPX-501 cases with high viral loads (CMV $>10^5$ copies/ml or AdV $>10,000$ copies/ml) not recorded among any of the patients (Table (3.3)). None of the patients developed viral induced disease. All patients responded to anti-viral therapy as noted in table 3.3 and figure 3.6. P006 had multiple courses of anti-viral treatment to control increasing AdV and CMV viral blood loads in the context of increasing steroid therapy to control aGvHD. Median time to clearance of blood virus was 40 days (26-87 days).

Early immune recovery- with the appearance of CD4 and CD8 cells in the first 2 months post-transplant- among TCR $\alpha\beta$ +BPX-501 influenced the capacity of the graft in handling the viruses as shown in Figure 3.6. Moreover, no increase in viral titre was seen in P002 and P007 after AP1903 infusion; figure 3.6.

Table 3.3. Pattern of viral reactivation among cases treated with BPX-501

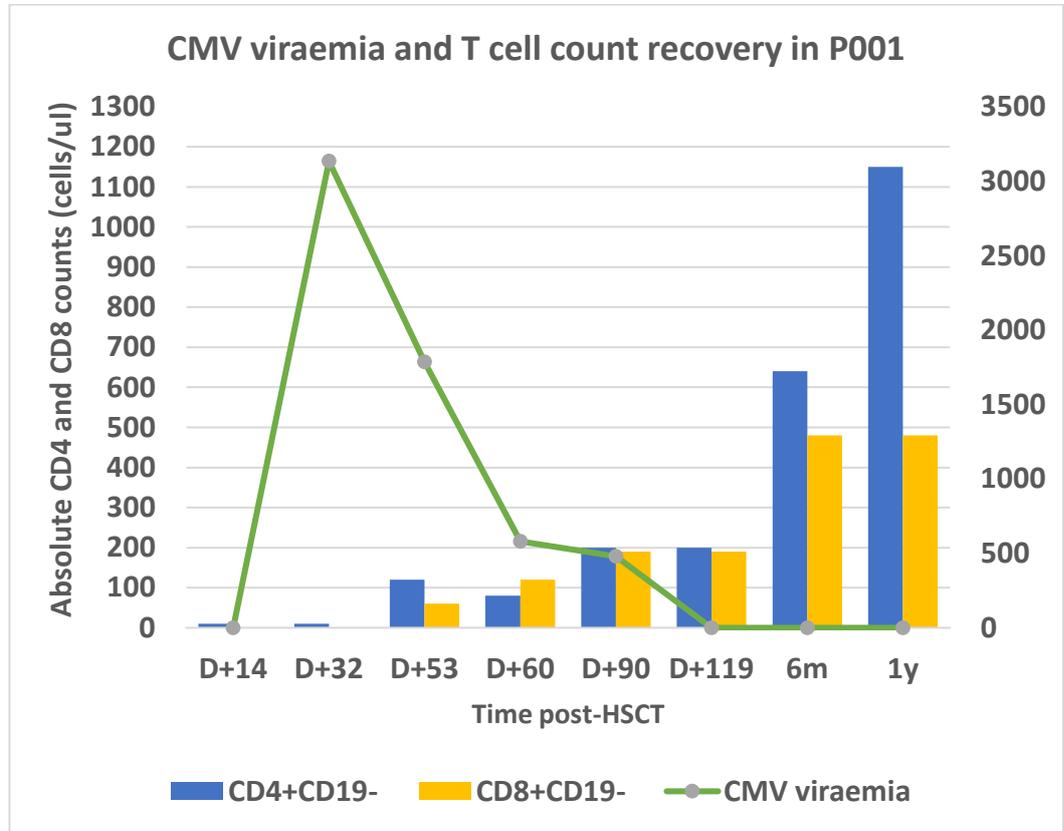
Pt ID	R/D CMV status	R/D EBV status	Pre-transplant viral reactivation	Post-transplant viral reactivation	Days from Onset to clearance	treatment	Sequel/ Outcome/ Last follow-up
P001	Pos/Pos	Neg/Pos	None	CMV viraemia (low grade)	D+32- D+119	None	None 36m
P002	Neg/Pos	Pos/Pos	? Gut EBV PTLD	CMV viraemia (X2) (Low grade)	D+30- D+67 D+120- D+150	Incomplete course of treatment 2 doses of Ganciclovir/ 2 doses of valganciclovir None	None 36m
P005	Pos/Pos	Pos/Pos	Previous CMV parotitis Previous EBV PTLD	CMV viraemia (Low grade viraemia)	D+14- D+40	Ganciclovir	None 8m

P006	Neg/Pos	Pos/Pos	None	CMV viraemia (X2 reactivation)	D+32- D+115 D+120 D+160	BPX-501 D+35 Foscarnet Valganciclovir Ganciclovir	Deceased uncontrolled aGvHD 5m
				AdV viraemia (X2 reactivation)	D+48- D+90 D+105- death (D+165)	Cidofovir BPX-501 Brincidofovir Cidofovir/Brincidofovir	
P007	Pos/Pos	Pos/Pos	CMV viraemia after 1 st haplo (Ganciclovir)	CMV viraemia (Low grade viraemia)	D+30- D+70	Ganciclovir/Foscarnet	None 8m

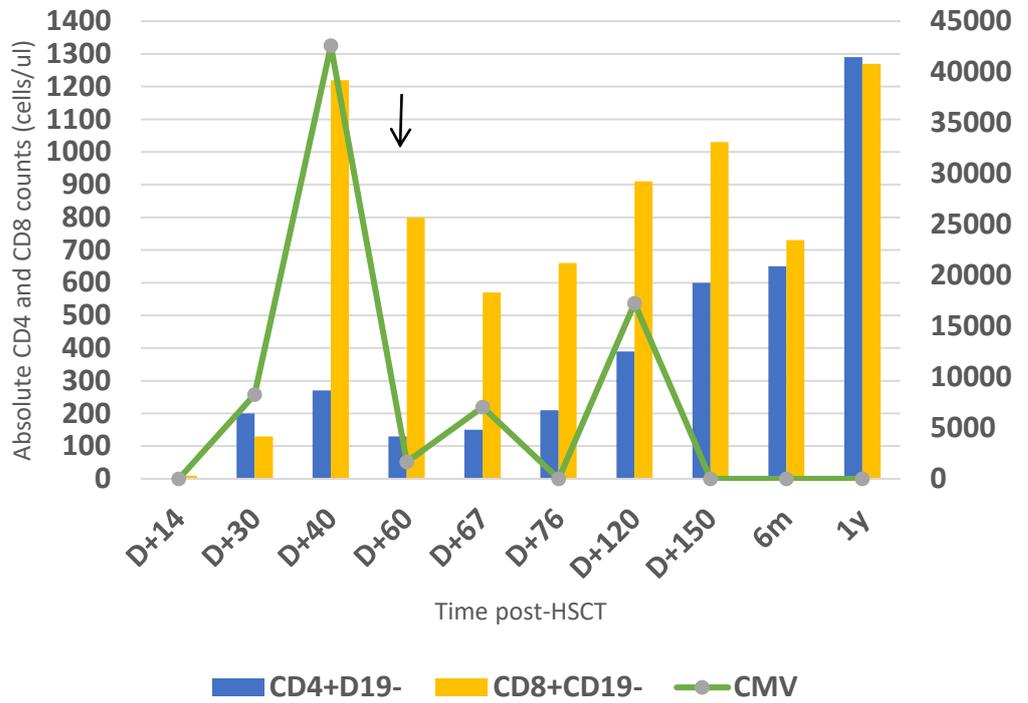
Abbreviations: Neg: negative, Pos: positive, AdV: adenovirus, CMV: Cytomegalovirus, PTLTD: post-transplant lymphoproliferative disease, EBV: Epstein Barr virus, RD: retinal detachment, CTLs: cytotoxic T lymphocyte infusion (s).

Figure 3.6. Viral reactivation among cases who received BPX-501±AP1903 in context of T cell recovery

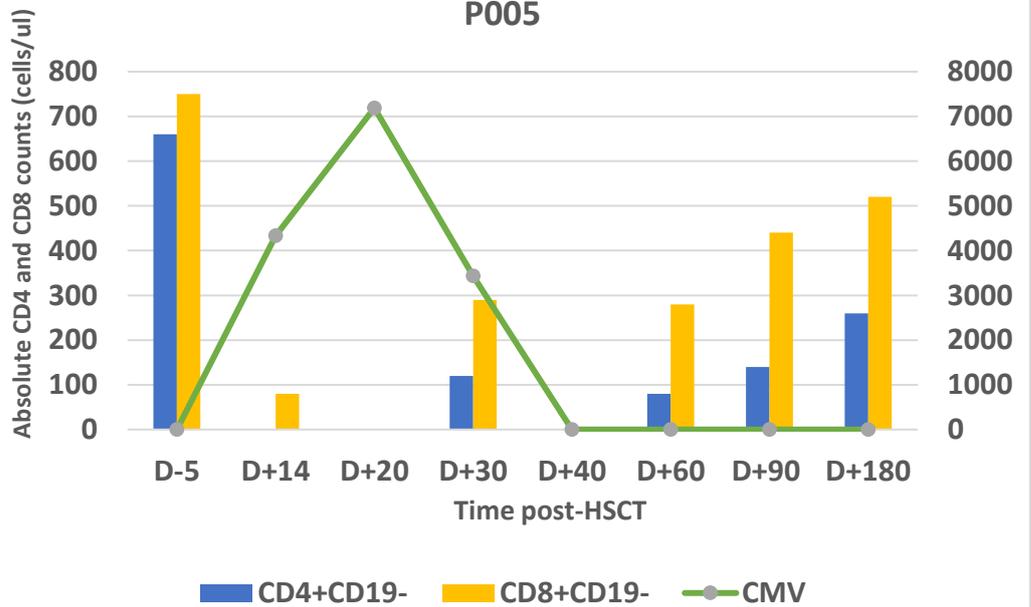
1) Low viral load in the context of early T cell recovery

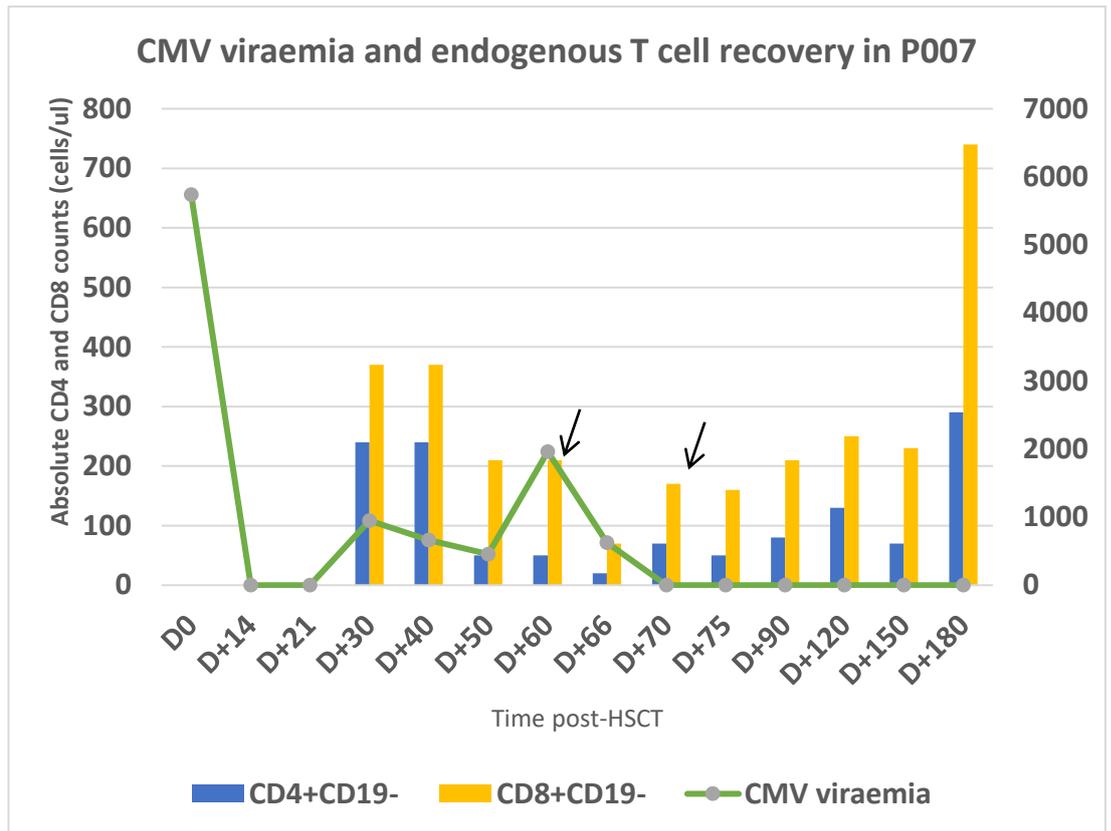


CMV viraemia and endogenous T cell recovery in P002

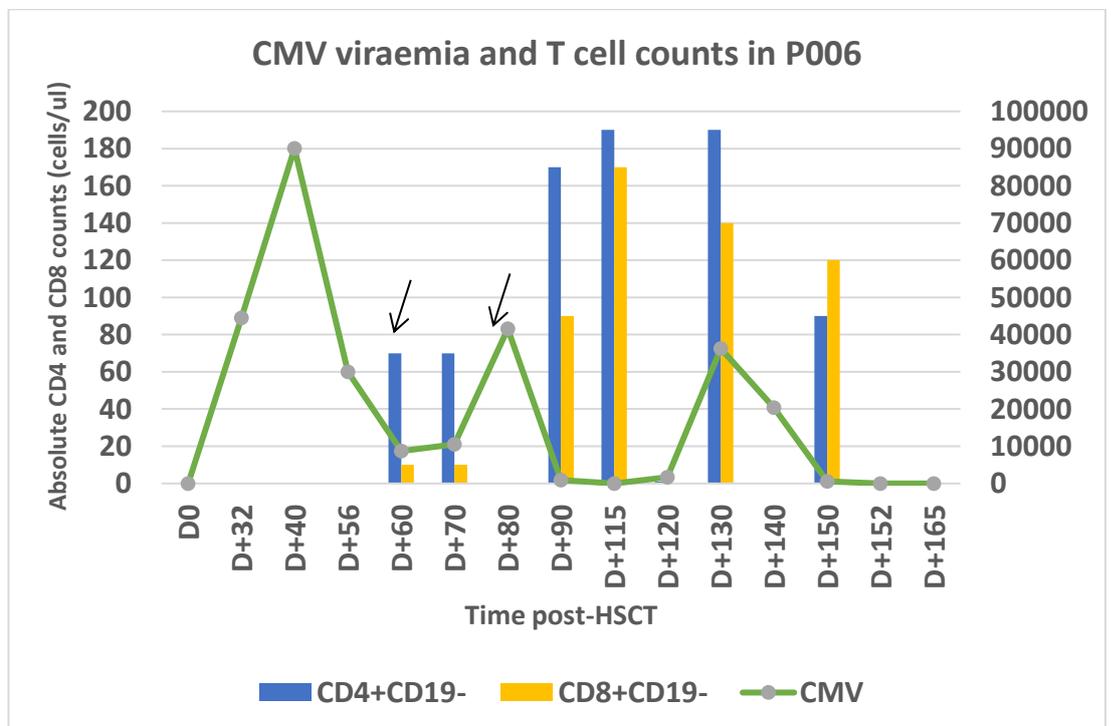


CMV viraemia and endogenous T cells recovery in P005

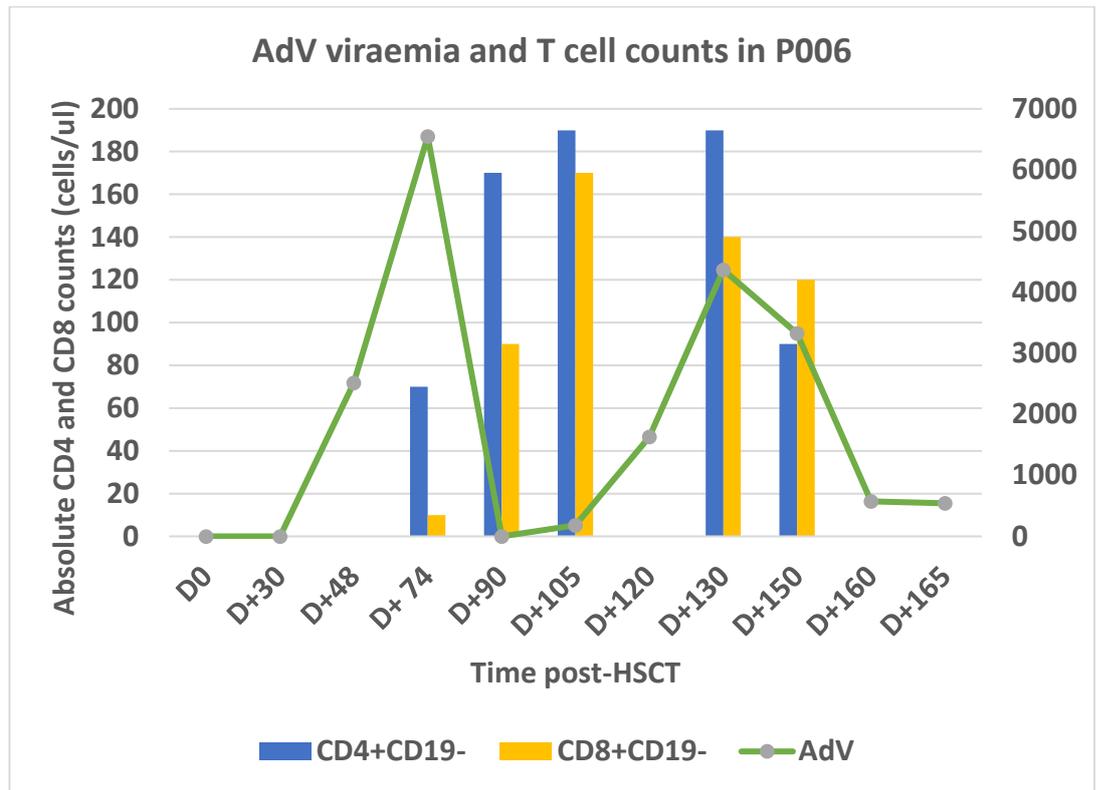




2) Pattern of CMV reactivations in P006



3) Pattern of AdV reactivation in P006



Arrow: denotes the time of AP1903 infusion. Abbreviations: CTLs: cytotoxic T lymphocytes infusion, CMV: cytomegaloviraemia, AdV: adenoviraemia

3.5 Summary of adverse events

List of adverse events seen post BPX-501 and AP1903 infusion were summarised in Table 3.4. Of note, aGvHD was recoded as possibly related to BPX-501 has been discussed with each case. There were no reported adverse events post-AP1903 infusion apart from mild thrombocytopenia (less than $150 \times 10^9/L$) being seen in P007 as shown in Figure 3.7. This pattern was not seen among the other cases who had received AP1903 infusion

Table 3.4: Reported AEs and SAEs post-BPX-501/ AP1903 administration

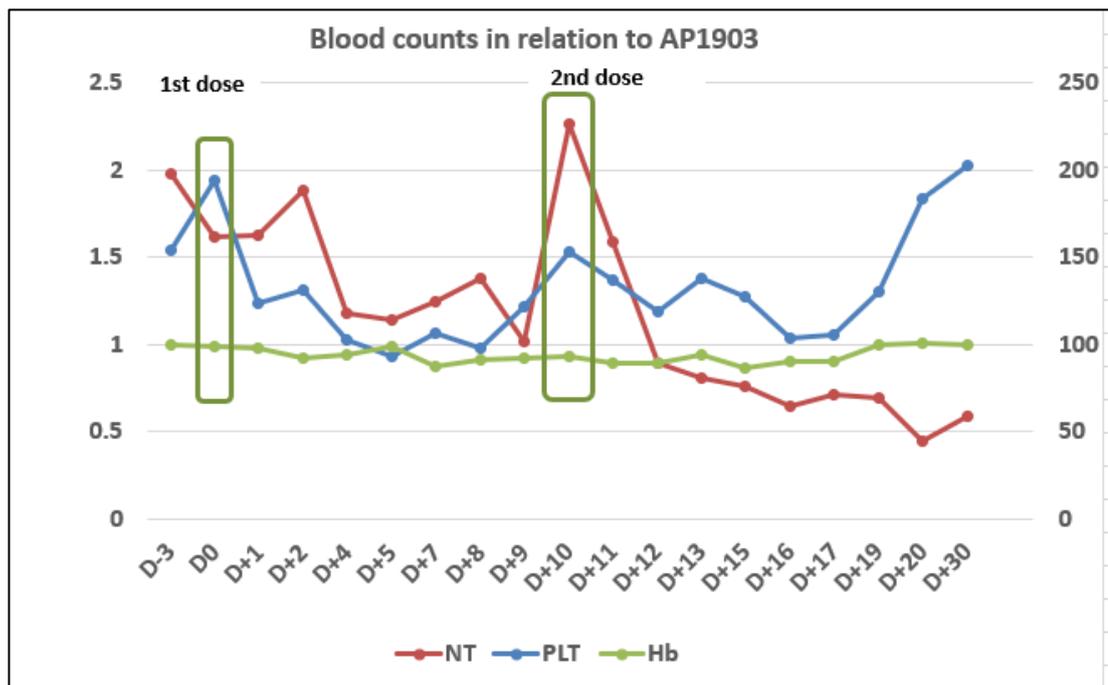
Pt ID	Adverse event	Grade	Relation BPX- 501 or AP1903 infusion	Intervention	Outcome
P001	Blurred vision	2 (Moderate)	NR	Discontinued Cyclizine	Resolved
	Abdominal pain/Vomiting	3 (Severe)	NR	IV fluids Nil by mouth	Resolved (Cyclic vomiting on Atenolol)
	BK in urine	2 (Mild)	NR	N	Resolved
	Urinary tract infection	3 (Severe)	NR	IV antibiotics	Resolved
	Hypertensive episodes	2 (Moderate)	NR	Monotherapy	Resolved
	CMV viraemia	1 (Mild)	NR	None	Resolved
	aGvHD stage 1-2 skin	2 (Moderate)	PR	Topical treatment	Resolved
P002	Blurred vision	2 (Moderate)	NR	Discontinued anti-emetic	Resolved
	CMV viraemia	1 (Mild)	NR	None	Resolved
	Fracture of left femur	3 (Severe)	NR	Surgical intervention	Resolved
	Osteosarcoma	3 (severe)	NR	Surgical intervention	Resolved
P003	aGvHD stage 3 skin	2 (Moderated)	PR	AP1903 administration	Resolved
	Elevated ALT	1 (Mild)	NR	None	Resolved
	Elevated GGT	3 (Severe)	NR	Liver biopsy	Resolving
	TMA	3 (Severe)	NR	Hospitalisation Eculizumab	Resolved
	Acute pancreatitis	4(life threatening)	NR	Nil by mouth Octreotide	Resolved

				PICU admission on MV	
	IRIS due to disseminated atypical mycobacteria	5 (lead to his death)	NR	PICU admission Steroids/anakinra	Death
P005	Late onset aGvHD skin 2	2 (Moderate)	PR	Topical therapy	Resolved
	CMV viraemia	1 (Mild)	NR	None	Resolved
P006	aGvHD skin 3/UG 1	2 (Moderate)	PR	AP1903	Resolved
	Adenoviral reactivation	2 (Moderate)	NR	IV Cidofovir Then Oral Brincidofovir	Was resolving at time of death (540 copies/ml)
	CMV reactivation	2(Moderate)	NR	IV Foscarnet. Then oral valganciclovir	Resolved
	Thrombocytopenia	1 (Mild)	PR (BM aGvHD)	None	Didn't resolve.
	aGvHD LG 2/ pneumatosis intestinalis	5 (led to his death)	PR	Steroids/CSA hospitalisation	Death
P007	CMV reactivation	2 (Moderate)	NR	Ganciclovir Foscarnet	Resolved
	Raised creatinine	1(Mild)	NR	Increased oral fluid intake	Ongoing
	Encephalopathy	3 (Severe)	PR	AP1903 Dexamethasone	Resolved
	aGvHD skin 1, UG1, LG 2	3 (Severe)	PR	Hospitalisation TPN	Resolved

				Prednisolone/CS A	
	cGvHD	1 (Mild)	PR	Topical	Resolved

Abbreviations: CSA: Cyclosporine A, aGvHD: acute graft versus host disease, UG: upper gut, LG: lower gut, CMV: cytomegalovirus, NR to either BPX-501 or AP1903: not related, PR to BPX-501 administration: probably related, PICU: pediatric intensive care unit, MV: mechanical ventilation, IV: intravenous.

Figure 3.7: Mild drop in platelets post-AP1903 infusion noted in patient P007



Primary Y-axis (to the left) represents neutrophil counts (X10⁹/L) and secondary Y axis (to the right) represents Hb blood levels (in grams/L) and platelet counts (X10⁹/L). X-axis represents Days post-AP1903 infusion. P007 received 2 doses of AP1903 (empty green boxes) Abbreviations: NT: neutrophils, PLT: platelets, Hb: hemoglobin blood level.

Table 3.5. Comparison of current study with previously published evidence using same method of T-cell depletion without BPX-501 add-back in GOSH/GNCH

	TCRαβ (n=25); Shah et al, 2017	TCRαβ + BPX-501 (n=6)
Diagnosis		
Malignant	N=0	N=2
PID	N=25	N=4
Stem cell source γ		
Maternal	13▲	2▲
Paternal	11	5
MMUD	2	0
TCRαβ dose X10 ⁴ /kg Median (range)	3.3 (0.075-9.5)	0.25 (0.07-1.11)
CD34+ cell dose X10 ⁶ /Kg Median (range)	17.8 (4.7-50.9)	13.52(8.81-24.06)
aGvHD prophylaxis γ		
CSA	11	0
CSA or Tacrolimus+ MMF	11	0
None	4▲	7▲
Days to neutrophil engraftment	15 (10-27)	18.5 (16-21)
CI of aGvHD		
aGvHD grade I-II	44.1%	83.3%
aGvHD grade III-IV	4.3%	16.6%
cGvHD	None	1 patient
CI of significant viral reactivation ¥	59.9%	16.6%
CI of graft loss	4.2%	0% ¥
1-year TRM (%)	16.4%	16.6%
Overall survival (%)	83.9%	66.6%

▲: A patient failed to engraft post a maternal haplo-HSCT and received either a MMUD (TCRαβ) or a paternal haplo-HSCT (TCRαβ+BPX-501)

¥: Significant viral infection (Shah et al, 2017) included rising blood titers, disseminated or symptomatic infection.

3.6 Discussion on clinical results (Table 3.5)

Historic data; from GOSH and Great North Children's hospital, on the outcome of TCR $\alpha\beta$ depleted transplant with no T cell add-back (TCR $\alpha\beta$) was published by Shah et al, 2017. Though the number of patients in the clinical trial was limited to set a comparative analysis, it was observed that significant viral infection dropped from 59.9% post-TCR $\alpha\beta$ to 16.6% with TCR $\alpha\beta$ +BPX-501. While all TCR $\alpha\beta$ +BPX-501 had achieved complete response or partial control (1 log drop of viral load) in response to anti-viral therapy, 4 out of 25 (16%) TCR $\alpha\beta$ patients had uncontrolled viraemia and received donor derived received cytotoxic T lymphocyte (CTLs) to control CMV or AdV viraemia. The median time to clear blood viral infection was shorter among TCR $\alpha\beta$ +BPX-501 patients than TCR $\alpha\beta$ patients; 40 versus 75.5 days. Moreover, while none of TCR $\alpha\beta$ +BPX-501 had viral induced organ disease, it was noted in 24% of TCR $\alpha\beta$ cases (6/25); including 2 patients with CMV retinitis, 2 with CMV pneumonitis, 1 with CMV enteropathy and 1 with enteroviral encephalitis (Shah et al, 2017). In most patient naïve T cells appeared at 2-3 months post-transplant after TCR $\alpha\beta$ +BPX-501 versus 4 months after TCR $\alpha\beta$ (Shah et al, 2017). BPX-501 deriving endogenous T cell reconstitution and immune recovery might have protected TCR $\alpha\beta$ +BPX-501 patients against significant viral infection.

Unfortunately, BPX-501 add-back was associated with development of aGvHD grades I-IV in all 6 patients. While two patients responded to topical steroid therapy, Four out of six required systemic therapy. AP1903 was administered to 3 patients with complete response in P002, partial response in P003 and P006. P003 had no recurrence of skin GvHD after AP1903 infusion however remained TPN dependent with uncontrolled enteropathy with persistent negative biopsies for aGvHD and barely detectable marked T cells. P006 had Stage 1 skin aGvHD at D+17 that required topical Tacrolimus in conjunction with topical Steroids to treat. BPX-501 infusion was deferred till D+35 when patient had CMV and AdV viral reactivation. Thus, patient was probably in an inflamed status (tissue breakdown) at time of BPX-501 infusion increasing his propensity for development of high grade aGvHD. AP1903 infusion was unable to allow a sustained remission of aGvHD -possibly reflecting that effect of allo-reactive CD3+CD19- cells in recurrence of GvHD. In comparison, TCR $\alpha\beta$ patients only developed grade II skin GvHD with no visceral involvement (Shah et al, 2017).

BPX-501 also potentially induced T cell neurotoxicity in P007 with complete remission in response to a combination of Dexamethasone and AP1903. There was no recorded toxicity to AP1903 administration.

Thus, addition of BPX-501 had boosted TCR $\alpha\beta$ + / CD19 depleted graft for a better control over viral infection however increased the risk of GvHD. Of note, in 3 out of 4 patients who received AP1903 that marked T cells either re-appeared (P002) or persisted after AP1903 infusion (P006, P007) raising concerns regarding a potential escape mechanism. Further investigation into the mechanism of resistance to AP1903 is discussed in chapter 4; section 4.9.

Chapter 4:
Production of a Lentivirus vector for transfer of icas9 in primary T cells

Introduction:

Lentiviral vectors are distinct members of Retroviridae family of viruses. They have many features of γ retroviral vectors with a stable ability to integrate within the host genome in addition to being able to stably transduce not only dividing cells as γ retroviral but also non-dividing cells (Naldini et al, 1996). Genotoxicity studies showed that these vectors harbour a reduced genotoxic potential compared to analogous γ retroviral constructs in HSC (Montini et al, 2009, Modlich et al, 2005). Lentiviral vectors have been used in several clinical trials in human with no reported toxicity or oncogenic transformation (Levine et al, 2006, Cavazzana-Calvo et al, 2010, Aiuti et al, 2013, Biffi et al, 2013). In an approach to use a safer vector for transfer of icas9 transgene to T cells, we examined the possibility of cloning icas9 transgene into a lentiviral vector.

Objectives:

1. Testing an alternative vector (lentivirus) for icas9 gene transfer in primary T cells.
2. To test the effect of the commonly used immune suppressive drugs on these genetically modified T cells together with the killing effect of AP1903.
3. To investigate resistance to AP1903.

4.1 Transfer of inducible gene (iCas9) into a lentiviral vector

icas9 transgene was successfully amplified by PCR from retrovirus plasmid with icas9 (MMLV. iCas9.T2A.CD19) as shown in Figure 4.1 and was subsequently cloned into PCCL1-hPGK vector as shown in Figure 4.2.

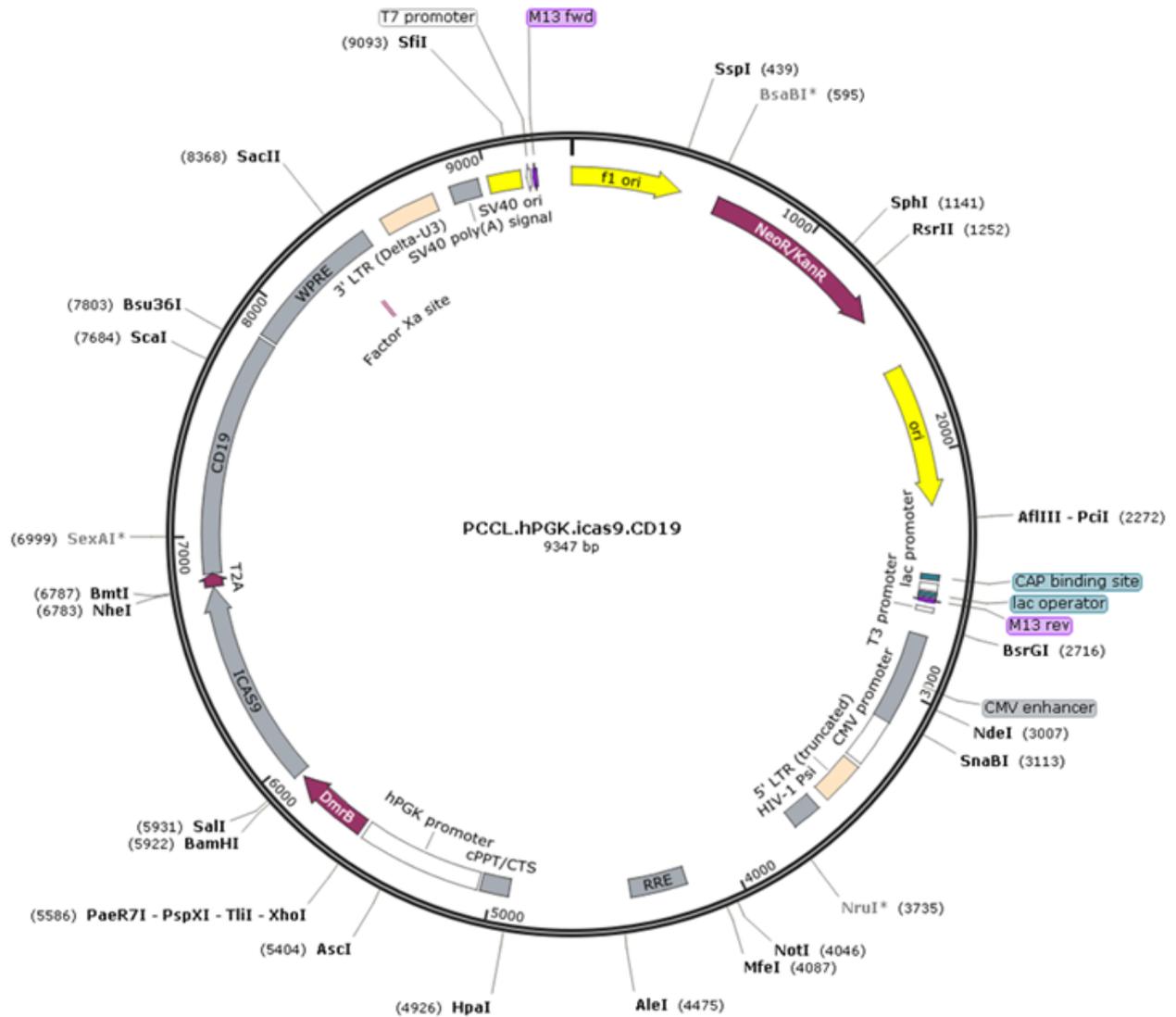
Good DNA yield was obtained for both miniprep and maxiprep of Lenti-icas9: 179 ng/ul and 1450 ng/ul, respectively. Production of a Lentivirus with icas9 (PCCL1-hPGK-icas9) was carried out twice and was successful in both experiments with a viral titre for harvest 1(H1) between $1.4-4.8 \times 10^8$ IU/ml.

Figure 4.1 Successful PCR amplification of icas9 transgene and results of test digest of PCCL1-HPGK-CD34



On the right of the ladder, results of test digest of PCCL1-HPGK-CD34 on the gel using Bam H1(7Kb). On the left of the ladder is PCR reaction for amplification of icas9 transgene (2.2 Kb). On the right side of the gel is a representation of 1Kb plus ladder.

Figure 4.2. Schematic representation of successful cloning of icas9 transgene into PCCL1-hPGK vector

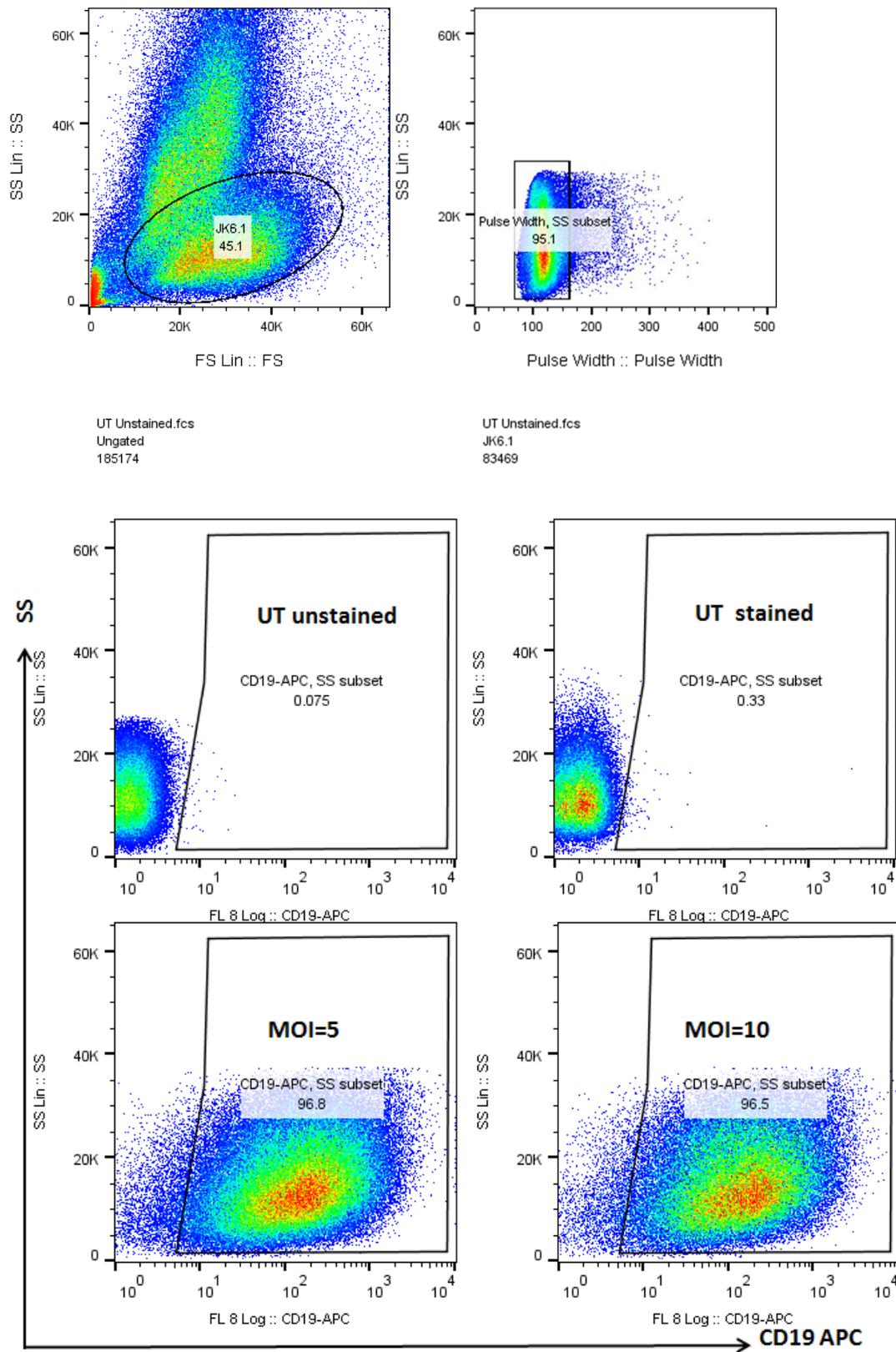


4.2. Successful transduction of Jurkats 6.1 and primary T cells with Lenti-icas9 virus:

4.2.1. Efficient transduction of Jurkats 6.1 with Lenti-icas9

At MOI of 5 and 10, transduction efficacy of Jurkats 6.1 with Lenti-icas9 was 96.47% and 96.17% as shown in Figure 4.3. Repeat assessment of transduction efficacy of Jurkats with Lenti-icas9 at MOI: 5 was carried out 3 times with a median of 97%. Based on these results an MOI of 5 was used to test the transduction efficacy in primary cells.

Figure 4.3. High transduction efficacy of JK 6.1 transduced with Lenti-icas9 at MOI of 5 and 10.



Abbreviations: UT: untransduced cells, SS: side scatter, MOI: multiplicity of infection.

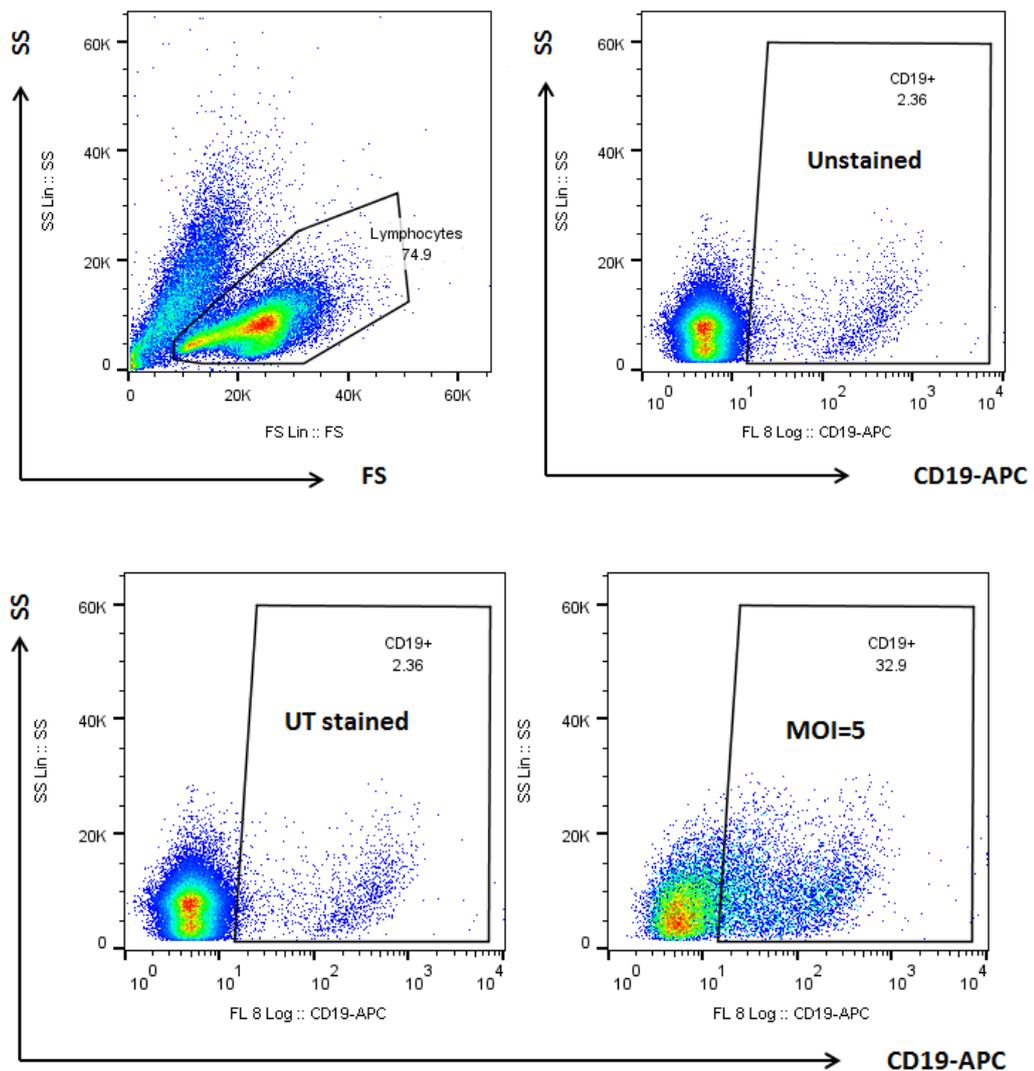
4.2.2 Efficient transduction of T cells with Lenti-icas9 virus:

At MOI of 5, using 6 different donors, the median transduction efficacy of primary cells with Lenti-icas9 was 40.9%; range: 18%-52.2% as shown in figure 4.4.

Conclusions:

1. Feasibility of production of a lentivirus vector having icas9 suicide gene.
2. High transduction efficacy was achieved in Jurkats 6.1 and T cells transduced with PCCL1-HPGK-FKBP-icas9-CD19 virus.

Figure 4.4: Example 1 of successful transduction of primary cells with Lenti-icas9 at MOI of 5



4.3 Identification of 2 populations of icas9 T cells based on CD19 expression

It was noted that the marked T cells that escaped the effect of AP1903 and survived AP1903 in P002, P003 and P007 had low expression of CD19 with median Fluorescent intensity (MFI) of less than 1000 (Figure 4.16). To investigate if the degree of CD19 expression would influence response to AP1903, an experiment was set to sort T cells transduced with lenti-icas9 into 2 populations based on MFI for CD19 APC, a, MFI population with high expression of CD19 (icas9); MFI of 18,627 and a population with low expression of CD19 (icas9); MFI of 1,168 as shown in figure 4.5.

These 2 populations were collected separately using Aria sorter. Efficiency of cell sorting for CD3+CD19+ T cells was checked using CyAn flowcytometry with purity results between 85%-90% as shown in figure 4.6.

4.4 Effective clearance of transduced Jurkats 6.1 with Lenti-icas9 in the presence of AP1903

Viability of transduced Jurkats 6.1 cells with Lenti-icas9 dropped from 100% in absence of AP1903 to 10% in a drug concentration of 1-10nM as shown in figure 4.7.

4.5 Low expressing icas9 T cells escaped the effect of AP1903

High expressing transduced T cells with Lenti- icas9 were sensitive to AP1903 addition with 80% killing in the vicinity of 10nM of AP1903 at 24-hour assessment. No effect was recorded on low expressing T cells with Lenti-icas9 at 3.5hrs and 24 hours., even at high drug concentrations; 1000nM and 3000nM (Figure 4.8).

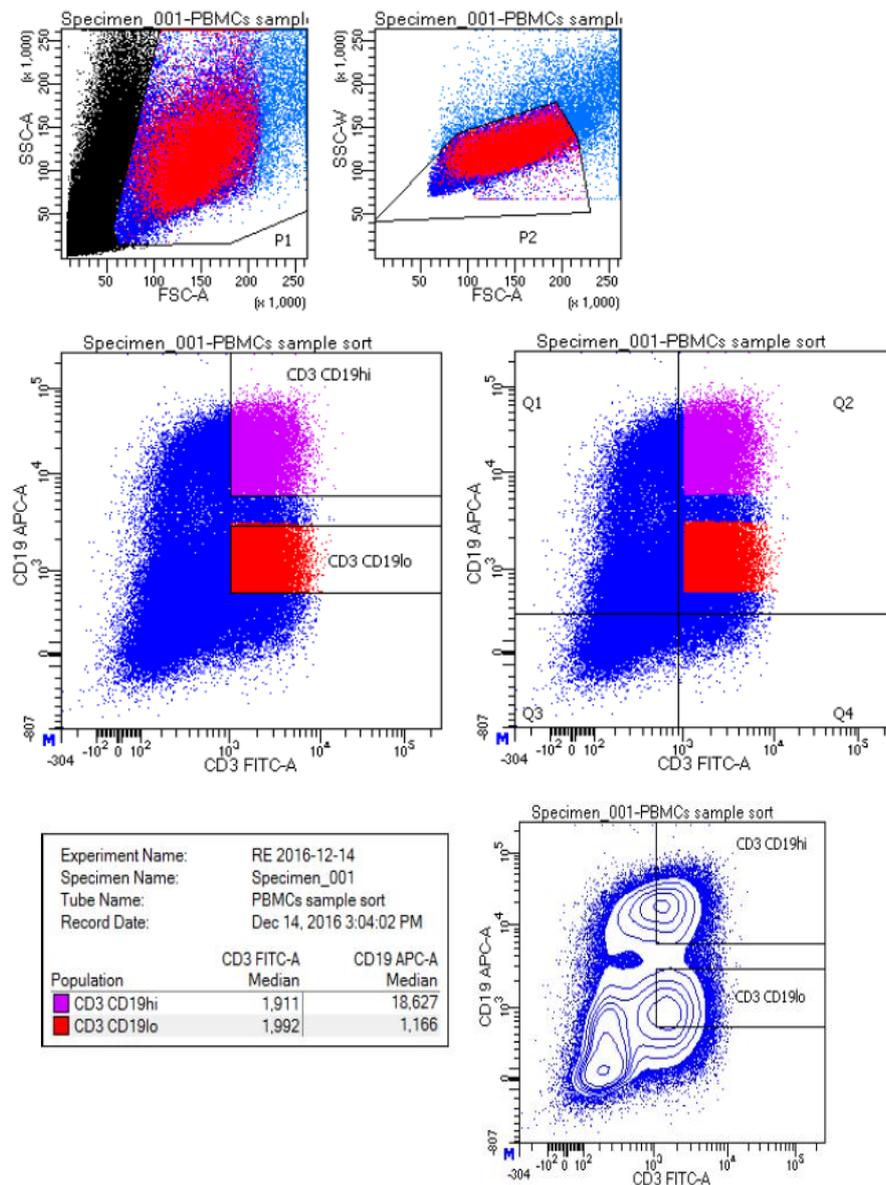
4.6 AP1903 induced early apoptosis followed by late necrosis of high expressing icas9 T cells

A distinguishable shift of cells at all drug concentrations was seen in high expressing population where cells showed early apoptosis at 3.5 hrs. shifting to late apoptosis/necrosis at 24 hours after addition of AP1903 as shown in Figure 4.9.

Conclusion:

1. AP1903 is an effective switch off medicine for clearance of transduced cells (Jurkats 6.1 or T cells) with lenti-icas9 with 80-90% clearance at a concentration as low as 10 nM.
2. The genetically modified T cells with Lenti-icas9 that survive AP1903 killing are the low expressing cell population.

Figure 4.5. Cell sorting for Lenti-icas9 using Aria sorter based on MFI



Abbreviation: CD3CD19hi: represents CD3+CD19+ cells with high MFI for CD19 APC. CD3CD19Lo: represents CD3+CD19+ cells with low MFI for CD19 APC

Figure 4.6. Gating for CD19+ APC using CyAn flowcytometry showed effective sorting of T cells with Lenti-icas9 low and Lenti-icas9 high

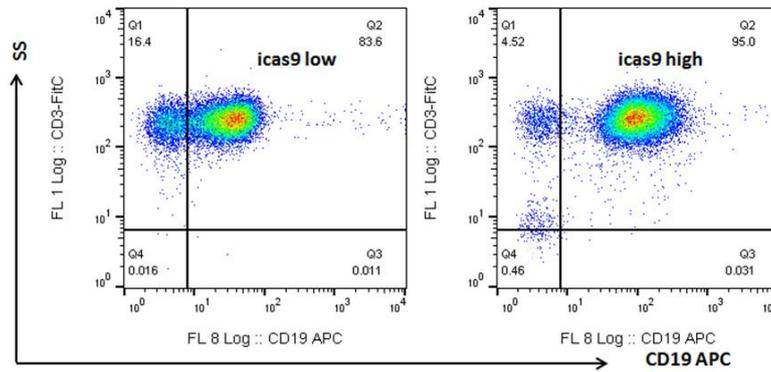
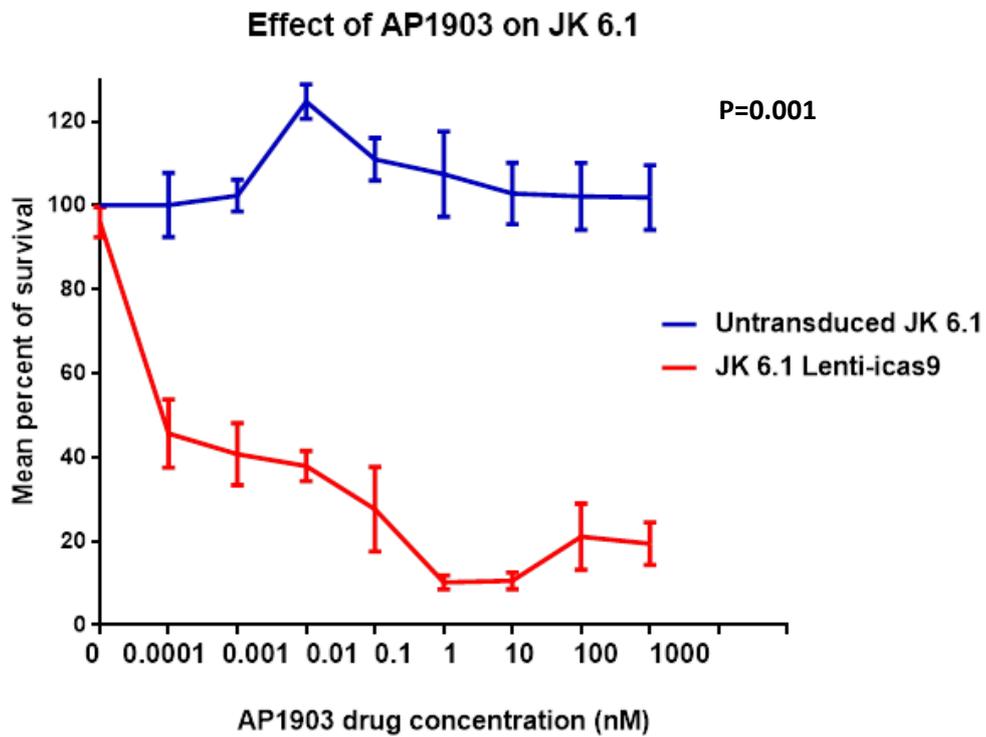


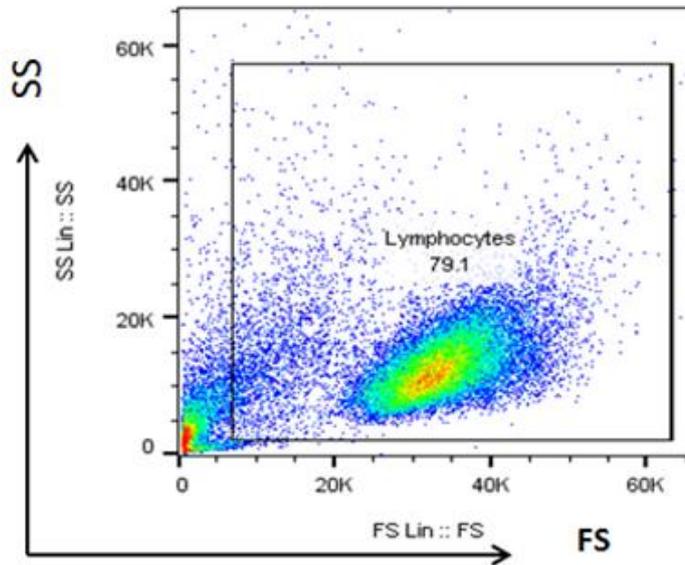
Figure 4.7: Reduced viability of transduced JK 6.1 with Lenti-icas9 in the presence of AP1903



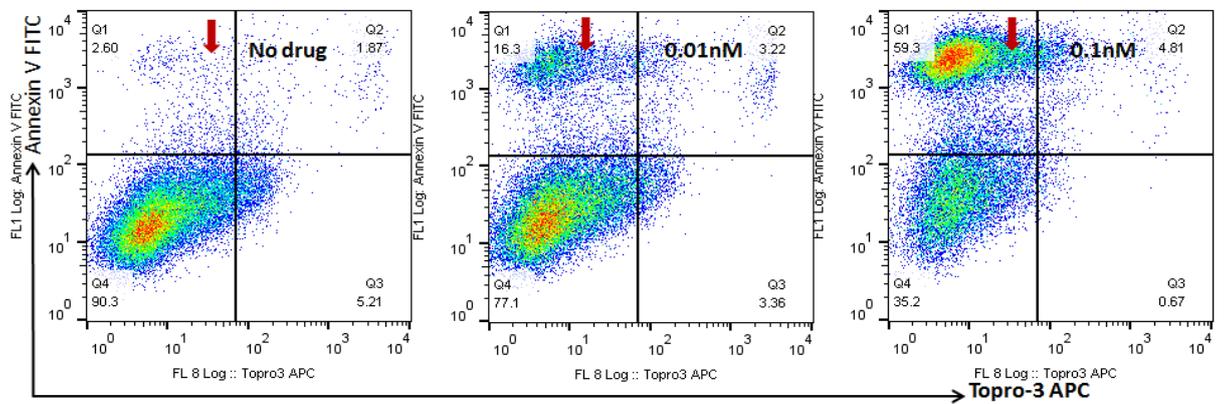
Data presented as mean and standard error of mean (SEM) of 3 readings

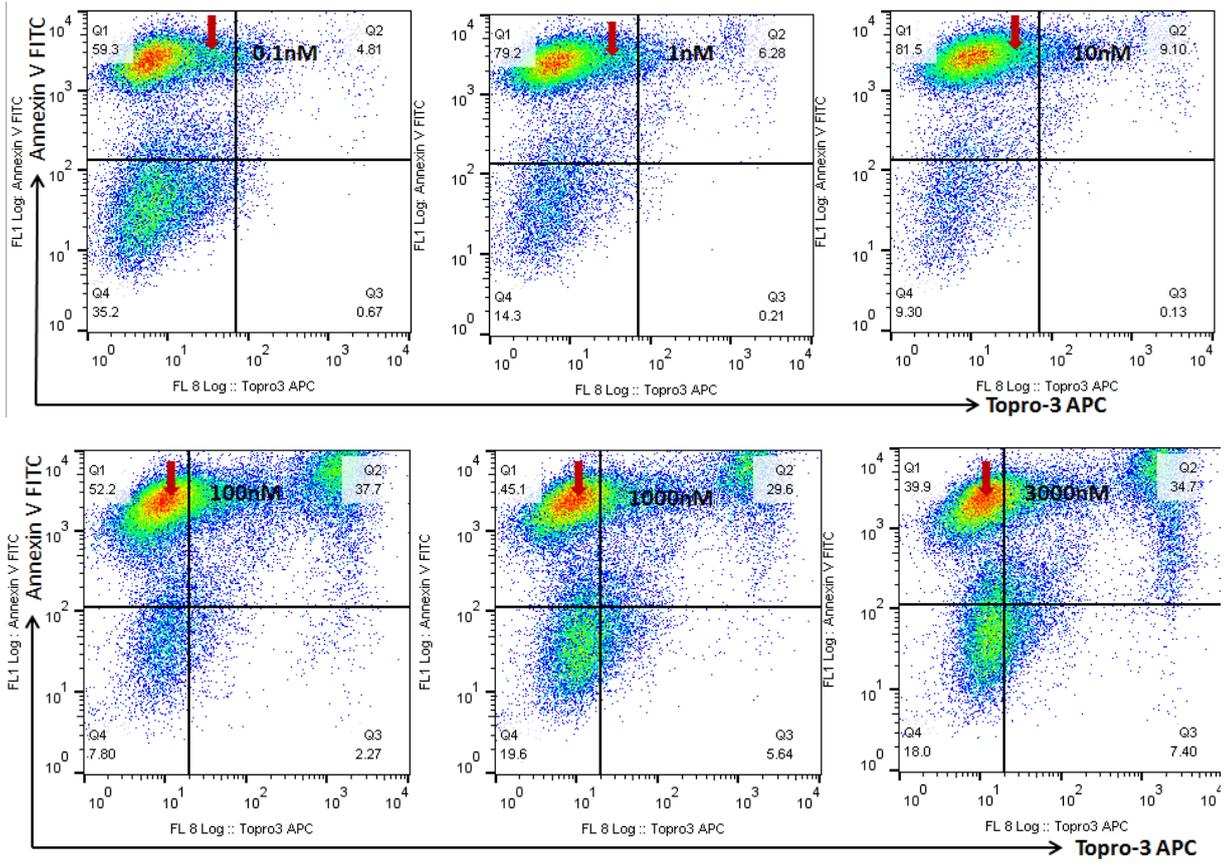
Figure 4.8. Effect of AP1903 on T cells with Lenti-icas9 high and Lenti-icas9 low at 3.5 and 24 hours of incubation

a) An example of gating for lymphocytes for apoptosis assay

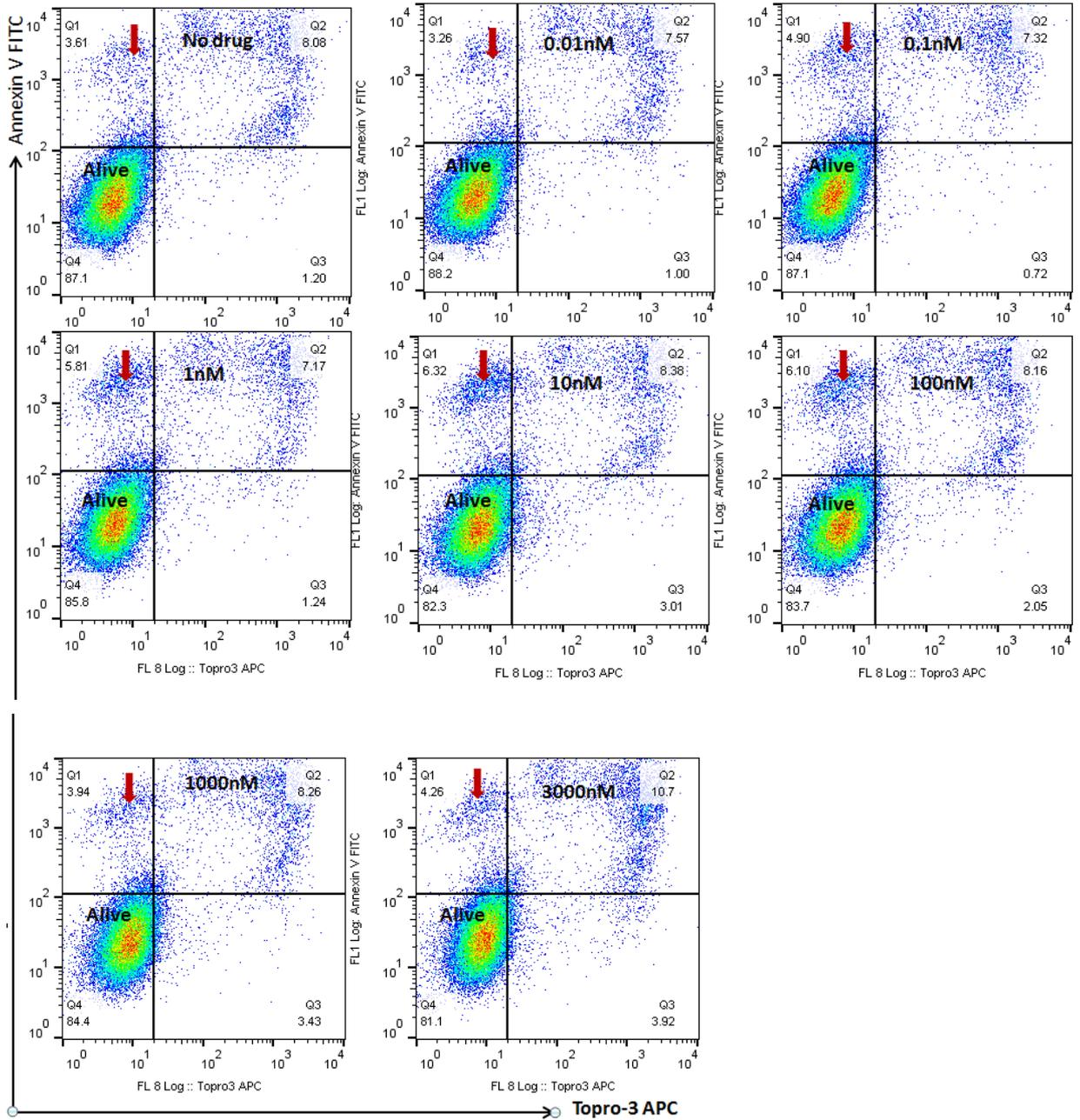


b) Apoptosis of T cells with Lenti-cas9 high at 3.5 hrs after exposure to AP1903

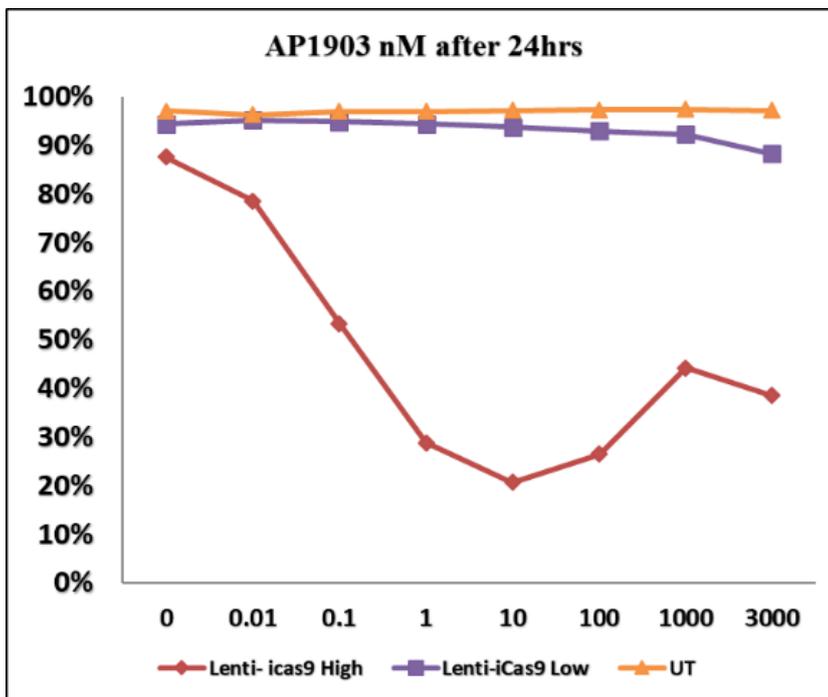
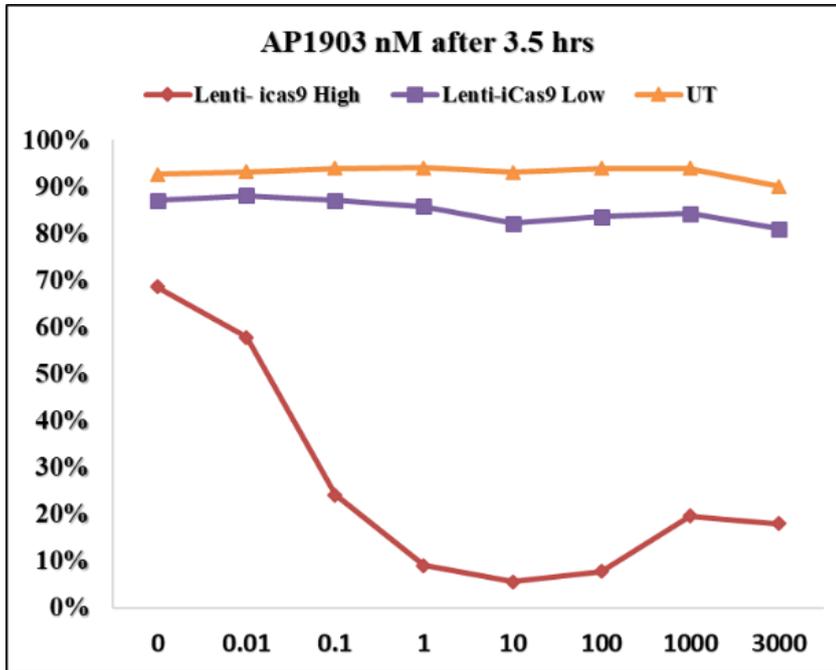




c) No response to AP1903 killing in T cells with Lenti-icas9 low at 3.5 hrs post-exposure even at high concentration of AP1903; 1000nM and 3000nM.

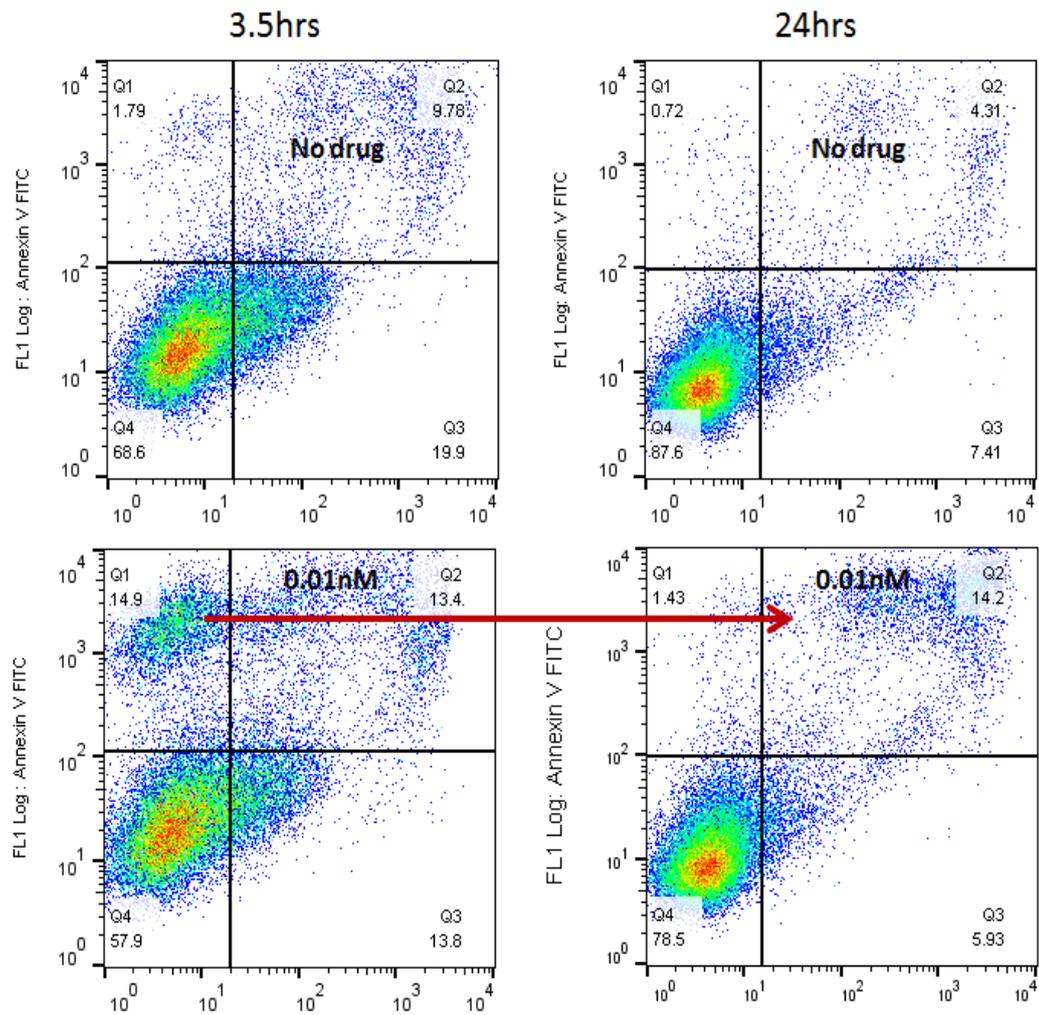


d) Effect of AP1903 on T cells transduced with Lent-icas9 at 3.5hr and 24hrs post-incubation in comparison to UT T cells



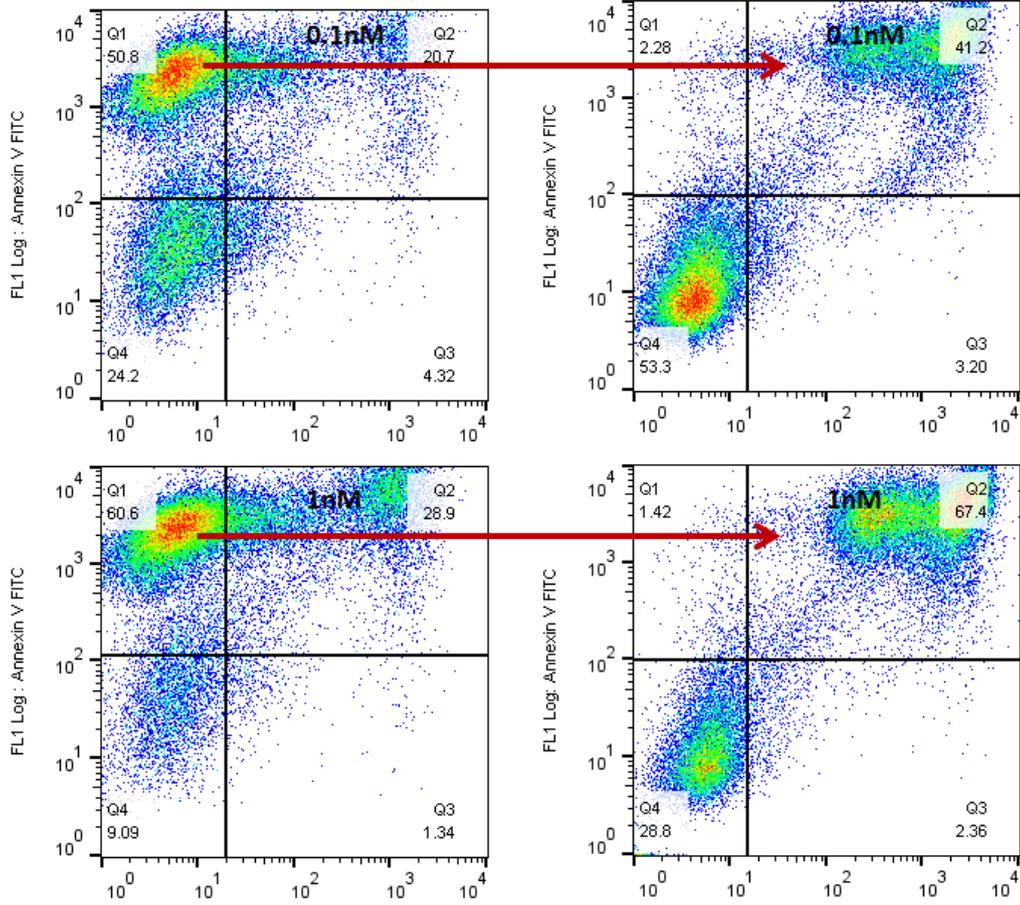
In figure (a), the gate was set to cover all cells in the field as dead cells will be scattered around and they need to be counted. Red arrow: denotes apoptotic cell population.

Figure 4.9. Under different concentrations of AP1903, T cells with Lenti-icas9 high shifted from early apoptosis at 3.5 hrs to late apoptosis/necrosis at 24 hrs (as the red arrow denotes)



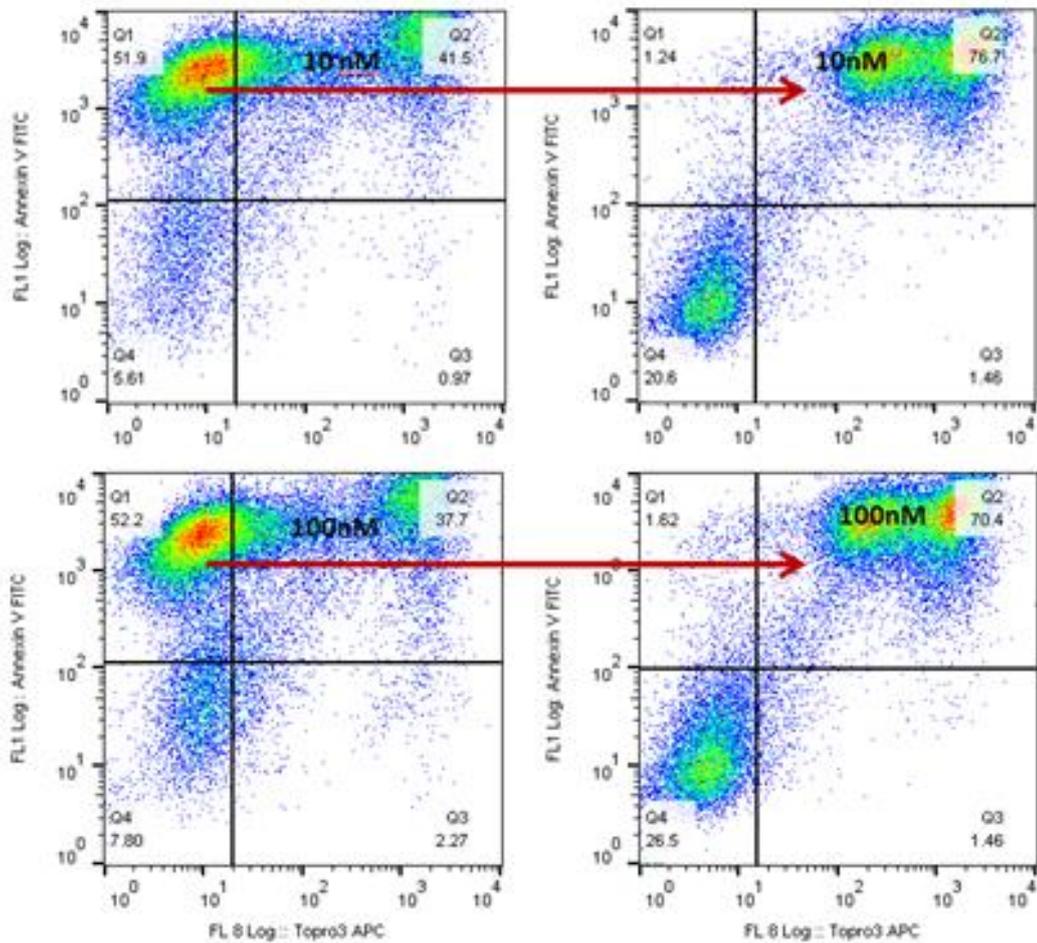
3.5 hrs

24 hrs



3.5 hrs

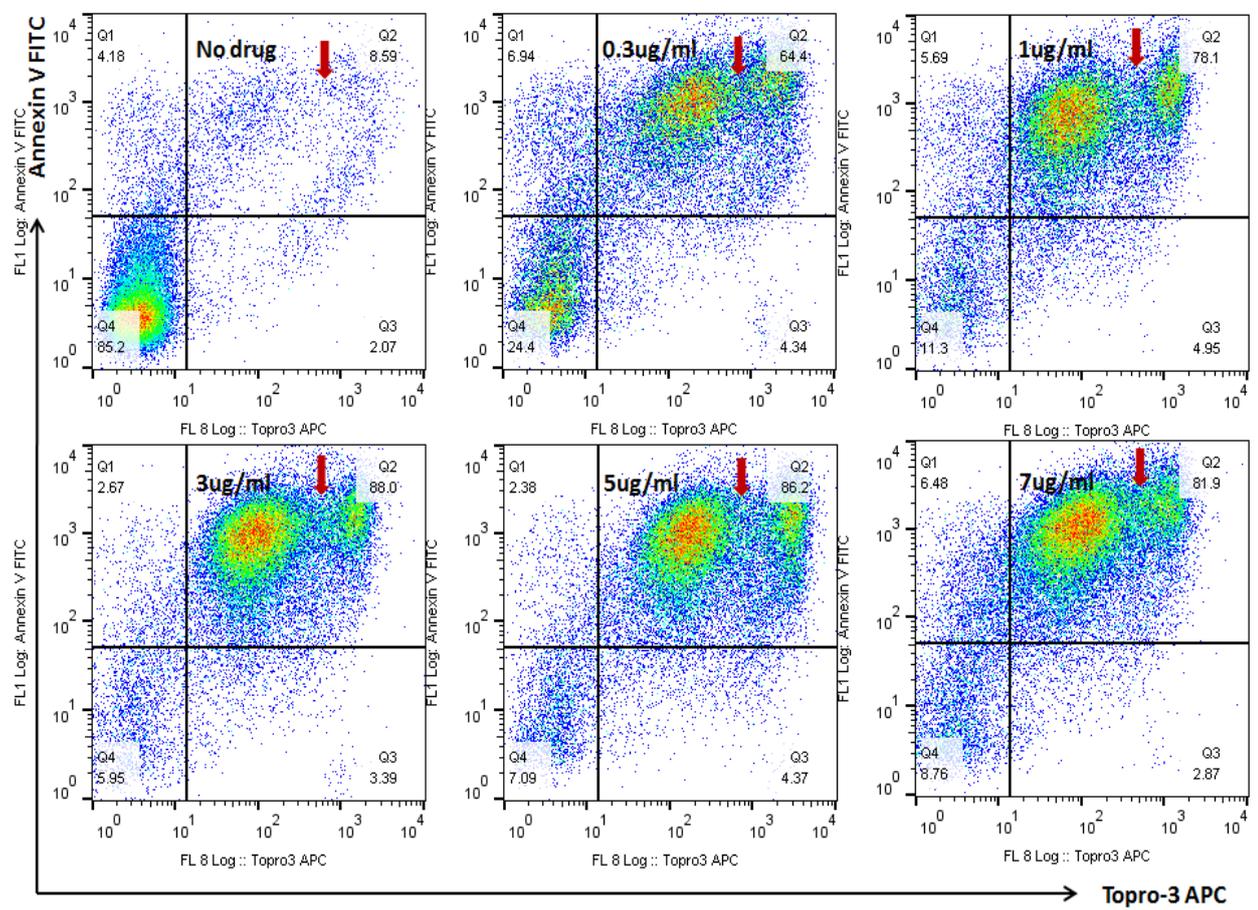
24 hrs



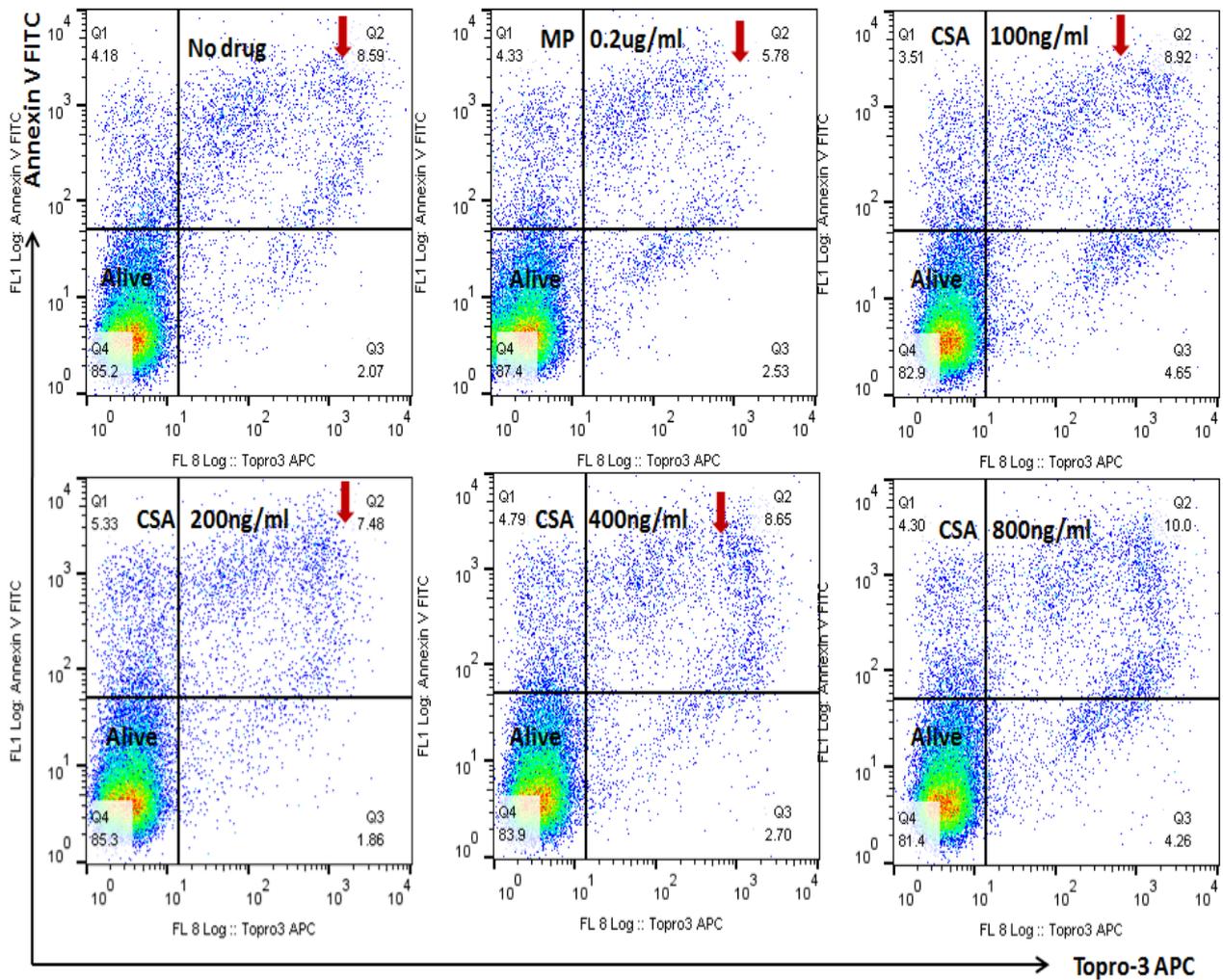
Y-axis represents Annexin V FITC and X-axis represents Topro-3 APC. Red arrow shows the shift of cells from early apoptosis (Annexin V+/Topro3-) to late necrosis (Annexin V+/Topro3+) at 3.5 hrs. and 24 hrs. of AP1903 exposure, respectively.

Figure 4.10. Effect of immune suppressive medications on survival of T cells transduced with Lenti-icas9 using Annexin V/Topro-3 apoptosis /necrosis assay

a) MMF (0.3-7ug/ml) induced apoptosis/necrosis of T cells with Lenti-icas9



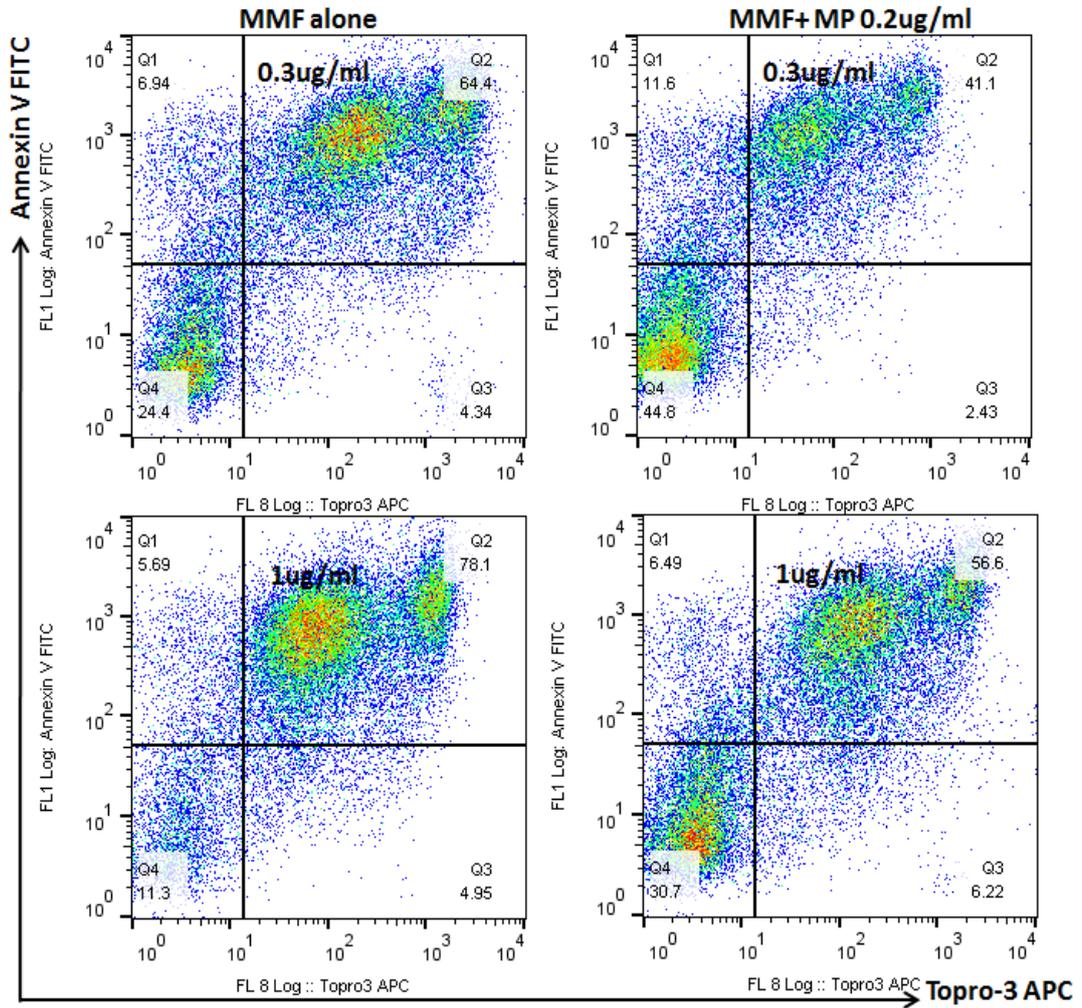
b) MP (0.2ug/ml) and CSA (100-800 ng/ml) had no effect on survival of T cells with Lenti-icas9

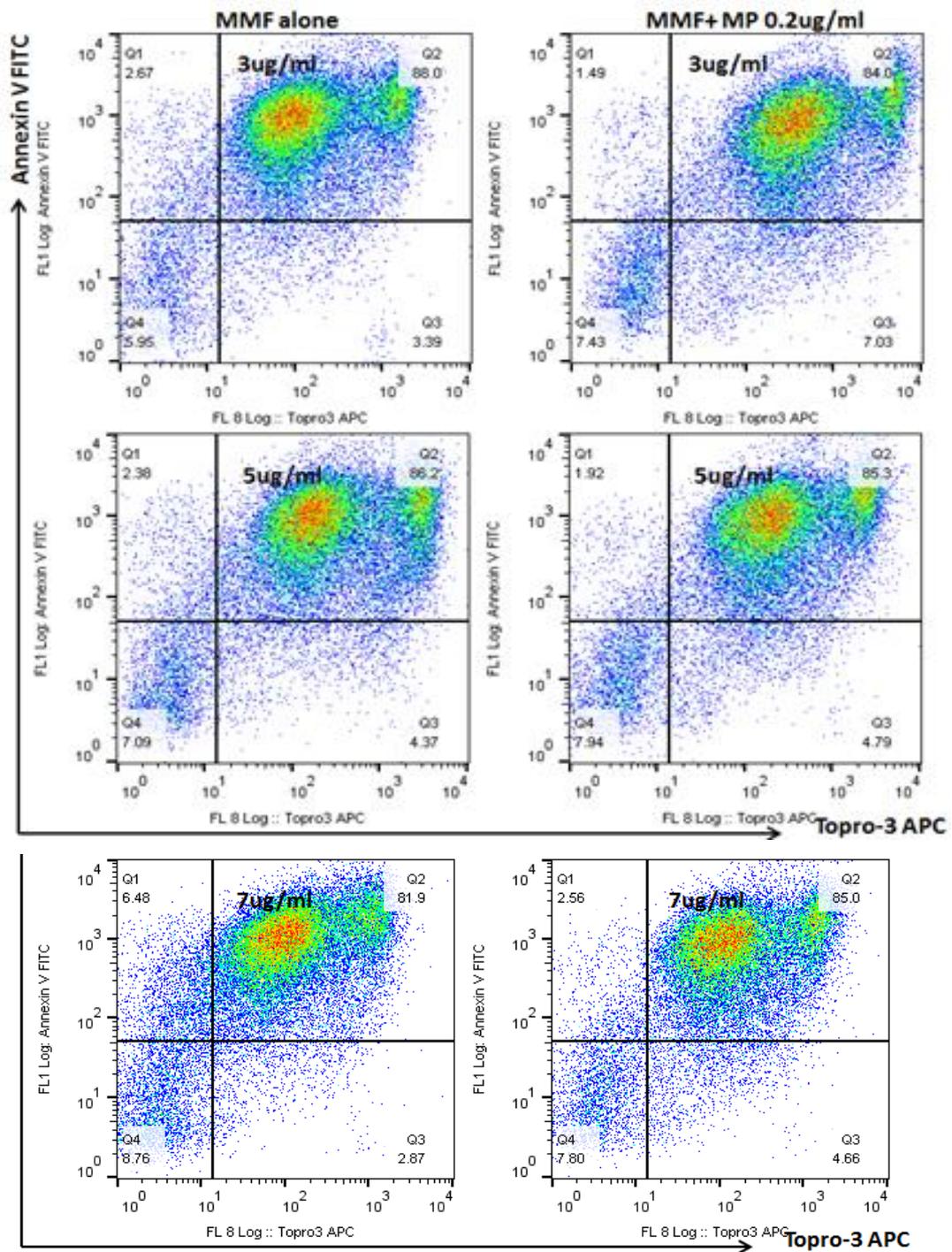


Red arrow: denotes late apoptotic/necrotic cell (Annexin V+ Topro-3 +) population

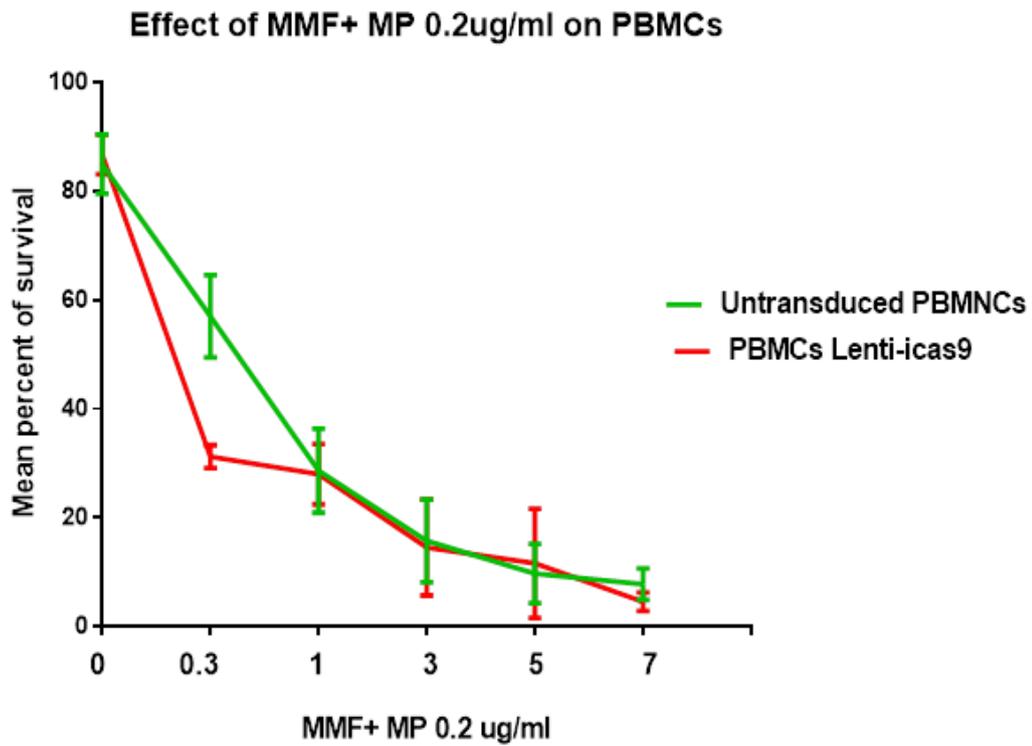
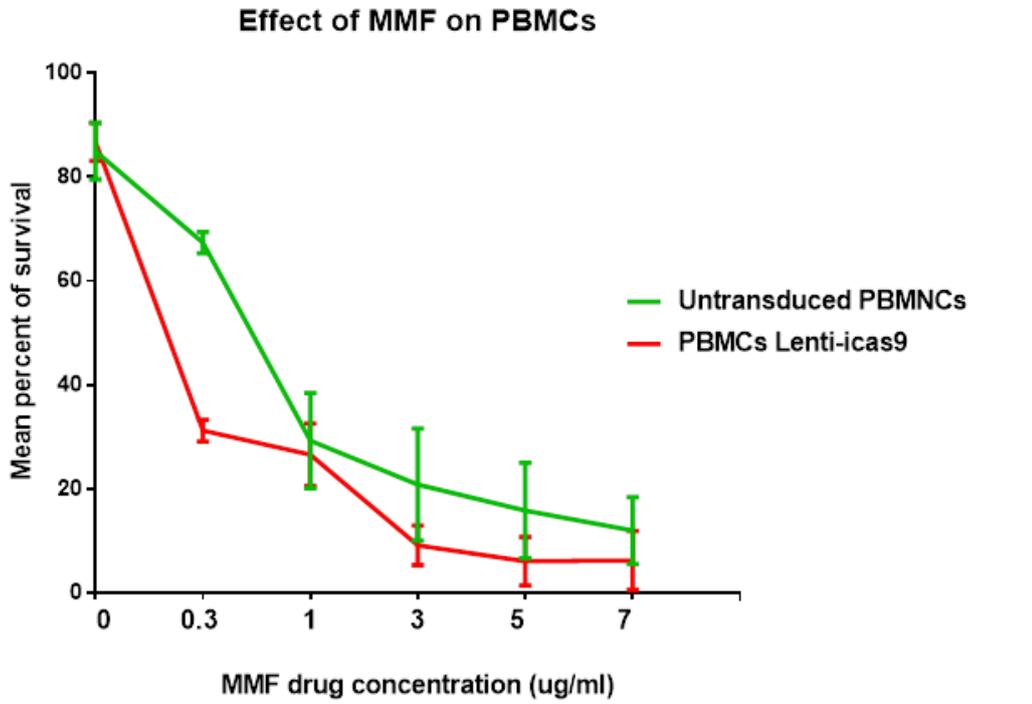
Figure 4.11. Effect of MMF (0-7ug/ml) ± MP 0.2ug/ml on survival of T cells with lenti-icas9 versus untransduced T cells

a) Flowcytometry showing the effect of MMF± MP 0.2ug/ml on survival of T cells with lenti-icas9





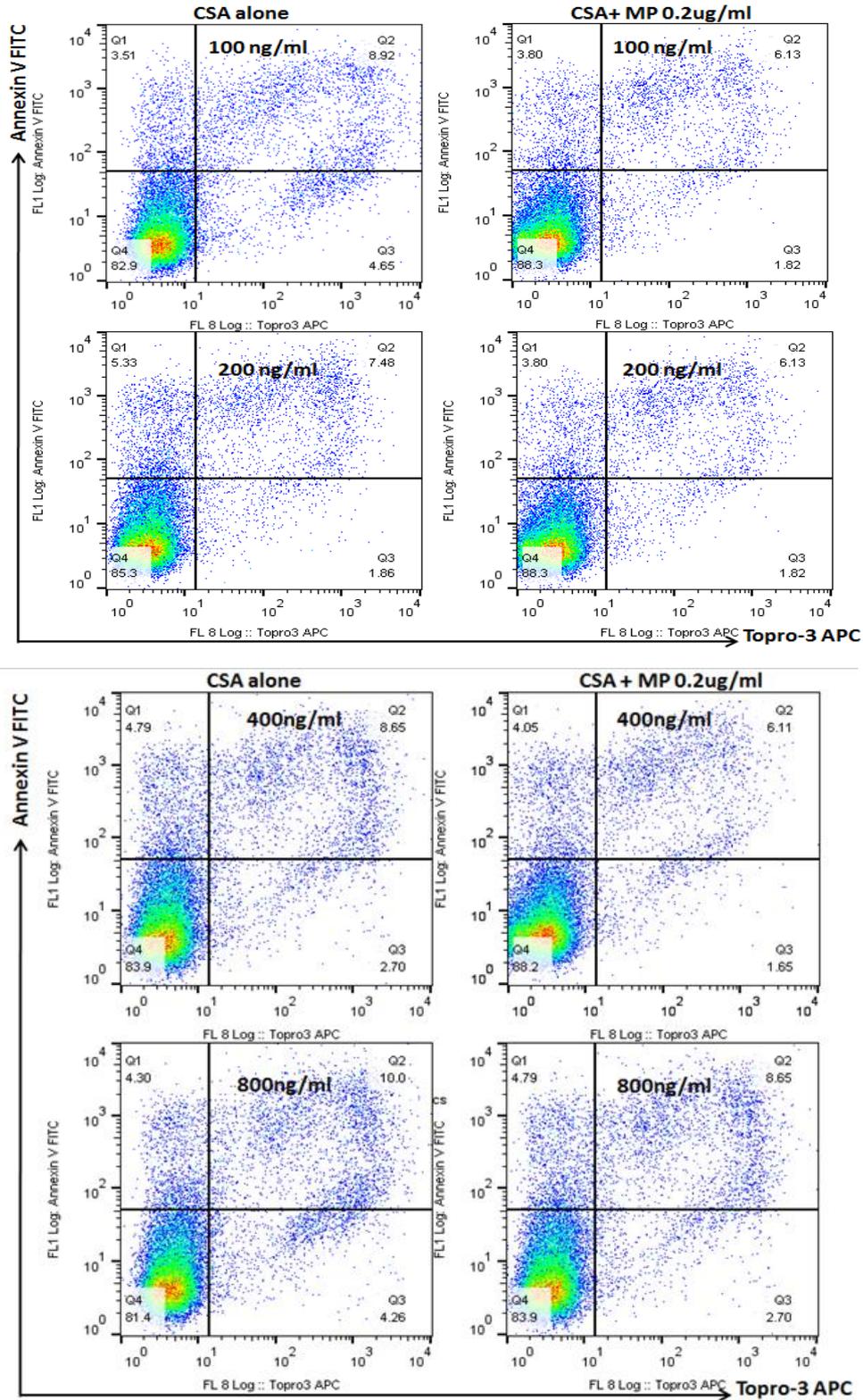
b) At different concentrations of MMF (0-7 ug/ml) in addition of MP (0.2 ug/ml) did not have any synergistic effect on T cells with Lenti-icas9 versus untransduced T cells



Mean and SEM of 3 readings were presented in figure.

Figure 4.12 Effect of CSA (0-800ng/ml) \pm MP 0.2ug/ml on T cells with Lenti-icas9 versus untransduced T cells

a) Flowcytometry showing effect of CSA \pm MP on T cells with Lenti-icas9



b) CSA (0-800 ng/ml) alone or in combination to MP (0.2ug/ml) did not affect the survival of either T cells with Lenti-icas9 or untransduced T cells.

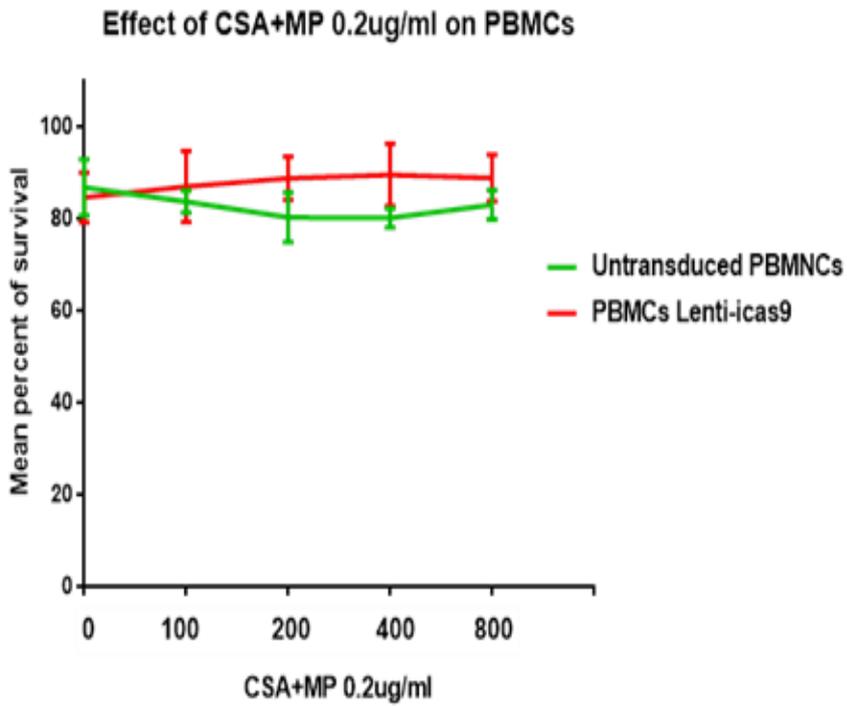
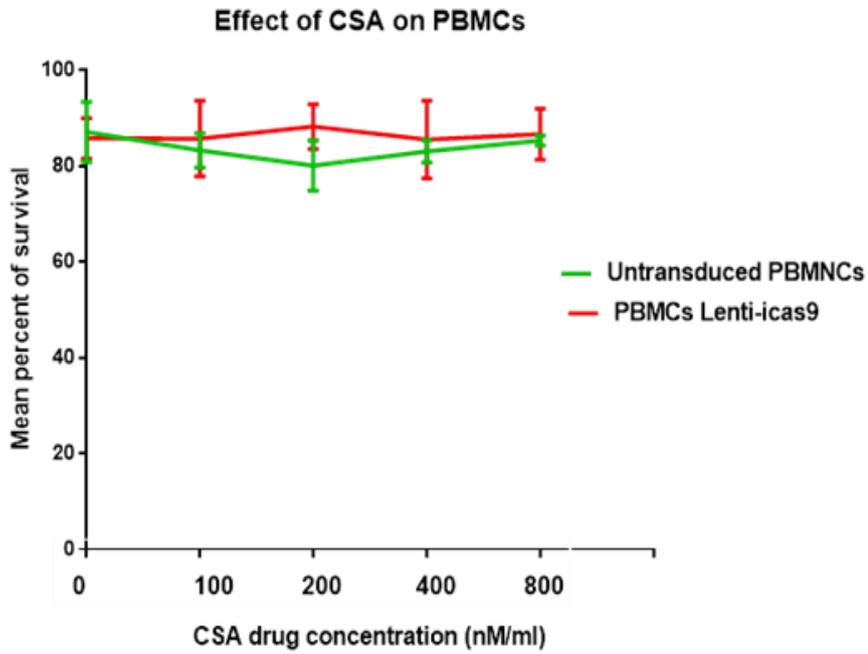
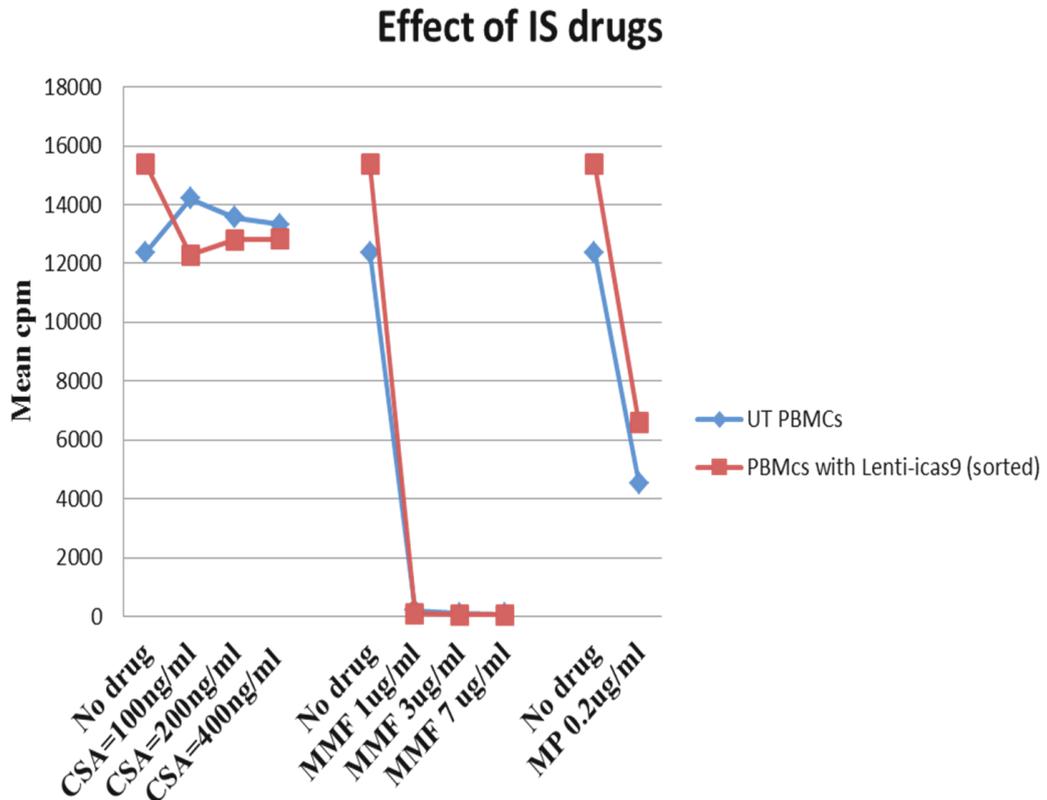


Figure 4.13. Effect of immune suppressive drugs on proliferative capacity of T cells with Lenti-icas9 versus untransduced T cells



4.7 The effect of immune suppressive medications on survival of T cells transduced with Lenti-icas9 using Annexin V/Topro-3 apoptosis /necrosis assay

Survival of the transduced T cells with lenti-icas9 dropped from 100% in absence of MMF reaching less than 20% survival at a concentration of 3 ug/ml of MMF as shown in figures 4.10 and 4.11. There was no reported difference between untransduced or transduced T cells with lenti-icas9 in response to CSA ± MP or MP alone; figures 4.10 and 4.12.

4.8 The effect of immune suppressive medications on proliferative capacity of T cells transduced with Lenti-icas9

Using radioactive thymidine assay, no proliferation was seen among both transduced and untransduced T cells with Lenti-icas9 upon exposure to 1ng/ml of Mycophenolate Mofetil as shown in figure 4.13. A drop in T cell proliferation to 20% was recorded among both untransduced T cells and transduced T cells with lenti-icas9 upon

exposure to 0.2 ug/ml of MP as shown in figure 4.13. On the contrary, different concentrations of CSA did not influence transduced and untransduced T cell proliferation after 96 hours of incubation with AP1903.

Conclusion:

1. MMF and MP inhibits the proliferation of the CD3+CD19+ T cells and non-transduced T cells.
2. MMF also affects their survival.

4.9 Investigate mechanism of resistance to AP1903

4.9.1 Resistance of P002 and P007 icas9 T cells to in vitro killing by AP1903

To understand the escape mechanism to AP1903, PMNCs from P002, P003 and P007 were sorted for CD3+CD19+ cells.

Marked T cells that escaped the effect of AP1903 and survived AP1903 in P002, P003 and P007 had low expression of CD19 with median Fluorescent intensity (MFI) of less than 1000 as in figure 4.15.

An experiment was set to check for in vitro response of these sorted T cells to AP1903. Sorted CD3+CD19+ T cells from P002 were resistant to killing by AP1903 with 100% survival in comparison to a drop of survival of transduced JK 6.1 with lenti-icas9 from 100% in the absence of drug exposure to less than 20% survival at concentrations of AP1903 from 10 nM to 3000nM (Figure 4.4). Sorted CD3+CD19+ T cells from P007 showed no response to AP1903 at 10nM and 14% killing at 3000nM. Few cells were available after sorting for P006 – reflecting steroid therapy- thus further analysis for in vitro response to AP1903 was not applicable (Figure 4.16).

Figure 4.14. Resistance of CD3+CD19+ cells from P002 to the killing effect of AP1903

a) CCK8 assay plate for P002



a) CCK8 assay results

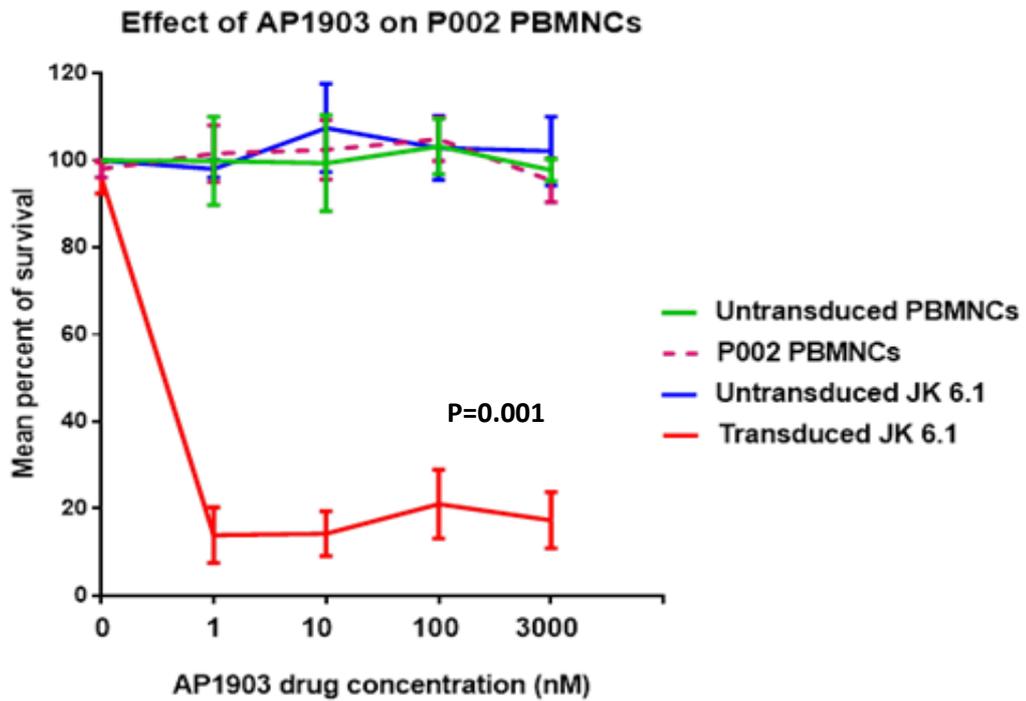
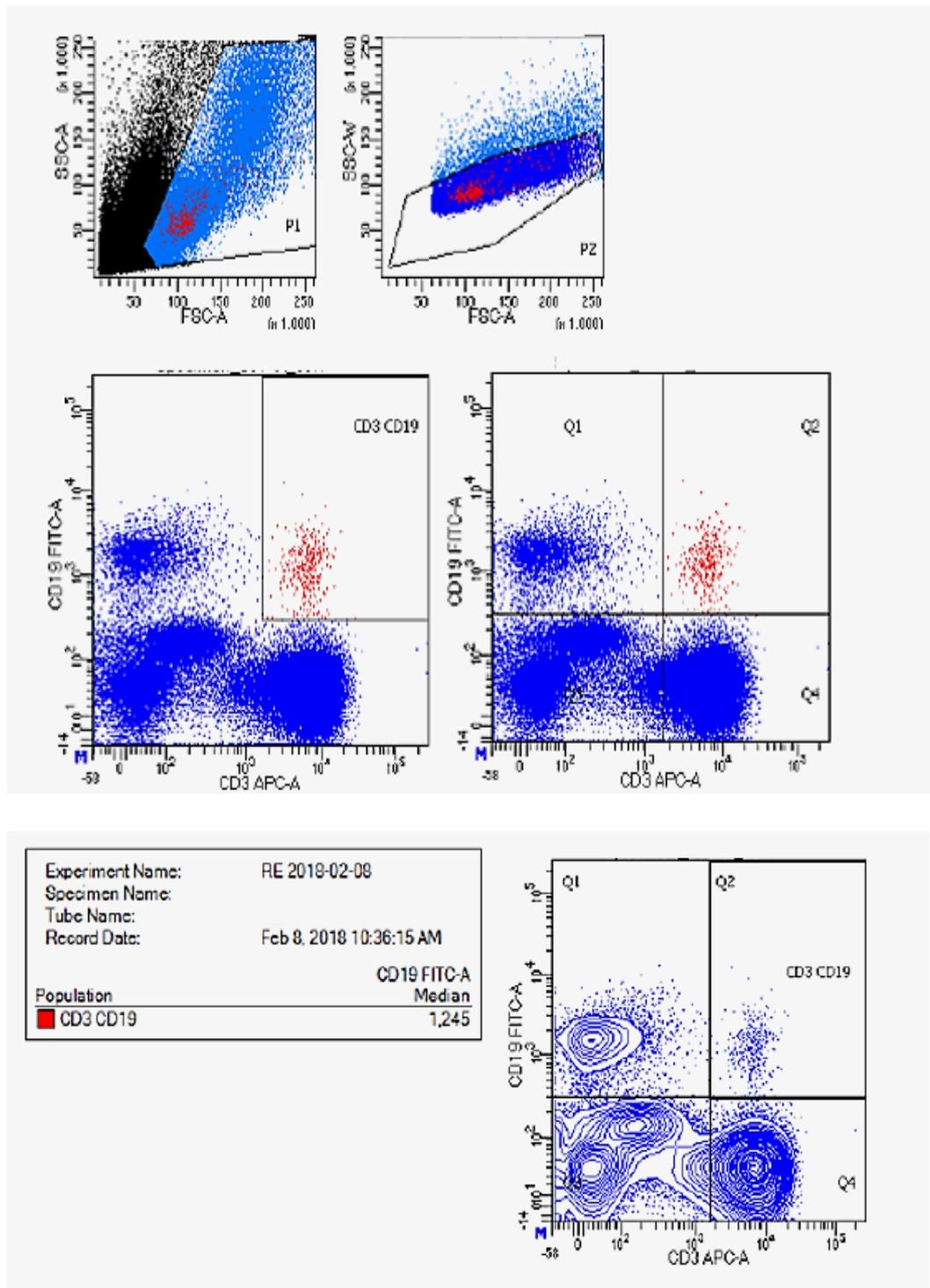


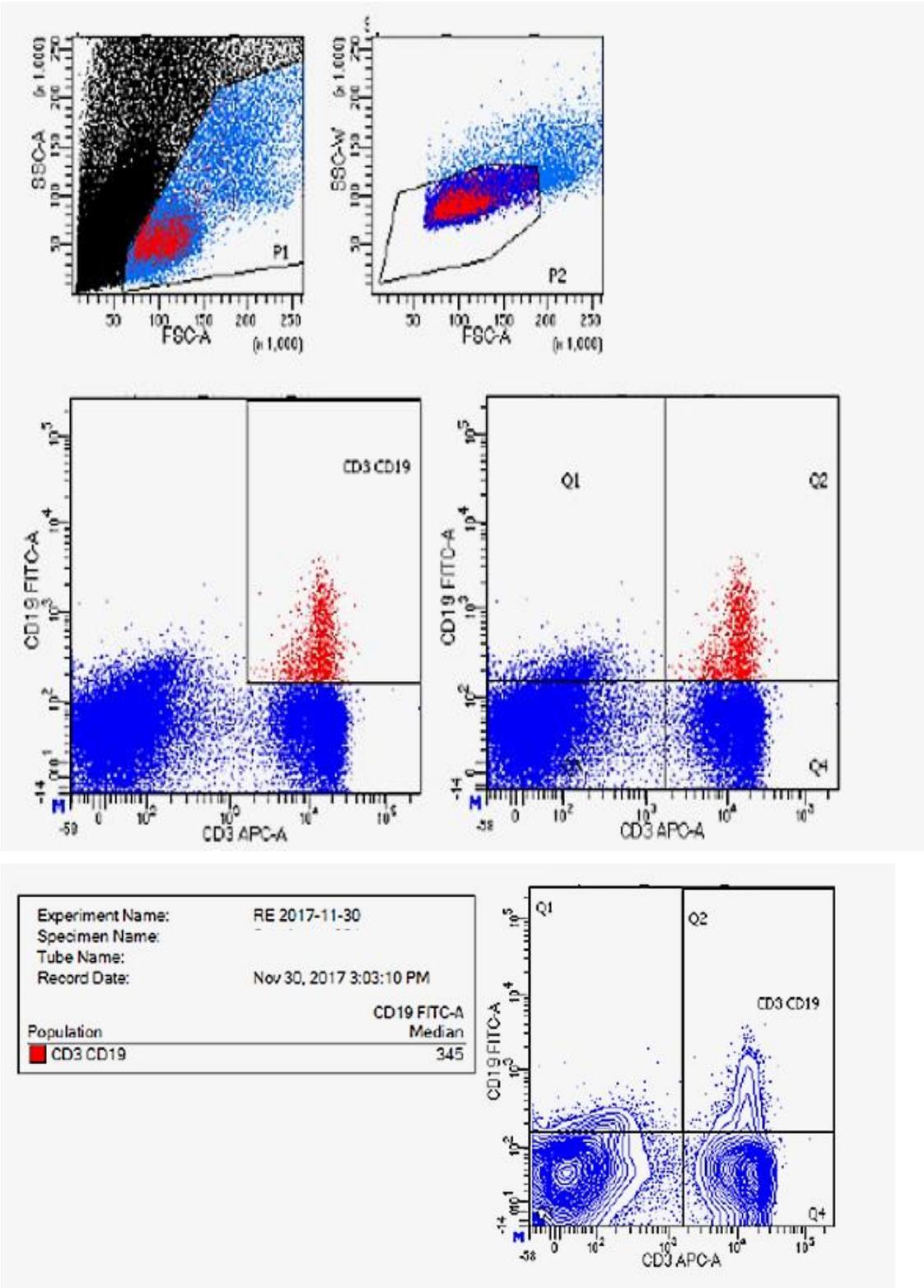
Figure (a) showed no change of colour of P002 cells, untransduced T cells, untransduced JK 6.1 in comparison to a colour change of JK 6.1 transduced with lenti-icas9 (denoting killing) upon exposure to various concentrations of AP1903.

Figure 4.15. Sorting of CD3+CD19+ cells from P002, P006, P007

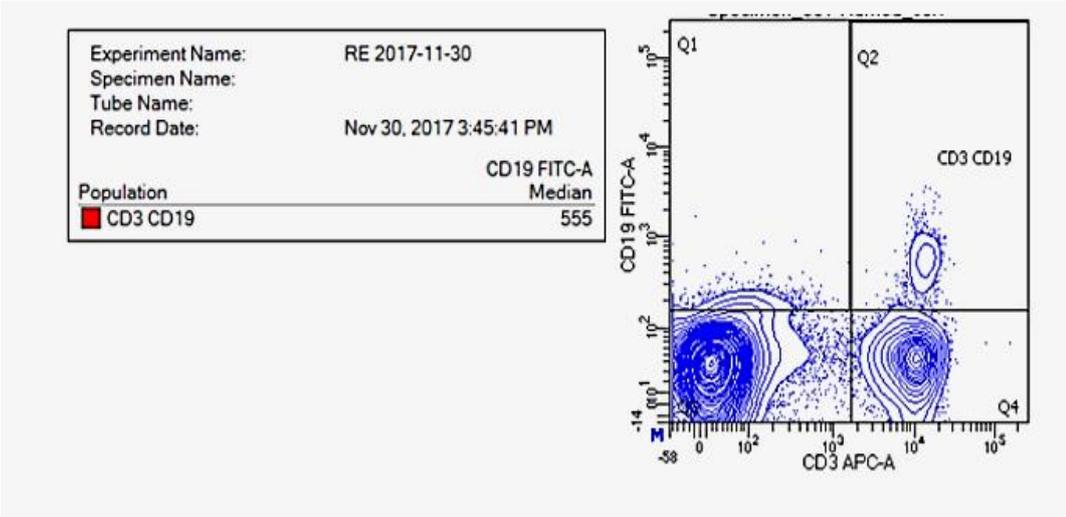
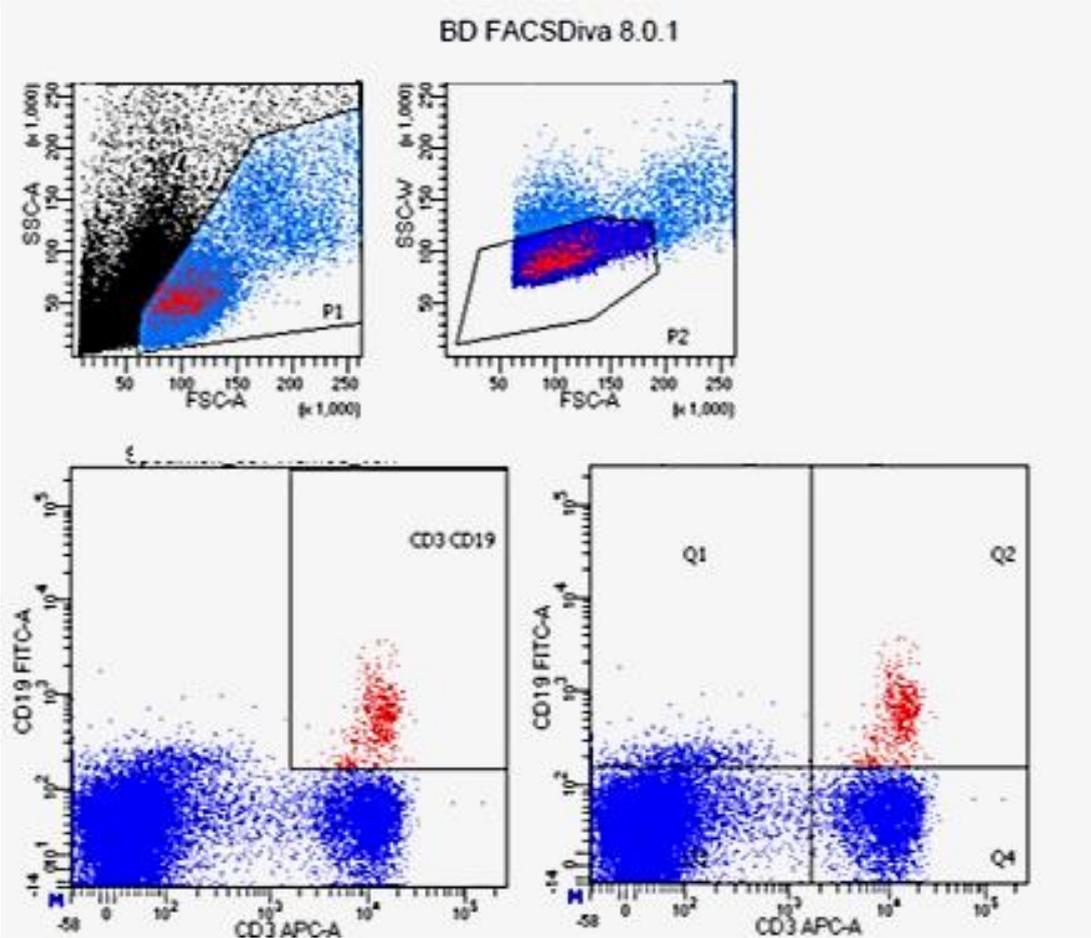
a) Flow cytometry plots for P002



b) Flow cytometry plots for P006



c) Flow cytometry plots for P007



d) Surviving icas9 T cells from P002, P006 and P007 have low expression of CD19+

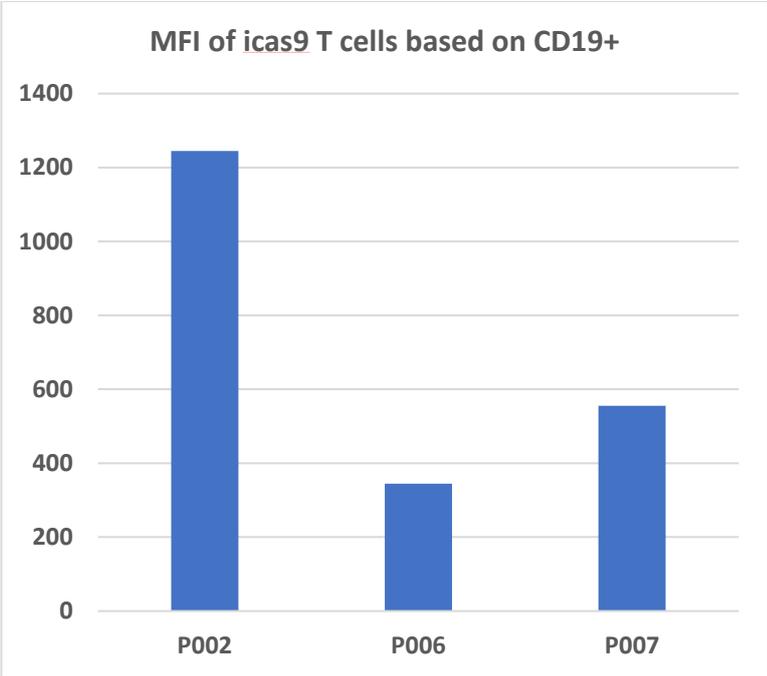


Figure 4.16. In vitro studies on the effect of AP1903 on CD3+CD19+ cells of P007

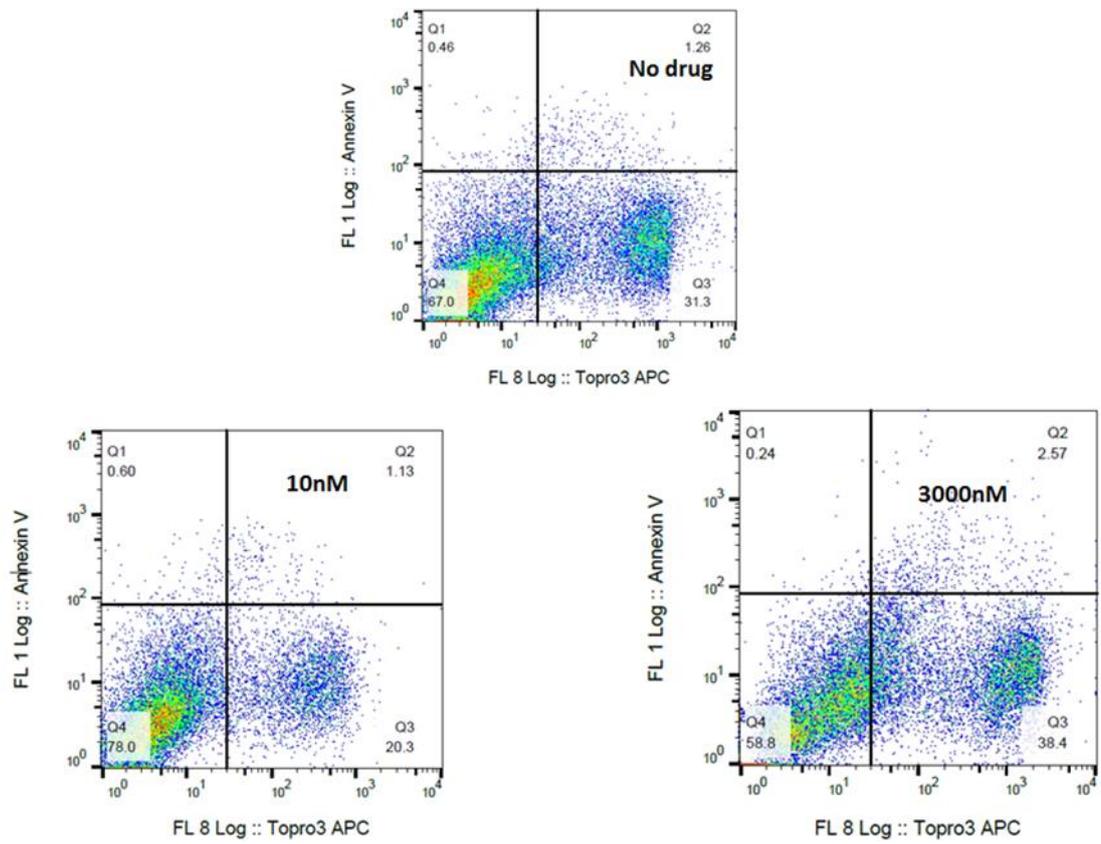


Figure 4.17. Schematic representation of successful cloning of icas9 transgene in PJet 1.2 vector

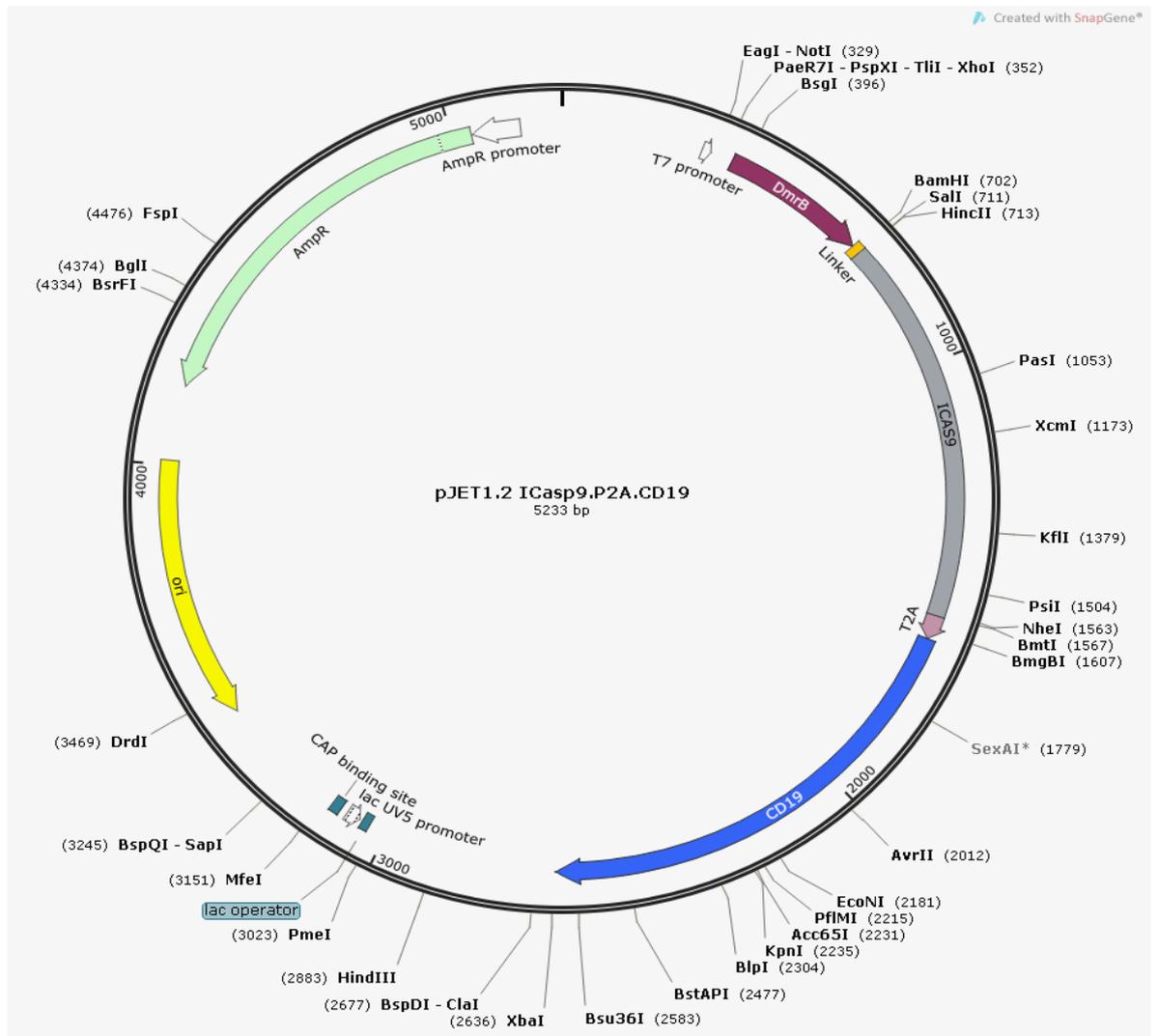


Figure 4.18. Schematic representation of the site of mutation found in P006 and P007



Drm: represents drug binding site.

4.9.2 Confirmed mutation in icas9 transgene in P006 and P007

To investigate for a potential mutation in icas9 transgene in P002, P006 and P007 that led to low expression of CD19+ and both in vivo and in vitro resistance to AP1903, sorted icas9 transgene from P002 was successfully cloned into Pjet 1.2 (Figure 4.17). A deletion of one nucleotide at position 271 amino-acid of icas9 transgene leading to a frame shift causing a premature stop codon in the iCas9 transgene was detected in 2 out of 10 PCR products in the DNA extracted from P 002 sorted T cells.

Next generation sequencing on genetically modified cells of P006 and P007 confirmed the presence of an insertion in icas9 transgene; c1,264insA (p. L411Tfs*4) causing a frameshift of human caspase 9 creating a stop codon at aminoacid position 414 at the end of icas9 transgene. The frequency of the mutation was 25% and 9% for P006 (1000 read depth) and P007 (800 read depth); respectively.

Figure 4.18 is a schematic representation of the mutation and its effect.

Conclusion:

1. Sporadic mutations in icas9 transgene could contribute to low expression and poor response to AP1903.
2. To confirm that this sporadic mutation (c1,264insA in icas9 transgene) might be responsible for low expression of CD3+CD19+ cells and resistance to AP1903, an experiment was set to produce a vector with the mutated transgene and test the difference in expression of icas9 transgene and response to AP1903 in 293T cell line transduced with a wild type icas9 transgene vector versus cells transduced with mutated icas9.

4.9.3 Production of a lenti-icas9 with mutant and wild icas9 transgene

Using site directed mutagenesis, mutation (c1,264 ins A) was introduced into icas9 transgene as shown in figure 4.19.

Mutant and wild icas9 construct was then cloned into a PCCL1-HPGK lentiviral vector- Based on VCN in 293 T cells, a lentivirus with icas9 mutant and icas9 wild was produced with a viral titer of 0.25×10^8 IU/ml and 1.7×10^8 IU/ml, respectively.

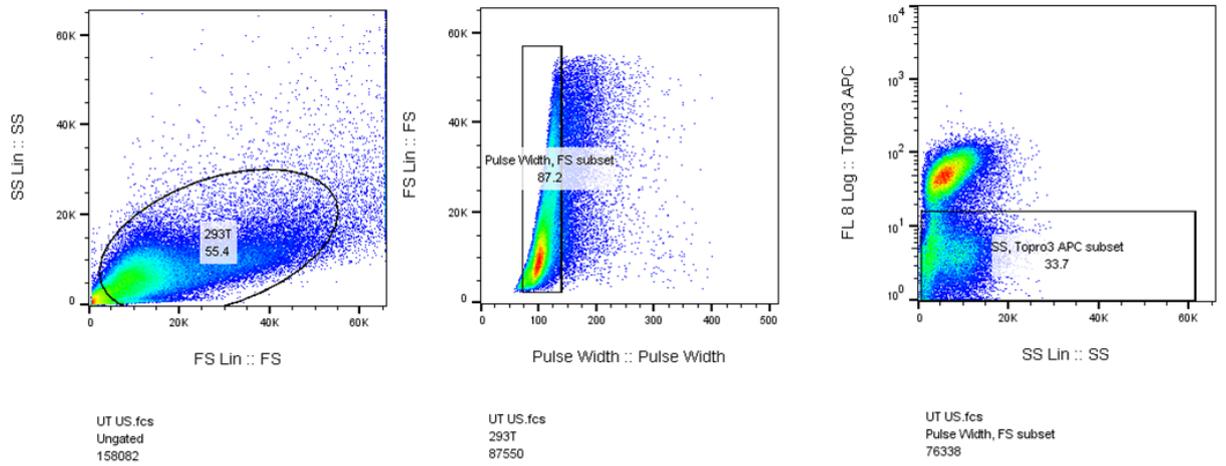
Table 4.1 summarises VCN in different dilutions of 293T transduced with Lenti-icas9 wild and Lenti-icas9 mutant. There was a discrepancy between flow results on viral titer and VCN denoting poor or low expression of CD19 + cells in 293T transduced with Lenti-icas9-mutant (Figure 4.20).

Figure 4.19: Schematic representation confirming the success of insertion of A at aminoacid position 411 of icas9 transgene

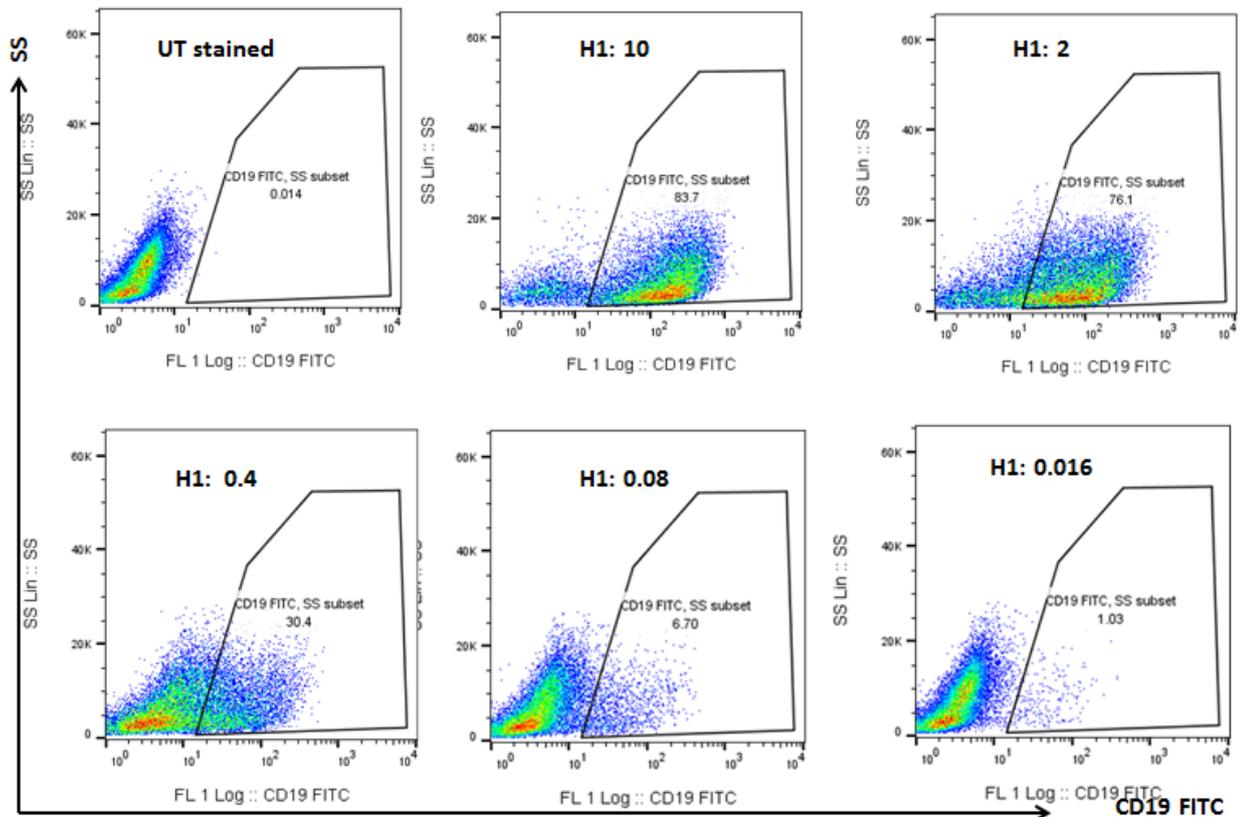


Figure 4.20: Production of a lentivirus vector with wild and mutant icas9

a) Setting the gate for CD19+ cells by flow cytometry



b) Harvest 1 for Lenti-icas9 wild



c) Harvest 1 for Lenti-icas9 mutant

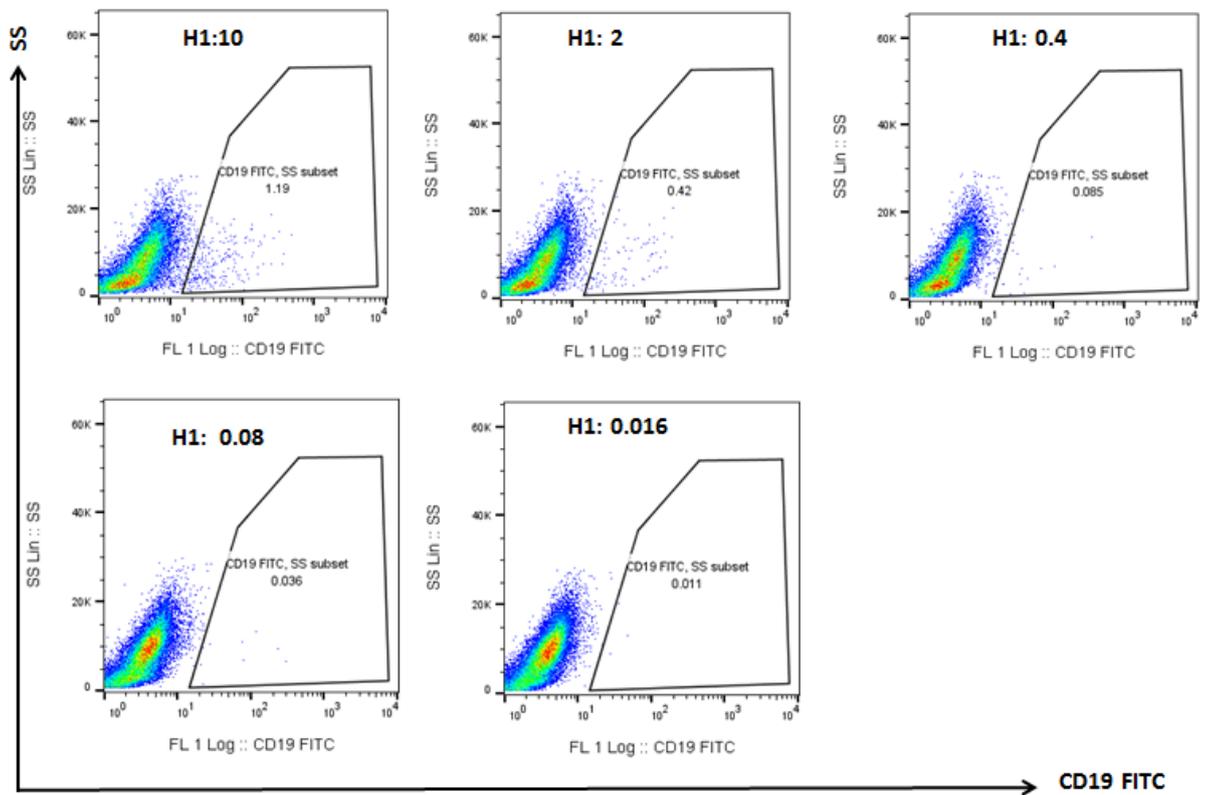


Table 4.1. Calculated VCN for Lenti-icas9 mutant and Lenti-icas9 wild

Viral dilution	VCN
Lenti-icas9 wild	
10	7.56
2	2
0.4	0.24
0.08	0.02
Lenti-icas9 mutant	
10	13.6
2	3
0.4	0.56
0.08	0.14

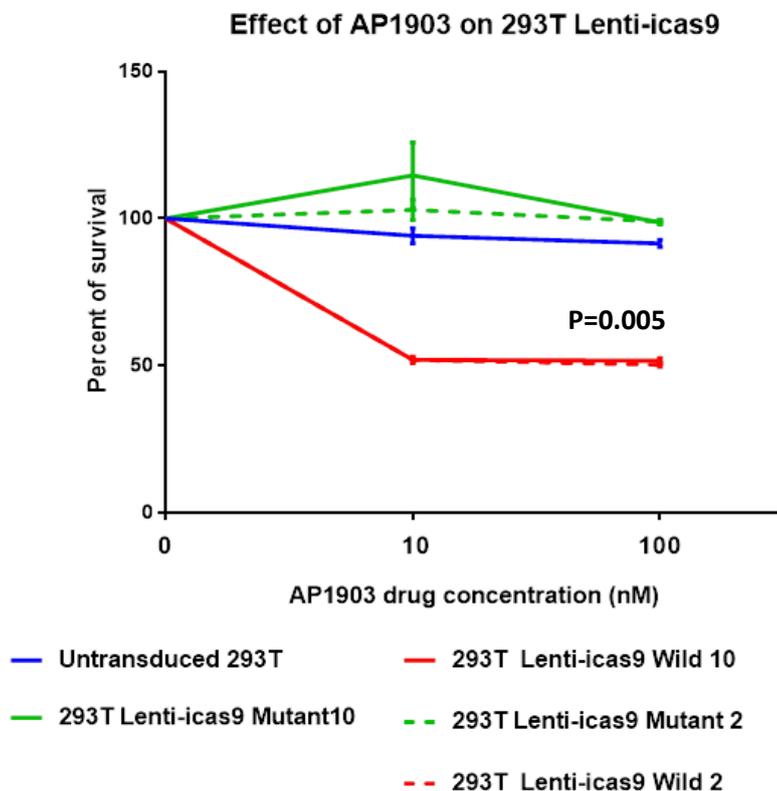
4.9.4 Transduced 293 T cells with Lenti-icas9 mutant were resistant to killing by AP1903

293T cells with Lenti-icas9 mutant 10 and 2 were resistant to AP1903 killing at therapeutic concentration (10nM) and 100 nM. While unsorted 293T cells with Lenti-icas9 mutant 10 and 2 had a drop of survival from 100% in absence of AP1903 to 50% in the vicinity of AP1903 10nM and 100nM (Figure 4.21).

Conclusion:

Frameshift mutation; c1,264insA in icas9 transgene contributed to the persistence of low expressing genetically modified T cells in P006 and P007 post-AP1903 infusion. and their in vivo (no response to a second dose of AP1903) and in vitro resistance to AP1903.

Figure 4.21: Effect of AP1903 on 293T cells transduced with Lenti-icas9 wild and Lenti-icas9 mutant



Mean and SEM of 3 readings was shown on Y-axis.

4.10: Discussion on mechanisms of resistance to AP1903

In our studied cohort of patients, a clear resurgence of icas9 T cells was seen in 4/4 patients who received AP1903 at 30 days, 209 days, 14 days and 10 days post-AP1903 infusion for P002, P003, P006 and P007. While P002 had no recurrence of GvHD coinciding with the survival of icas9 T cells, P006 and P007 had either recurrence or appearance of GvHD requiring steroid therapy. P003 had no recurrence of skin GvHD post-AP1903 infusion however, he had persistent GvHD biopsy negative enteropathy that might be disease related. For P006 and P007, there was no drop in icas9 T cell after 2nd infusion of AP1903. Thus, 2 out of 4 patients had allo-immunity in the presence of the surviving icas9 T cells. This observation of incomplete killing of icas9 T cells were previously reported in earlier studies.

In CASPALLO trial, AP1903 was administered to 4 patients who developed aGvHD. In three patients icas9 T cells re-appeared after 17 days of AP1903 infusion while icas9 T cells remained undetectable for at least 31 days post-infusion for the fourth patient. Interestingly, aGvHD disappeared in all 4 patients within 24-48 hours post-AP1903 infusion with no recurrence up to 1 year in patients whose icas9 T cells resurged. DOTTI trial showed the same pattern in all 3 patients who received AP1903 for treatment of aGvHD with the resurgence of icas9 T cells at 14 days post-AP1903 infusion and again with no recurrence of aGvHD. In vitro tests clearly demonstrated susceptibility of these resurgent icas9 T cells to AP1903 with a kill rate of approximately 85% and a significant drop in icas9 VCN. However, this still means that some of the cells escaped killing (Di Stasi et al, 2011, Zhou et al, 2016). It was noted that the cells that escaped killing by AP1903 have reduced transgene expression (measured by assessment of a surface marker linked to icas9) despite having an icas9 VCN like parental line (Yagyu et al, 2016).

Our in vitro studies showed clearly that while high expressing T cells with Lenti-icas9 (MFI of CD19 in icas9 T cells being above 18,000) were sensitive to AP1903 administration, low expressing icas9 T cells (MFI of CD19 in icas9 T cells was approximately 1000) showed resistance to killing. Different groups tried to unveil the mechanism of resistance to killing.

Investigators hypothesised that icas9 transgene might have been epigenetically silenced. Yagyu et al, 2016 established human induced pluripotent stem cells (hiPSCs) that express the icas9 transgene and eGFP driven by EF1 α core promotor. Upon exposure to AP1903, 1-5% of transduced cells were resistant to AP1903

clearance. In comparison to AP1903- sensitive icas9 cells, AP1903-resistant icas9 cells showed lower expression of GFP though similar icas9 VCN to AP1903-sensitive icas9 cells. Bisulfite sequencing demonstrated higher levels of methylation in AP1903- resistant than -sensitive icas9 hiPSCs. Forty-eight-hour culturing of AP1903- resistant icas9 cells with an epigenetic modifier; histone deacetyl transferase inhibitor upregulated icas9 mRNA expression, though killing slightly improved, it remained incomplete with some cells escaping the effect of AP1903. While icas9 low expression might be induced by epigenetic silencing, another mechanism is responsible for the resistance to AP1903.

icas9 lie in lower stream of apoptosis pathway with studies showing no effect of anti-apoptotic molecules namely c-Flip and Bcl-XL on downregulation of caspase 9 (Tey et al, 2007) and potential role of XIAP in direct inhibition of both caspase 9 and caspase 3 (Jones et al, 2014). Nevertheless, AP1903- resistant icas9 hiPSCs were found to have similar level of XIAP as AP1903- sensitive yet surprisingly higher levels of Bcl2 were recorded in AP1903- resistant cells. Bcl2 inhibitor augmented sensitivity of AP1903- resistant icas9 cells to AP1903 yet still with incomplete clearance.

Zhou et al, 2016 reported a patient who developed skin and probably liver aGvHD post-BPX-501 infusion and received 3 doses of AP1903 (3 months apart) for control of GvHD. Though, there was a drop in icas9 T cells post-1st and 2nd infusion. There was no drop in icas9 T after the 3rd infusion and complete elimination of icas9 T cells was not achieved. Of note, immediately prior to the first AP1903 infusion, MFI of CD19 in cas9 T cells was above 12000. The recovering icas9 cells had an MFI of 4000. Following subsequent infusions, MFI of surviving cells was always <3000-denoting that low expressing icas9 T cells were able to escape the effect of AP1903. Transgene expression derived from γ retroviral vectors is known to be highly dependent on state of T cell activation (Pollok et al, 1998). The surviving icas9 T cells were exposed in vitro to CD3/CD28 monoclonal antibodies to stimulate TCR activation. After 48 hours of activation, cells showed increase CD19 expression with MFI for CD19 rising to above 30,000. Upon exposure to AP1903, percentage of surviving icas9 was reduced only by 50%. A nonsense mutation in drug binding site of icas9 transgene causing a premature stop codon in icas9 transgene was detected in 2 of 10 cloned PCR products of patient's PBMCs post-AP1903 infusion. However, they did not investigate for the existence of the mutation by next generation sequencing and did not run any invitro studies to investigate if this mutation was responsible for resistance of AP1903.

In P002, P006 and P007 MFI for CD19+ in surviving icas9 T cells post-AP1903 was below 1000 confirming that surviving cells were low expressing cells. Next generation sequencing on icas9 T cells of P006 and P007 confirmed the presence of an insertion in icas9 transgene; c1,264insA (p. L411Tfs*4) causing a frameshift of human caspase 9 creating a stop codon. To further confirm that this mutation led to low expression of CD19+ in icas9 T cells and resistance to killing, we cloned 2 constructs; one with the mutation and one with the wild form of icas9 transgene into a Lenti-viral vector and tested the transduction efficacy in 293T cells. While VCN was equivalent, 293T with Lenti-icas9 mutant showed limited expression of CD19+ in comparison to high expression of CD19+ in 293T transduced with Lenti-icas9 wild. Furthermore, 293T cells with Lenti-icas9 wild were sensitive to AP1903 while 293T with Lenti-icas9 mutant showed similar survival to untransduced 293T cells. These results thus confirm that a sporadic mutation in icas9 transgene might be responsible for low icas9 expression and resistance to AP1903.

**Chapter 5:
Discussion and implications.**

Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) constitutes a curative treatment for children with malignant and non-malignant disorders. HLA-partially matched haploidentical (haplo) donors represent a viable alternative option for those children who lack an HLA-compatible donor. T-cell depletion approaches with positive (CD34 selection) or negative selection (alpha/beta T-cell and CD19+ B-cell-depletion) allow engraftment of donor cells with a low risk of GvHD; however, success is limited by delayed immune recovery, increasing the risk of fatal infections. Infusion of unmanipulated donor T cells (DLI) to accelerate immune recovery is associated with high risk of fatal GVHD. In contrast, adoptive transfer of donor T cells genetically manipulated to include a safety switch can be a suitable strategy to render DLI safer and more widely applicable.

In our centre, the use of BPX-501 (icas9 T cells) facilitated engraftment, earlier CD4 and CD8 count recovery in TCR $\alpha\beta$ /CD19 depleted Haplo-HSCT in comparison to historic cohort with no T cell add-back (TCR $\alpha\beta$). This was associated with a significant drop of severe viral infections to 16% with TCR $\alpha\beta$ /CD19 depleted HSCT+BPX-501. Difficulty in handling viral infections among TCR $\alpha\beta$ /CD19 depleted grafts was universally reported across different studies to occur between 50-70% (Balashov et al, 2015, Maschan et al, 2016, Laberko et al, 2017, Elfeky et al, 2019).

Multicentre data on the use of BPX-501 from USA (US) and Europe (EU) supported these findings. To date, 229 patients (including our cohort of patients) with malignant and non-malignant disease were treated with TCR $\alpha\beta$ /CD19 depleted haploidentical transplant followed by BPX-501 add-back as part of 2 multicentre US (NCT03301168) and EU (NCT02065869) prospective trials (Elfeky et al, 2018 (b)). Among 166 evaluable patients, at a median follow-up of 17.6 months, none had graft failure, overall survival and disease-free survival rates were 94.2% and 89.4%; respectively. CD3+CD4+ T cells above 500 cells/ul were achieved by D+100. The percentage of circulating CD3+CD19+ cells at D+100 were 9.06 \pm 10.52%. (Algeri et al, 2018). On the contrary, achieving CD4 counts above 300 cells/ul was consistently reported to be delayed till 5-9 months after TCR $\alpha\beta$ /CD19 depleted HSCT with no T cell addback (Shah et al, 2018, Locatelli et al, 2015, Elfeky et al, 2019). BPX-501 infusion was clearly shown to improve viral immunity and allow timely viral clearance in patients who had a CD34+ selection followed by icas9 T cell add-back (Di Stasi et al, 2011, Zhou et al, 2015 (b)). Published data on viral immunity from the current multicentre study are sparse. However, in phase 1 study, analysis of the function of T cells from 16 recruits showed that the proliferative response to a polyclonal mitogen or to CMV lysate was comparable to that of a healthy control in 50% of patients as early as

day+60 after a haplo-HSCT with BPX-501 infusion. By day+150, all patients had a normal proliferative response. Response to both EBV and AdV antigens was slightly delayed, but progressively improved overtime. Moreover, Lucarelli et al, 2015 described 3 patients with SCID (n=2) and WAS (n=1) who had evidence of significant peri-transplant CMV or AdV viraemia. All received TCR $\alpha\beta$ + BPX-501 between D+15-D+17 as part of phase 1 dose escalation trial. $\alpha\beta$ CD3+ cells were above 500 cells/ul by 30-90 days post-HSCT. This early immune recovery was associated with clearance of viral infection between 50-100 days post-HSCT. All 3 patients were alive and well with no recurrences of viral reactivation by D+180. Interestingly, these 3 patients were given different doses of BPX-501; 2.5X10⁵/ kg, 5X10⁵/Kg and 1X10⁶/kg. These reports support data from DOTTI and CASPALLO trial that a dose of 2.5 X10⁵/kg was enough to confer viral mediated immunity. However, this low dose was shown not to confer enough GvL effect to prevent tumour relapse (Zhou et al, 2016, Di Stasi et al, 2011).

Merli et al, 2018 have recently presented single centre data on 111 patients who received TCR $\alpha\beta$ /CD19 depleted haplo-HSCT with no T cell add-back for acute leukaemia. While TRM was impressively low 6%, the main cause of treatment failure was leukaemia relapse with 5-year CI of relapse free survival (RFS) and relapse being 70% and 24%, respectively. Other centres have reported a higher relapse rate between 30-47% which might in part reflect the type of preparative conditioning used (Im et al, 2016 (b), Maschan et al, 2016, Laberko et al, 2017). Locatelli et al, 2017 has clearly shown that use of TBI during conditioning regimen in a TCR $\alpha\beta$ /CD19-haplo HSCT was associated with relapse rate of 18% vs 45% in patients who did not receive TBI. Among 100 evaluable patients with acute leukaemia who received TCR $\alpha\beta$ + BPX-501 as part of the current multicentre study (including patient P006), TRM was reported as 4.8% and 8.8% among AML and ALL patients respectively- which was equivalent to the use of TCR $\alpha\beta$ with no T cell add-back. However, RFS and overall survival rose from 70% and 70% with no T cell-addback to 82.2% and 90.1% with the use of BPX-501; respectively. Of note, TBI was included in conditioning of 62% of patient with TCR $\alpha\beta$ + BPX-501 versus 76% in patients who only received a TCR $\alpha\beta$ HSCT. Hence, a dose of 1X10⁶/Kg of BPX-501 supported the TCR $\alpha\beta$ /CD19 depleted graft to confer anti-leukaemia in patients with acute leukaemia. Giving the encouraging results from the multicentre trial, recently Bellicum pharmaceutical has launched a phase 2/3 trial in US (THRIVE) to investigate the use of BPX-501 in adult and adolescent patients 12 years-17 years with intermediate and high-risk acute myeloid leukaemia (AML) or myelodysplastic syndromes (MDS). Following

completion of phase 2, participant will be randomly enrolled to either a TCR $\alpha\beta$ haplo-HSCT +BPX-501 or Haplo-HSCT post-cyclophosphamide (NCT03699475).

BPX-501 add-back with TCR $\alpha\beta$ haplo-HSCT has thus abrogated the risk of viral reactivation and improved survival among patients with malignant and non-malignant disease. However, in our studied group we noted increased risk of aGvHD; all patients developed aGvHD grade I-III with two out of 6 patients having aGvHD grade III. Yet, the small number of patients recruited from our centre limit further analysis. On the contrary, data from the current multicentre study (including our cohort of patients), demonstrated no increase in GvHD rates with BPX-501 infusion in a TCR $\alpha\beta$ /CD19 depleted Haplo-HSCT. CI of aGvHD grade I-IV, aGvHD grade III-IV and cGvHD were 21.8% ,2.1% and 4.6% respectively (Elfeky et al, 2018 (b)). 24 patients-including P002, P006 and P007- received 1-2 doses of Rimiducid (AP1903) to control GvHD with an overall response rate of 92% observed. Median time to initial response was 2 days. At a median follow-up of 7.8 months, 77% of responders were still in either complete remission or partial response (Elfeky et al, 2018 (b)).

Another reported side effect post-BPX-501 add-back was the development of neurotoxicity as been seen in P007. Subsequently, 2 other patients in the multicenter trial (EU; (NCT02065869)) developed neurological complication at 46 days and 40 days post-BPX-501 administration (personal communication). Both patients became sleepy and entered into deep coma till death. RNA sequencing for viruses and icas9 T cell vector on post-mortem brain biopsy was carried out for both patients (at Great Ormond Street hospital laboratory). In both cases, there was no evidence of vector signature in brain biopsy. While no evidence of a viral element was seen in patient 1, patient 2 had confirmed Astovirus VA1/HMO-C strain.

Neurotoxicity is a well-documented complication post-CAR T cell therapy that usually occur between 4-14 days post-CAR T cell administration and typically present with memory loss, aphasia and tremors that lasts for 2-4 days in the majority of cases (Neelapu et al, 2017). The mechanism of CAR T cell driven neurotoxicity is not yet understood. However, due to the correlation between severe cytokine release syndrome (CRS) and neurotoxicity with the expansion of CAR T cells and production of IL6 in the periphery, a possible rapid transfer of inflammatory cytokines to CSF was thought to be a potential mechanism. Though CAR-T cells were seen in the CSF of patients with neurotoxicity without CNS malignancy (Lee et al, 2015, Hu et al, 2016), it is unlikely that CNS invasion by CAR T cells could lead directly to neuronal

cytotoxicity. Most patients exhibit a complete neurological remission which would be impossible with widespread neuronal destruction (Dholaria et al, 2018).

While CRS and neurotoxicity are common complications post-CAR T cell expansion in response to tumor antigens, there are limited data in the literature that GvHD from adoptive transfer of alloreactive T cells can induce CRS and neurotoxicity. In DOTTI trial, one out of 12 patients developed fever, rash and diarrhea associated with elevated cytokines including IL10, TNF α , INF γ and IL6 18 days post-icas9 T cell infusion. Symptoms completely resolved, and cytokines dropped 2 hours after AP1903 infusion (Zhou et al, 2015 (b)).

Evidence of icas9 T cell infiltration to the CNS was previously reported among 2 patients in the DOTTI trial. One patient had varicella zoster (VZV) meningitis with >5 million copies of VZV /ml of cerebrospinal fluid (CSF). Within one week of icas9 T cell infusion, viral load dropped to 6900 copies/ml in CSF. CSF examination showed a considerable population of CD3+CD19+ cells (25%) consistent with presence of icas9 T cells in peripheral blood (33%). Icas9 T cells were thus able to traffic to the CNS and cross the inflamed blood brain barrier to clear CNS viral infection. Another patient developed unexplained encephalopathy with no obvious infection, drug or vascular related etiology. This patient received AP1903 for a possible cerebral localised GvHD. Peripheral blood showed 96% drop in circulating CD3+CD19+ T cells in periphery by flow cytometry with evidence of equivalent drop in PB VCN of icas9 T cells by qPCR. However, encephalopathy progressed, and the patient passed away in a week time post-AP1903 infusion. Post-mortem brain biopsy showed 552 copies/ul of DNA of icas9 transgene by qPCR in the frontal lobe cortex. Though authors reported this neurotoxicity as being unrelated to icas9 infusion based on low number of icas9 T cells in brain biopsy, low numbers of icas9 T cells might be attributed to AP1903 administration a week before death (Zhou et al, 2015 (b)).

In our centre, P007 developed unexplained fever 19 days post-BPX-501 infusion followed by neurotoxicity. Unfortunately, cytokine levels were not measured at the start of the fever- so we cannot exclude that this fever was a form of CRS. Parents declined to consent for a brain biopsy when the patient developed neurotoxicity but the detection of icas9 T cells in CSF by flow cytometry- even in low numbers- was considered as a potential cause for the neurotoxicity. Thus, patient was given AP1903 in addition to steroids. In fact, 5 days later he showed signs of improvement and in one month he was back to normal with no sequel.

CAR T cell neurotoxicity is also noted to be more frequent in patients with pre-existing neurological conditions, younger age and heavily pre-treated patients (Gust et al, 2017, DeAngelo et al, 2017).

Both our patient (P007) and the patient who developed encephalopathy in the DOTTI trial had multiple common factors. Both had difficult to treat tumors; JMML and refractory AML that required more than one HSCT; 2 in P007 and 3 in the latter. Moreover, both received TBI as part of the conditioning protocol. Thus, both patients were heavily treated and potentially their blood brain barrier was not intact to protect against icas9 T cell trafficking and potential neurotoxicity.

In my studied cohort, I noted the survival of low CD19+ expressing T cells post-AP1903 infusion. Other trial centers (personal communication) had similar observation but in absence of toxicity no further analysis was carried out. In my studied cohort, in vitro experiments confirmed resistance to AP1903 and a mutation in icas9 transgene to be a potential escape mechanism.

In an approach to use a safer vector for transfer of icas9 transgene to T cells, we cloned icas9 transgene into a lentiviral vector.

Lentiviral vectors have been extensively investigated and optimised over the past two decades. Third generation lentiviral vectors mitigate the risk of replication competent lentivirus (RCL) production 1) as they lack both regulatory and accessory HIV viral genes that mediate viral survival and 2) their production is carried out through a 3 plasmid-packaging system thus severely reducing the risk for RCL as multiple recombination events will then be necessary to create a virus that harbours all the sequences required for replication (Melone and O'Doherty et al, 2018).

Herein, we demonstrated the feasibility of production of a third-generation lentiviral vector with hPGK promoter driving icas9 transgene expression. High transduction efficacy was achieved in both Jurkats 6.1 and T cells transduced with PCCL1-HPGK-FKBP-icas9-CD19 virus (Lenti-icas9) with transduction efficacy titres of 90% and 40% respectively. Using Lenti-icas9 ensured effective activation of icas9 transgene by the dimerizer (AP1903) with a clearance rate of 80% of transduced T cells in the presence of 10nM of AP1903 equivalent to results of pre-clinical and clinical trials using γ retroviral vectors for icas9 gene transfer (Marin et al, 2012, Zhou et al, 2015 (a), Zhou et al, 2015 (b)).

We further investigated the effect of commonly used immune suppressive drugs -for management of GvHD and allo-immunity post-HSCT - on lenti-icas9 T cells and demonstrated impaired T cell proliferation in response to methylprednisolone and mycophenolate mofetil (MMF). Moreover, MMF led to apoptosis of lenti-icas9 T cells.

MMF is a selective inhibitor of inosine monophosphate dehydrogenase (IMPDH). Inhibition of IMPDH enzyme leads to reduction of xanthine monophosphate, guanosine triphosphate (GTP) and deoxy GTP (dGTP) thus impair both RNA and DNA synthesis in T and B cells who rely on guanosine synthesis for their proliferation. An early study from 1999 (Cohn et al, 1999) demonstrated increased apoptosis in human lymphoid and monocytic cell lines. Recent studies investigated the apoptosis pathway activated by MMF. Chaigne-Delalande et al, 2008 tested both JK 6.1 and PBMNCs activated by PHA and demonstrated increased cell death in response to increased in vitro concentrations of MMF. Interestingly they demonstrated an increase in caspase 3 as early as 30 hours after the addition of MMF with evidence of mitochondrial disruption as the initial trigger for apoptosis. Autophagy was responsible for the subsequent cell death. Takebe et al, 2006 studied the effect of MMF in multiple myeloma cell lines that are known to overexpress IMPDH. MMF-induced G1-S phase cycle arrest and induction of apoptosis through a caspase-dependent pathway involving mitochondrial membrane disruption, cytochrome c release and caspases activation including caspase 3 and 9.

In my current experiment, there was no increased apoptosis of icas9 T cells versus untransduced T cells which indicates that the current icas9 construct doesn't allow any activation of caspase 9 pathway except on cell exposure to the dimerizer; AP1903.

As previously discussed in section 4.10, we noted that in all 4 patients who received AP1903, a resurgence of cells was observed at variable intervals and in at least 2 of the four patients this resurgence was associated with allo-reactivity. A second dose of AP1903 in P006 failed to clear marked T cells with persistence of gut GvHD. Given the above data on susceptibility of BPX-501 cells to immune suppressive medications support their potential use in case of BPX-501 driven allo-immunity when the dimerizer fails to clear the marked T cells.

To sum up, data from the current trial showed that the adoptive transfer of BPX-501 T cells following TCR $\alpha\beta$ /CD19 depleted haplo-HSCT present a novel and highly effective transplantation strategy for paediatric patients with malignant or non-malignant disorders. BPX-501 administration improved the outcomes of

TCR $\alpha\beta$ /CD19 depleted grafts with better handling of post-HSCT viral infections and low relapse rates associated with accelerated T cell immune reconstitution. Overall survival rates of 94% was achieved. These rates are equivalent to rates reported with the use matched unrelated donor grafts. Despite the addition of BPX-501, overall rates of GvHD were low with few cases of high-grade aGvHD or cGvHD. AP1903 (Rimiducid) was shown to be an effective treatment for patients who developed GvHD. However, incomplete clearance or resurgence of icas9 T cells post-administration of a dimerizer noted in some patients is a potential risk for recurrence of alloreactivity or potential toxicity. c1,264insA mutation in icas9 transgene is a potential cause for persistence of low-expressing icas9 T cells and their escape mechanism from the effect of the dimerizer drug. I have demonstrated the feasibility of transfer of icas9 transgene into a third-generation lentiviral vector with efficient transduction of primary cells. In the context of this vector, I have demonstrated effective activation of icas9 transgene upon exposure to the dimerizer. These engineered T cells are sensitive to the effect of common immune suppressive drugs including steroids, ciclosporin A and mycophenolate mofetil.

Future perspectives and potential clinical implications

Monitoring the location of genetically modified cells in patients could have important ramifications in terms of safety and could potentially allow understanding the biology of these therapeutic intervention; understand their biodistribution, migration to different sites and capacity to proliferate in response to tumor.

While investigators were able to track marked cells through the presence of a gene marker as truncated CD34 in HSV TK or truncated CD19 in icas9, however, this can only reflect levels of marked cells in blood and tissue fluids. Eissenberg et al, 2014 used a non-invasive reporter (8F-9-(4-fluoro 3hydroxymethylbutyl) guanine (18FHBG) and positron emission tomography (PET)/CT scans to follow human T cells retrovirally transduced with HSV-TK (CD34-TK75). 18FHBG is a substrate for TK enzyme. TK75 is a mutant form of TK (Black et al, 1996) with high sensitivity to ganciclovir and more accumulated phosphorylation of 18FHBG for imaging. TK75 was fused to truncated CD34 to allow selection of transduced cells through Miltenyie biotechnology.

Two NSG mice were infused by the GMP prepared CD34-TK75. As expected xenogenic CD34-TK-75 induced GvHD in NSG mice. PET scan showed evidence of trafficking of marked cells to the thymus before the development of GvHD. Nervi et

al, 2007 have previously demonstrated that the thymus is one of the preferred sites for migration and proliferation of human T cells during xenogenic GvHD in NSG mice.

Finding such a pattern in patients could provide both a non-invasive means to track these cells in human DLI recipients and possible surrogate marker to predict GvHD. In the phase I clinical trial, 8 patients received these marked T cells ; 6 had baseline PET/CT scan at baseline, D+14 and D+30. Only one out of the six monitored patients had GvHD. Unfortunately, there was no discernable difference in the biodistribution of ¹⁸FHBG in this patient or any other patient at baseline and later time points. A threshold of radiotracer might be required for the marked cells to be detected. In this pilot trial, patients were administered 0.1-1.3X10⁶/Kg DLI which the authors considered as a very limited number of transduced T cells. Other possible causes behind the failure to track the marked cells in the clinical trial might include diffuse distribution of marked cells or in appropriate choice of time point.

One should consider the possibility of engineering a substrate for caspase 9 to be used as a radiotracer to track human T cells retroviral or lentiviral transduced with icas9 suicide gene. Tracking the cells will give us more insight into safety and could potentially be used as a surrogate marker for GvHD.

Recent years has witnessed advances in CAR T cell therapies to target B -cell malignancies with 2 anti-CD19 CAR T schemes being approved by FDA for management of acute leukemia in pediatric and young adults (Novartis's Kymriah) or relapsed/refractory large B cell lymphoma in adults (Gilead's Yescarta) (Leyfman, 2018). However, the application of CAR-T cells is obviously hampered by the adverse effects, such as cytokines release syndrome and on-target off-tumor toxicity. In some clinical trials, patients quitted the treatment of CAR-T cells due to life-threatening toxicity. Seeking to alleviate these toxicities or prevent the occurrence, researchers have developed a number of safety strategies of CAR-T cells, including use of suicide genes (Yu et al, 2019). Moreover, researchers have been investigating the use of suicide gene technology in targeting solid tumors (Ando et al, 2014 and Xie et al, 2001). In this context, 100% clearance of target cells after activation of suicide gene will be desired to achieve tumor clearance and limit relapse.

Many factors govern which suicide gene is optimal. Among them is rapid and complete clearance in case of toxicity or to achieve tumor clearance.

Section 1.6 demonstrated the superiority of icas9 over other pro-caspases and thymidine kinase as a suicide gene. While the use of icas9 suicide gene technology

has shown in vitro and in vivo evidence of rapid response in clearance of marked cells, incomplete clearance represents a concern that need tackling. While there are different possible mechanistic factors leading to resistance to killing as discussed in section 4.10, a mutation in icas9 transgene is one of the potential factors that can lead to poor expression of suicide gene and resistance (current study and Zhou et al, 2016). The raised question here is could we induce a change (mutation) in icas9 transgene to increase expression of the gene and thus improve the sensitivity to the dimerizer.

In a similar scenario, 2 independent investigators investigated the possibility of inducing a mutant thymidine kinase (TK) to enhance activity to ganciclovir. Balzarini et al; 2006 reported a mutant TK (A168H mutation) with 4-fold increase in activity towards ganciclovir. Subsequently, Preuss et al, 2011 splice corrected the A168H mutant to make a new mutant TK called TK007 with in vitro studies showing improved cancer killing efficiency and in vivo studies demonstrated complete remission of glioblastoma xenograft tumors in the presence of ganciclovir with doses as low as 10mg/kg. Further studies are needed to look into the possibility of inducing a mutation in icas9 transgene to improve expression and increase sensitivity for the dimerizer.

Chapter 6:
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