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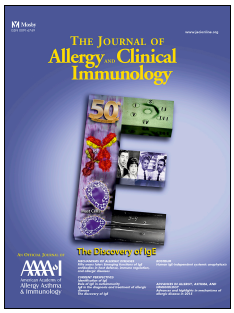

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Transfer of gene corrected T cells corrects humoral and cytotoxic defects in X-linked lymphoproliferative disease (XLP1)

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Abstract

Background: XLP1 arises from mutations in the *SH2D1A* gene encoding SAP, an adaptor protein expressed in T, NK and NKT cells. Defects lead to abnormalities of T and NK cell cytotoxicity and T cell dependent humoral function. Clinical manifestations include haemophagocytic lymphohistiocytosis (HLH), lymphoma and dysgammaglobulinaemia. Curative treatment is limited to haematopoietic stem cell transplant with outcome reliant on a good donor match.

Objectives: As most symptoms arise from defective T cell function, we investigated whether transfer of SAP gene corrected T cells could reconstitute known effector cell defects.

Methods: $CD3^+$ lymphocytes from sap-deficient mice were transduced with a gammaretroviral vector encoding human *SAP* cDNA before transfer into sub-lethally irradiated sap-deficient recipients. Following immunisation with the T-dependent antigen NP-CGG, recovery of humoral function was evaluated through germinal centre formation and antigen specific responses. To efficiently transduce patient CD3+ cells, we generated an equivalent lentiviral SAP vector. Functional recovery was demonstrated using *in vitro* cytotoxicity and T_{FH} cell function assays, alongside tumour clearance in an *in vivo* LCL lymphoma xenograft model.

Results: In sap-deficient mice, 20-40% engraftment of gene modified T cells led to significant recovery of germinal centre formation and NP-specific antibody responses. Gene corrected patient T cells demonstrated improved cytotoxicity and T_{FH} cell function in vitro. Adoptive transfer of gene corrected patient CTLs reduced tumour burden to a level comparable with healthy donor CTLs in an *in vivo* lymphoma model.

Conclusions: These data demonstrate that autologous T cell gene therapy corrects SAP dependent defects and may offer an alternative therapeutic option for XLP1 patients.

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Key message

This study demonstrates that adoptive transfer of gene corrected T cells corrects humoral and cytotoxic abnormalities seen in XLP1 establishing potential therapeutic benefit of this approach for patients with XLP1.

Capsule summary

XLP arising from mutations in a gene encoding SAP is a combined immunodeficiency with limited therapeutic options that arises from defects of effector cell function. Correction of autologous T cells using gene transfer can restore T cell dependent immune function in SAP-deficient mice and humans.

Key words

XLP, T cell gene therapy, T follicular helper cells, T cell cytotoxicity,

Abbreviations

CTL – cytotoxic T lymphocyte

EBV – Epstein Barr virus

FHL - familial haemophagocytic lymphohistiocytosis

HLA – human leukocyte antigen

HLH - haemophagocytic lymphohistiocytosis

HSCT - haematopoietic stem cell transplantation

LCL – lymphoblastoid cell line

PBMC - peripheral blood mononuclear cell

SAP – SLAM associated protein

T_{FH} cell – T follicular helper cell

WT - wild type

XLP – X-linked lymphoproliferative disease

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Introduction

X-linked lymphoproliferative disease 1 (XLP1) is a severe primary immunodeficiency arising from mutations in the *SH2D1A* gene which encodes an intracellular adaptor protein called SAP (SLAM associated protein). The absence of SAP leads to multiple immunological defects including impaired T and NK cell cytotoxicity(1-4), lack of NKT cell development(5, 6) and defective CD4+ T follicular cell (T_{FH}) help(7-9) which lead to abnormal humoral function. The clinical disease phenotype is characterised by severe immune dysregulatory phenomena including abnormalities in immunoglobulin production and T dependent humoral immune responses, T cell effector defects leading to haemophagocytic lymphohistiocytosis (HLH), and development of lymphoma.

Specific disease manifestations may be treated supportively with replacement immunoglobulin for dysgammaglobulinaemia, HLH chemotherapeutic protocols, monoclonal serotherapy for EBV driven disease and appropriate chemotherapy regimens for malignancy, but curative treatment for patients with XLP1 is limited to allogeneic haematopoietic stem cell transplant (HSCT). Results are highly dependent on a good donor match and the absence of active disease at transplant with survival falling to 50% if patients enter transplant with HLH(10). For over two decades, autologous haematopoietic stem cell (HSC) gene therapy has been shown to be a successful treatment option for specific immune deficiencies(11) and this experience supports the development of therapeutic gene therapy strategies for other monogenic immune deficiencies.

In a sap-deficient mouse model we previously demonstrated correction of cellular and humoral defects through lentiviral (LV) mediated gene transfer into haematopoietic progenitor cells thereby providing proof of concept for gene therapy in XLP1(12). One concern about this approach was that the non-physiological expression of SAP in progenitor cell populations after stem cell gene transfer might be associated with certain risks due to the role of SAP as an important signalling molecule and its tightly regulated expression profile. Although no adverse effects were seen when SAP was expressed in HSCs or other haematopoietic compartments where expression is usually limited, we wanted to evaluate whether transfer of gene corrected T cells may offer a potentially safer treatment option. We evaluated a number of regulatory elements in the context of an HSC gene therapy approach to provide lineage specific SAP expression but were unable to identify a promoter capable of affording specificity and sufficient protein expression to restore immune function (unpublished data).

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Autologous T cell gene therapy would diminish concerns over ectopic SAP expression and has an established safety profile with hundreds of patients treated to date for haematological malignancies in cancer immunotherapy trials with no reported transformational events(13-17). Further, important manifestations of XLP1 such as HLH, lymphoma development and dysgammaglobulinaemia arise from defective T cell function and would be potentially corrected through this approach. We therefore sought to investigate whether infusion of gene modified T cells could correct both humoral and cytotoxic immune defects in a sap-deficient murine model and an *in vivo* tumour model using corrected patient cells. Here we show, for the first time, that viral vector mediated gene correction of the T cell compartment can recover these immune defects both *in vitro* and *in vivo*. This work provides evidence that an autologous gene corrected T cell approach may offer therapeutic benefit to patients with XLP1.

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Materials and Methods

Mice:

sap-deficient mice (*sap*$^{y/-}$, *sap*$^{-/-}$) have been previously described(18-20) . NSG (NOD/SCID/IL2rγ^{null}) mice were obtained from Jackson Laboratory. Animals were raised in specific pathogen free conditions and all studies were licensed under the Animals (Scientific Procedures) Act 1986 (Home Office, London, United Kingdom).

Vector constructs:

For murine experiments, a gammaretroviral vector on the SF91 backbone containing codon optimised human *SAP* cDNA with an internal ribosomal entry site (IRES) element and enhanced green fluorescent protein (eGFP) was used. Human primary cell experiments were carried out using third generation lentiviral vector on a pCCL backbone containing codon optimised human *SAP* cDNA driven by the elongation factor 1α short (EFS) promoter, IRES and eGFP or eGFP alone (EFS-SAP-eGFP; EFS-eGFP).

Murine CD3$^+$ T cell selection and transduction:

CD3+ T cells were isolated by negative magnetic selection (Pan T cells) MicroBeads, Miltenyi, Bergisch Gladbach, Germany) from harvested splenocytes and cultured in RPMI 1640, 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin, 1 mM betamercaptoethanol and 1 mM sodium pyruvate (all Life Technologies) and stimulated with 20U/ml murine Interleukin (IL) 2 (Peptrotech, Rocky Hill, NJ, USA) and 100µg/mL anti-CD3e and 100µg/mL anti-CD28 T cell activation/expansion (Miltenyi, Bergisch Gladbach, Germany)

Transduction was performed 24 hours later with retroviral supernatant via spinoculation (90 minutes at 1000g) in recombinant human fibronectin fragment (RetroNectin®, Takara Bio Europe S.A.S, Saint-Germain-en-Laye, France) pre-coated plates. 72h post-transduction cells were harvested and analysed using Flow Cytometry (LSRII BD) for transduction efficiency and CD4$^+$/8$^+$ phenotype using Rat anti-Mouse CD8a (APC) and Rat anti-Mouse CD4 (BV510) (BD Bioscience). CD3$^+$ cells were injected into sub-lethally (6Gy) irradiated sap-deficient recipient mice. Eight weeks post-reconstitution mice were challenged via intraperitoneal injections of T cell dependent antigen; chicken gamma globulin conjugated to hapten: 4-Hydroxy-3-nitrophenylacetly (NP-CGG, 150ug/ml)

Patient samples

Consent was obtained to use samples from four unrelated patients. All patients had proven mutations in SH2D1A (P1 c.57_59 duplication, P2 hemizygous deletion of exon 2, P3 large deletion spanning from exon 2, P4 large deletion spanning exons 2-4). Samples from P1, P2, P4 were used for T_{FH} assays and samples from P1, P3 and P4 were used in cytotoxicity experiments.

In vitro T_{FH} cell assay:

PBMCs were isolated by Ficoll centrifugation (GE Healthcare, Amersham, UK). Naive $CD4^+$ T cells were isolated from non-transformed healthy donor PBMCs and *Herpesvirus Saimiri* (HVS) transformed XLP1 patient samples using Miltenyi MACs negative selection following manufacturer's protocol. Selected cells were activated using either anti-CD3/CD28 Dynabeads™ at a 1:1 ratio with X-VIVO (SIGMA) media supplemented with 5% human serum and human recombinant IL-2 at a concentration of 20U/mL, or with human recombinant IL-6 (100U/mL); IL-7 (20U/mL); IL-21 (200U/mL). Cells were incubated for 72h and transduced at a multiplicity of infection (MOI) of 50. Cells were subsequently cultured maintaining *in vitro* conditions at a density of $5.0x10^4$ cells/well of a 96-well round bottom plate in the presence of 15ng/mL Staphylococcal enteroxin B (SEB), either alone or with allogeneic memory B cells isolated from tonsillar mononuclear cells at a ratio of 1:1. Cells were co-cultured for 10 days before immunophenotyping using LSRII and BD conjugated antibodies; mouse anti-human CD4 (BV650), mouse anti-human PD-1 (CD279) (BV510) and mouse anti-human CXCR5 (CD189) (BV41). ELISA assays to analyse IL-21 and IgM/IgG/IgG_{1-3} concentrations were performed on supernatant using Platinum ELISA kits (ebioscience).

Generation of LCLs and CTLs:

PBMCs were isolated, as above, from EBV seropositive healthy donors for generation of autologous and allogeneic LCLs using EBV B95.8 supernatant (kindly provided by Ida Ricciardelli) in the presence of 50ug cyclosporine. PBMCs were stimulated with 40Gy irradiated LCLs in vitro over a period of 4 weeks with weekly stimulations.

In vitro cytotoxicity and CTL phenotyping:

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Generated CTLs were transduced using EFS-SAP-eGFP or EFS-eGFP vectors at MOI 50 and phenotyped using; PE Mouse anti-CD4; APC Mouse anti-CD8; BV650 Mouse anti-Human CD45RA; BV510 Mouse anti-Human CD62L (BD Bioscience) to establish memory phenotype and transduction efficiency prior to NSG tumour model. CTL function was determined using *in vitro* ^{51}Cr ($Na_2{}^{51}CrO_4$, PerkinElmer, Waltham, MA, USA) release assay with allogeneic LCL target and non-specific P185 murine mastocytoma cell target with anti-CD3 conjugation (BD Biosciences) to determine EBV directed and redirected killing. An effector to target ratio of 30:1 was used and serial dilutions performed to determine cytotoxicity range and incubated for 4 hours at 37°C. ^{51}Cr release in the supernatant was measured with a beta counter (Trilux, 1450 MicroBeta, PerkinElmer).

NSG tumour model:

Allogeneic LCLs were transduced with a lentiviral vector expressing fire-fly luciferase and blue fluorescent protein reporter gene to select on highly expressing cells. A cell dosage of $5.0x10^6$ was prepared in Matrigel™ Matrix (Corning) with equal amounts of PBS and injected into NSG mice subcutaneously at the nape of the neck. 48h post-LCL injections xenografted tumour burden was established using IVIS *in vivo* imagining system (Xenogen; Caliper Life Sciences, Hopkinton, MA) in the presence of 15mg/kg intraperitoneally injected D-Luciferin. Healthy donor and XLP1 patient CTLs were injected intravenously via the tail vein at a ratio of 1:1 CTL:LCL ratio ($5x10^6$ total cells per mouse) and tumour regression was monitored using IVIS every 48h for 10 days post tumour establishment.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, CA, USA). Statistical significance for murine experiments was determined using two way analysis of variance (ANOVA). Statistical significance for cytotoxicity and T_{FH} In vitro assays was calculated using appropriate non-parametric testing including Sidak's,Dunnett's and Kruskall-Wallis multiple comparison tests, Mann-Whitney two tailed student t tests assuming non-gaussian distribution.

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Results

Transfer of SAP replete T cells restores humoral immunity

To demonstrate that SAP expression in the T cell compartment can correct humoral immunity in a sap-deficient murine model, we adoptively transferred gene corrected sap-deficient $CD3^+$ T cells into sap-deficient recipients following sub-lethal irradiation (6Gy). A retroviral construct was used to transduce T cells in this context as murine T cells are not efficiently transduced with lentiviral vectors, due to restriction factors present in this population (21). We generated gammaretroviral vectors containing a codon optimised human SAP cDNA and eGFP or eGFP alone (Figure 1A) and efficiently transduced sap-deficient $CD3^+$ lymphocytes with a standard activation protocol using anti-CD3/28 beads and IL-2 (approximately 60% transduction efficiency for both SAP and GFP control vectors with an average vector copy number of 2.4). As expected, there was a slight predominance of $CD8^+$ cells in transduced populations but high levels of transduction were achieved in both the $CD4^+$ and $CD8^+$ compartments (Supplementary Figure 1). To determine T cell engraftment levels, transduced cells from female sap-deficient donors were adoptively transferred into male sap-deficient recipients, following sub-lethal irradiation. At 8 weeks post-infusion of gene modified T cells, animals were challenged with NP-CGG and analysed 10 days later to assess response to immunisation (Figure 1B). We demonstrated significantly higher levels of $GL7^+/CD19^+$ cells (a germinal centre marker) in the spleens of animals receiving the GRV-SAP-eGFP vector compared to those receiving the control eGFP vector ($p< 0.01$) with levels comparable to WT animals (Figure 2A). GC formation was confirmed by PNA staining of splenic sections in sap-deficient animals receiving gene corrected T cells (Figure 1B). We also found significantly improved NP-specific IgG_1 levels ($p< 0.01$) in the GRV-SAP-eGFP vector treated group in comparison to animals in the sap-deficient or eGFP control group demonstrating functional humoral reconstitution following correction of the T cell compartment (Figure 1C, sup Figure 2). Higher levels of eGFP were seen in the T_{FH} population of immunised animals receiving both vectors (Supplemental Figure 2C) consistent with expansion of this population in response to antigen challenge confirming that SAP is required only for T_{FH} cell function and not development. Together these data suggest that infusion of gene corrected sap-deficient T cells can lead to restoration of functional humoral responses to T dependent antigens.

SAP gene transfer restores T_{FH} cell function

To explore the clinical relevance of the data generated in the sap-deficient murine model, we investigated whether restoration of SAP expression in T_{FH} cells from XLP1 patients could ameliorate defective T_{FH} activity using an established *in vitro* T_{FH} cell functional assay (22). In patients with XLP1,

the T_{FH} population secreted less IL-21 indicative of T_{FH} dysfunction and B cells failed to secrete immunoglobulins. For transduction of human primary T cells we used a lentiviral vector with SAP expression driven by the EFS promoter (Figure 3A). This vector had been previously used in our proof of concept HSC gene therapy studies (12). Given the scarcity of primary patient samples, we generated patient T cell lines through transformation with *Herpesvirus Saimiri* (HVS). We were able to demonstrate that the function of HVS transformed lymphocytes was comparable to that of non-transformed T cells in terms of cytotoxicity and memory phenotype (Supplementary Figure 3).

Naive $CD4^+CD45RA^+$ cells from both patient HVS transformed cells and healthy donor PBMCs (untransformed) were selected then cultured in two different conditions to determine whether maintenance and transduction of the T_{FH} population could be optimised through cytokine stimulation. A standard activation protocol using anti-CD3/CD28 Dynabeads© and human recombinant IL-2 at 100U/mL was compared to a predicted pro-T_{FH} cytokine cocktail of IL-6 and IL-21(23-28) with media supplement of IL-7 to allow proliferation of cells, prior to lentiviral transduction with either EFS-eGFP or EFS-SAPeGFP vectors. Transduced cells (transduced at 35-40% efficiency determined by eGFP expression) were either cultured alone or co-cultured with allogeneic memory B cells derived from healthy donor tonsillar samples at a ratio of 1:1 for a 10-day incubation period. Cells were then analysed to determine the overall T_{FH} percentage ($CD4^+CXCR5^+PD\text{-}1^+$) (Figure 3) and supernatant tested to quantify the concentration of secreted IL-21 and immunoglobulin levels (Figure 4). The T_{FH} population was significantly reduced in XLP1 patients compared to healthy controls (Fig 3b and c) and this was associated with lower levels of IL-21 secretion and immunoglobulin production (Fig 4a and b). In contrast, we found that the T_{FH} population, as characterised by $CD4^+CXCR5^+PD\text{-}1^+$ expression, was restored to normal levels when XLP patient cells were gene corrected with SAP expressing vector (Fig 3b and c). There was no difference in the percentage of T_{FH} population between cells transduced using the anti-CD3/CD28 Dynabeads© or using pro-T_{FH} cytokine cocktail of IL-6 and IL-21. We also demonstrated functional recovery of the T_{FH} population in gene corrected XLP1 patient cells with significantly increased IL-21 secretion, IgM levels and IgG levels (including IgG_1, IgG_2 and IgG_3 subclasses, Supplementary Figure 4) ($p<0.01$) that were similar to healthy controls (Fig 4 a and b). In these functional studies, there is a difference between the T_{FH} activity of cells transduced using the different transduction conditions in comparison to uncorrected cells. Although an overall improvement is seen across all conditions, this is most pronounced in, XLP cells transduced with the pro-T_{FH} cytokine cocktail of IL-6,7 and IL-21 with significantly greater IL-21 secretion and Ig production in comparison to XLP uncorrected cells. XLP cells transduced using the anti-CD3/CD28 Dynabeads© show increased function but did not reach statistical significance. These results demonstrate that upon SAP correction, naïve T cells from

XLP1 patients can be stimulated to differentiate into T_{FH} cells, which in turn are capable of providing adequate B cell help to allow for immunoglobulin secretion *in vitro*.

Lentiviral mediated SAP gene transfer in XLP1 CD8+ T cells restores cytotoxicity

SAP-deficient cytotoxic lymphocytes (CTLs) are unable to efficiently kill EBV infected lymphoblastoid cell lines (LCLs) (3, 29). We investigated whether restoration of SAP expression in deficient T cells could restore cytotoxicity in this context. To examine the effect of lentiviral mediated *SAP* gene transfer on cytotoxicity in human $CD8^+$ EBV specific CTLs we again used HVS transformed cells from both healthy donors and XLP patients. Prior to gene modification we were able to demonstrate that these transformed cells maintained cytotoxic function with no significant difference in killing compared to EBV-CTLs derived from fresh PBMCs (from the same donor) using a standard *in vitro* ^{51}Chromium release assay (Supplementary Figure 5).

To demonstrate that this response is mediated via a SAP-dependent pathway and not through CD3, we co-cultured healthy donor and non-corrected and corrected XLP1 T cells against p815 tumour cells incubated with soluble CD3 (Figure 5). All three lines demonstrated cytotoxicity against p815 cells suggesting that in XLP1, T cell cytotoxicity is maintained against non-LCL targets but expression of SAP is required for cytotoxicity against EBV-LCLs.

Patient derived HVS cells were transduced with the EFS-SAPeGFP vector and EBV-CTL cytotoxicity against both autologous and allogeneic LCL targets assessed using a ^{51}Cr release assay. Our results demonstrate recovery of killing activity in gene corrected patient CTLs with transduction efficiency ranging from 24-48% and average final vector copy number of 1.9 viral copies per cell (Figure 5, Supplementary Figure 5), and no significant differences in cytotoxic function when using an autologous and allogeneic target

Adoptive transfer of gene corrected XLP1 patient EBV-CTLs leads to regression of EBV-lymphoma in an *in vivo* xenografted NSG mouse model

We sought to determine whether adoptive transfer of gene corrected T cells could mediate tumour clearance in NSG mice engrafted with EBV+ LCL tumours. EBV-LCLs from a healthy donor were transduced with a luciferase expressing cassette and transplanted into NSG mice to form a palpable tumour. CTLs were generated from PBMCs from healthy donors and two XLP1 patients. Patient CTLs were transduced with the EFS-SAPeGFP vector post allogeneic LCL stimulation. 72 hours post-transduction cells were phenotyped and transduction efficiency determined using flow cytometry

(Figure 6A and B). Phenotyping of transduced CD8+ CTLs demonstrated persistence of both central memory ($CD45RA^-$ $CD62L^+$) and naïve populations ($CD45RA^+$ $CD62L^+$) across donors (ranges 40-70% and 8-20% respectively) suggesting maintenance of long lived T cell populations after transduction with our lentiviral vectors. Transduction efficiencies in the range of 24-50% were achieved at an MOI of 50, which was associated with an increase in intracellular SAP expression (Figure 6C) and restoration of cytotoxic function (Figure 5).

EBV-CTLs were injected intravenously 48 hours after establishment of tumours at ratio of 1:1 LCL:CTL and bioluminescence analysed every 48 hours subsequently (Figure 7A). Regression of tumours could be visualised by Day 2 post CTL infusion with complete clearance observed in both healthy control and gene corrected XLP1 patients by day 10, in stark contrast to animals receiving uncorrected patient cells (Figure 7B and C). We observed tumour persistence in untreated (n=3) and patient uncorrected (n=6) (EFS-eGFP transduced) CTL treated mice within in a range of $3.0\text{-}6.0\text{x}10^6$ $p/s/cm^2sr$ by day 10 post LCL infusion. In contrast mice treated with healthy donor CTLs (n=9, CTLs from 3 donors) demonstrated a significant reduction in tumour burden 48h post-CTL treatment with bioluminescence values dropping from $3.0\text{x}10^6$ $p/s/cm^2/sr$ to $4.0\text{x}10^4$ $p/s/cm^2/sr$ and even further by day 10 to a final value of $1.0\text{x}10^3$ $p/s/cm^2/sr$ equivalent to that of an animal which was not subjected to tumour engraftment. Similarly, we found a considerable reduction in tumour burden in animals infused with gene corrected patient CTLs (n=6, CTLs from 2 patients) which, by day 10, was equivalent to the level of tumour clearance observed in animals treated with healthy donor CTLs and significantly better than CTL mediated tumour clearance in animals receiving uncorrected patient CTLs ($p < 0.0001$).

Discussion

Haematopoietic stem cell gene therapy has been successfully used to treat a number of monogenic haematologic and immunological diseases and offers a curative treatment option for patients lacking a suitable donor for HSCT. XLP1 is a monogenic primary immune deficiency with a range of severe manifestations and even in cases of early diagnosis, provision of prophylactic therapies and close monitoring, the condition can be fatal due to the development of HLH or malignancy (10, 30). Outcome following HSCT from mismatched donors is significantly worse in this population than from HLA-identical donors(10) and as such, the development of novel gene therapy strategies may offer

patients lacking suitable donors an alternative management option. We have previously described correction of a sap-deficient murine model using gene corrected HSCs with *SAP* transgene expression controlled by the ubiquitously active EFS promoter(12) , but given the role of SAP as an intracellular signalling molecule and the lack of normal SAP expression in HSCs, we explored the use of *SAP* gene transfer in T cells, where it is normally expressed, as an alternative option.

Here we provide first evidence that adoptive transfer of gene corrected T cells can correct the humoral and cytotoxic defects associated with XLP1 and this could represent a clinically applicable therapy for patients with this condition. Immune abnormalities in XLP1 arise predominantly from defects in the T cell compartment. Correction of $CD3^+$ lymphocytes may therefore afford protection from HLH, caused by dysregulated T cell activation and impaired cytotoxicity, alongside reduced risk of malignancy through improved tumour surveillance, and improve humoral immunity through correction of T_{FH} function. Although the NK cell population will remain uncorrected there is evidence of redundancy in the role these cells play in the pathophysiology of HLH and correction of the CD8 compartment alone may therefore be sufficient[32].

In an established sap-deficient murine model we have shown, *in vivo*, that transfer of SAP corrected T lymphocytes leads to recovery of functional humoral immunity following T cell dependent antigen challenge through formation of germinal centres and specific antibody responses, both absent in sap-deficient animals. This is achievable at levels of engraftment close to 40%, which is clinically feasible with current lentiviral transduction platforms in human primary cells. Moreover, we were able to demonstrate functional correction of patient T_{FH} cells, *in vitro*, with significantly improved IL-21 and immunoglobulin secretion profiles. The functional profile of gene corrected T_{FH} cells from XLP patients was enhanced when cells were cultured with IL-6, IL-7 and IL-21; cytokines which had been chosen based on their role in the differentiation and maintenance of the T_{FH} cell population(27). Together this data suggest that T cell gene therapy could be of considerable clinical benefit to XLP1 patients with dysgammaglobulinaemia.

Our data also confirms that lentiviral mediated *SAP* gene transfer can correct cytotoxic defects in patient $CD8^+$ CTLs in the context of EBV; essential in preventing and treating HLH and lymphoproliferative complications. This has been demonstrated through both *in vitro* cytotoxicity assays and an *in vivo* EBV-LCL lymphoma tumour model in NSG mice. We convincingly show that

gene corrected patient CTLs are able to induce tumour regression to the same level as healthy donor CTLs. We may therefore assume that gene modified patient $CD8^+$ T cells would be functional in the context of EBV viral challenge and potentially HLH in XLP1 patients.

The anticipated level of engraftment of gene corrected T cells required to permit functional immune reconstitution in patients is an important question but there is limited clinical experience of patients post HSCT with low level mixed chimerism. We have shown here that patient T lymphocytes can be efficiently transduced leading to restoration of SAP expression and, although levels of expression are below that seen in healthy donor cells, a modest increase in protein correlates with correction of T_{FH} function and cytotoxicity. Although it is challenging to define the exact level of engraftment required we can extrapolate from the data presented here that as little as 15% correction can restore immune function. Taken in the clinical context these results are encouraging, suggesting that low levels of engraftment may be sufficient to rescue phenotype.

The long-term persistence of autologous gene modified T cells, and thus the longevity of clinical benefit, also remains to be determined. Early clinical trials for Adenosine deaminase deficient severe combined immune deficiency (ADA-SCID) employed peripheral T cells transduced with a gammaretroviral vector and gene marked cells were detectable over 10 years post-infusion(31) . Similar findings have also been reported in the context of suicide gene therapy clinical trials(32) . It has now been established that specific T cell subsets are capable of sustaining the T cell compartment, namely T stem cell memory (T_{SCM}) and central memory (T_{CM}) cells(33). Here we have shown maintenance of a central memory population following transduction with our lentiviral construct, supporting the idea that SAP corrected patient cells may persist for several years. Whether T cell gene therapy offers a definitive treatment for patients with XLP1 lacking a donor for transplant will only become evident through a clinical trial. Although a T cell strategy should provide long term benefit it also does not prevent patients from receiving a subsequent stem cell procedure and in this context, may also be used to treat XLP1 patients prior to HSCT if a suitable donor is unavailable within the necessary time period.

In conclusion, the data presented here strongly supports the clinical translation of a lentiviral mediated T cell gene therapy approach to treat patients with XLP1 who lack a suitable donor for HSCT. Given that we have shown amelioration of both humoral and cellular immune defects, patients with a range of clinical phenotypes may benefit from this therapeutic strategy.

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Authorship

Contributions: C.B., N.P., H.B.G. designed the experiments; N.P, B.H., B.D., performed research and analysed data; I.R., S.G. helped design tumour model experiments and analyse data. N.P. and C.B. wrote the manuscript. All authors read and commented on the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests

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FIGURE LEGENDS

Figure 1: Experimental design of T cell adoptive transfer model (A) Schematic representation of long terminal repeat (LTR) driven gammaretroviral vector used for ex-vivo transduction of murine donor splenic $CD3^+$ selected T cells (i) mock vector containing enhanced green fluorescent protein (eGFP) reporter gene only (ii) vector containing codon optimised SAP cDNA and eGFP (B) Time line of adoptive T cell transfer experiments following sub-lethal (6Gy) irradiation at D0 with intravenous infusion of transduced murine CD3+ T cells. Animals underwent tail vein bleeds at 3 and 6 weeks to assess peripheral T cell donor engraftment prior to immunological challenge at week 8 with T cell dependent antigen NP-CGG.

Figure 2: Analysis of humoral function following immunological challenge (A) Analysis germinal centre B cells by flow cytometry in splenic lymphocytes stained with anti-CD19 and anti-GL7 antibodies. Results from individual mice are represented by dots with mean value represented by a horizontal bar. (** $p<0.05$) **(B)** Germinal centre staining in splenic follicles using peanut agglutinin (PNA) marking for germinal centre B cells, 10 days after immunization with NP-CGG at a magnification of x40. Slides are representative of results seen in wild type (WT) and sap-deficient control animals and animals receiving gene modified T cells (transduced with GRV-eGFP or GRV-SAP-eGFP vectors) demonstrating recovery of germinal centres in sap deficient mice receiving SAP containing vector. **(C)** NP-specific antibody production analysis using an ELISA assay performed on blood serum samples of all cohorts post NP-CGG vaccination demonstrating functional restoration of germinal centre activity in SAP reconstituted animals comparable to that of WT littermates (**$p<0.05$).

Figure 3: Correction of XLP1 patient PBMC derived T Follicular Helper cells (A) Schematic representation of lentiviral vectors containing codon optimised human *SAP* cDNA driven by elongation factor 1α short (EFS) promoter and eGFP or EFS-eGFP only. An MOI of 20 was used to transduce PBMC derived selected naive $CD4^+$ cells prior to *in vitro* B-cell co-culture assay. Transduction efficiency ranged between 30-45% (data not shown) with vector copy number of 2-3 copies per cell **(B)** Representative flow cytometry contour plots of differentiated $CD4^+$ cell phenotype 10 days post co-culture with allogeneic tonsillar memory B cells or CD4 cell culture alone. Top two panels, middle to right are healthy donor cells pre-stimulated with standard activatory conditions (1.0×10^6 cells/ml with a 1:1 anti CD3/CD28 bead ratio + hIL-2 (10ng/mL) or T_{FH} polarising cytokines

IL-6 (100ng/mL) /7 (10ng/mL) /21 (20ng/mL). Bottom two panels, left to right are of PBMC derived and HVS transformed XLP1 patient differentiated $CD4^+$ T cells using the conditions as above. Cells were transduced 3 days post stimulation and cultured with or without allogeneic B cells. **(C)** Recovery of T_{FH} population as determined by CXCR5 and PD-1 expression in XLP1 patient corrected cells post B cell co-culture assay in cells cultured in both anti-CD3/28/IL-2 and IL-6/7/21 culture conditions(n=3) (** $p<0.05$).

Figure 4: Functional correction of XLP1 patient T_{FH} cells *in vitro*. (A) Quantification of IL-21 concentration and **(B)** IgG levels and **(C)** IgM levels in supernatant 10 days after co-culture of naïve CD4+ T cells and allogeneic B cells by ELISA. (**$p<0.05$, ***$p<0.001$, ****$p<0.0001$).

Figure 5: SAP gene correction of XLP1 patient CTLs restores cytotoxicity in vitro: (A) (i) *In vitro* cytotoxic activity of CTLs generated from both healthy donors and EBV seropositive XLP1 patients (gene corrected and uncorrected) against allogeneic LCL target cells prior to intravenous infusion into NSG mice as measured in a ^{51}Chromium release assay. Assays were performed in triplicate and data shown are mean ± SEM of all values; **(ii)** Specificity of SAP function was determined using non-LCL targets in a cytotoxicity assay in parallel. Murine mastocytoma P815 cells were co-cultured with healthy donor and patient corrected and uncorrected effector cells incubated with soluble anti-CD3. CTLS from all donors, including XLP1 patients, displayed cytotoxic activity suggesting that EBV^+ LCL targeted killing is SAP and not CD3 mediated.

Figure 6: Phenotype of transduced and gene corrected cytotoxic T lymphocytes: (A) Representative flow cytometry contour plots of CTL phenotype following *in vitro* stimulation with allogeneic LCLs in mock transduced healthy donor and patient cells and patient cells transduced with a corrective lentiviral SAP vector (CM, central memory; EM, effector memory; TEMRA, CD45RA+ effector memory; N, naïve). Transduction efficiency was assessed by flow cytometry; eGFP expression ranged from (24-50%). **(B)** SAP expression GFP+ selected patient uncorrected and patient gene corrected cells as analysed by intracellular FACS staining (control IgG2b, solid line; anti-SAP antibody, dotted line).

Figure 7: Adoptive transfer of gene corrected XLP1 patient CTLs induces regression of EBV^+ LCL generated tumours in an NSG mouse model: (A) EBV^+ B-LCL xenograft tumour model experimental

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design. **(B) (i)** Bioluminescence images of NSG mice 48 hours after subcutaneous LCL injections displaying formation of localised solid tumours **(ii)** Tumour burden after 10 days in untreated mice (top left hand panel, n=3), and mice treated with healthy donor CTLs (3 donors, 3 animals per donor) (iii) Tumour burden after 10 days in mice receiving uncorrected XLP1 patient CTLs (top panel) or gene corrected patient CTLs (bottom panel), (2 patient donors, 3 animals per donor) showing reduction in tumour burden in animals receiving gene corrected XLP1 patient cells. **(C)** Dot plot representing quantification of tumour burden determined using average photon density per second per cm^2 per steradian ($p/s/cm^2/sr$) on day of CTL infusions (D0), after 48 hours (D2) and after 10 days (D10). ***$p<0.0001$

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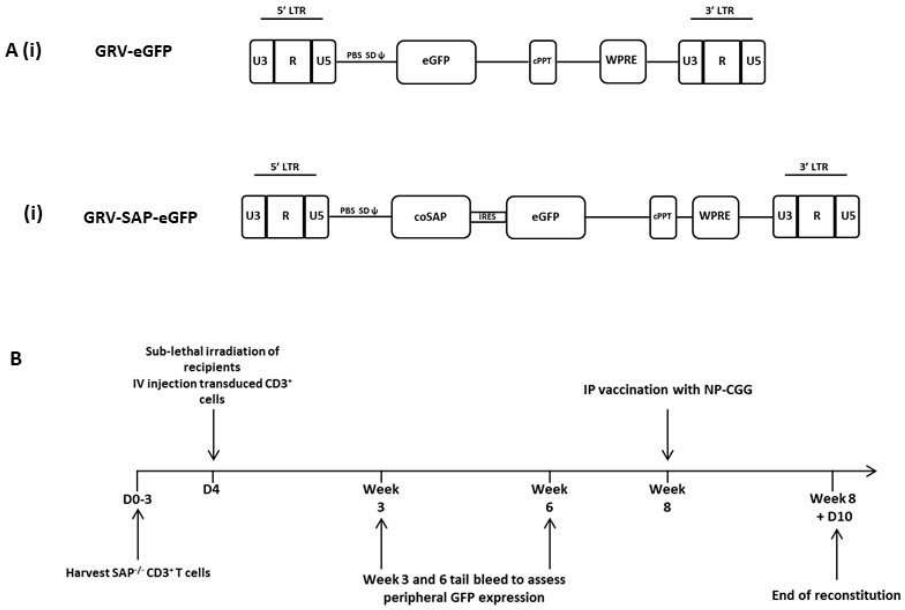
Figure 1



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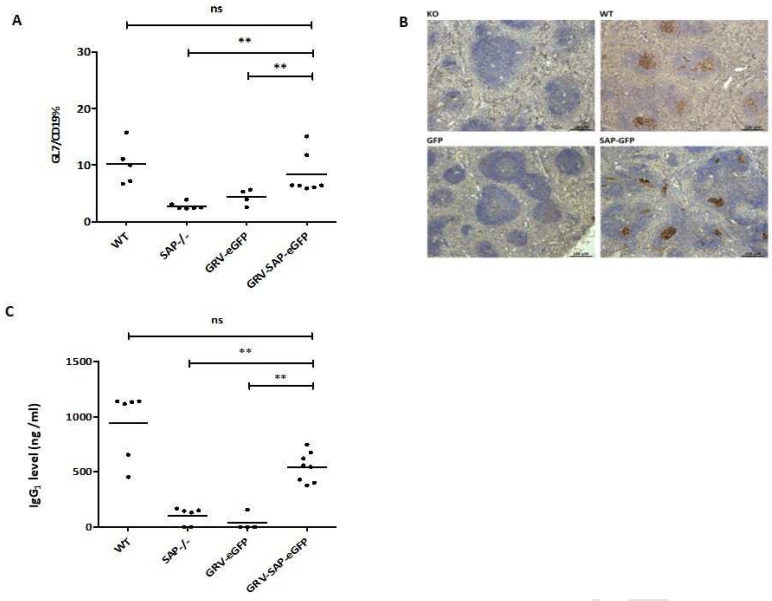

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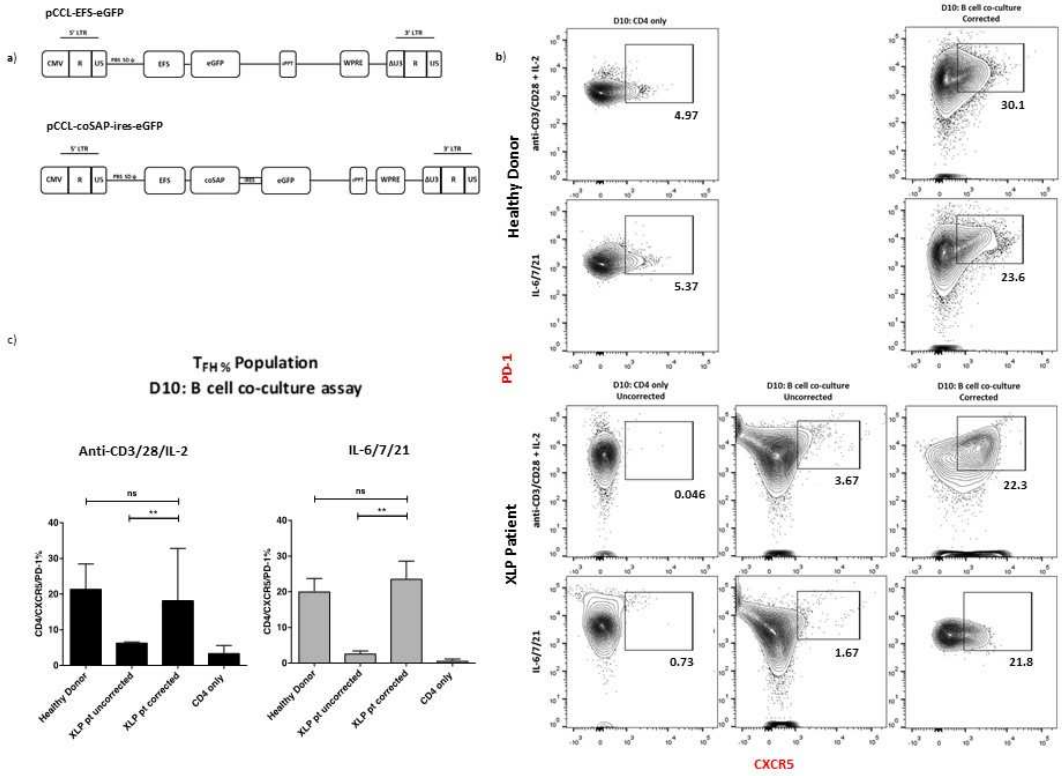

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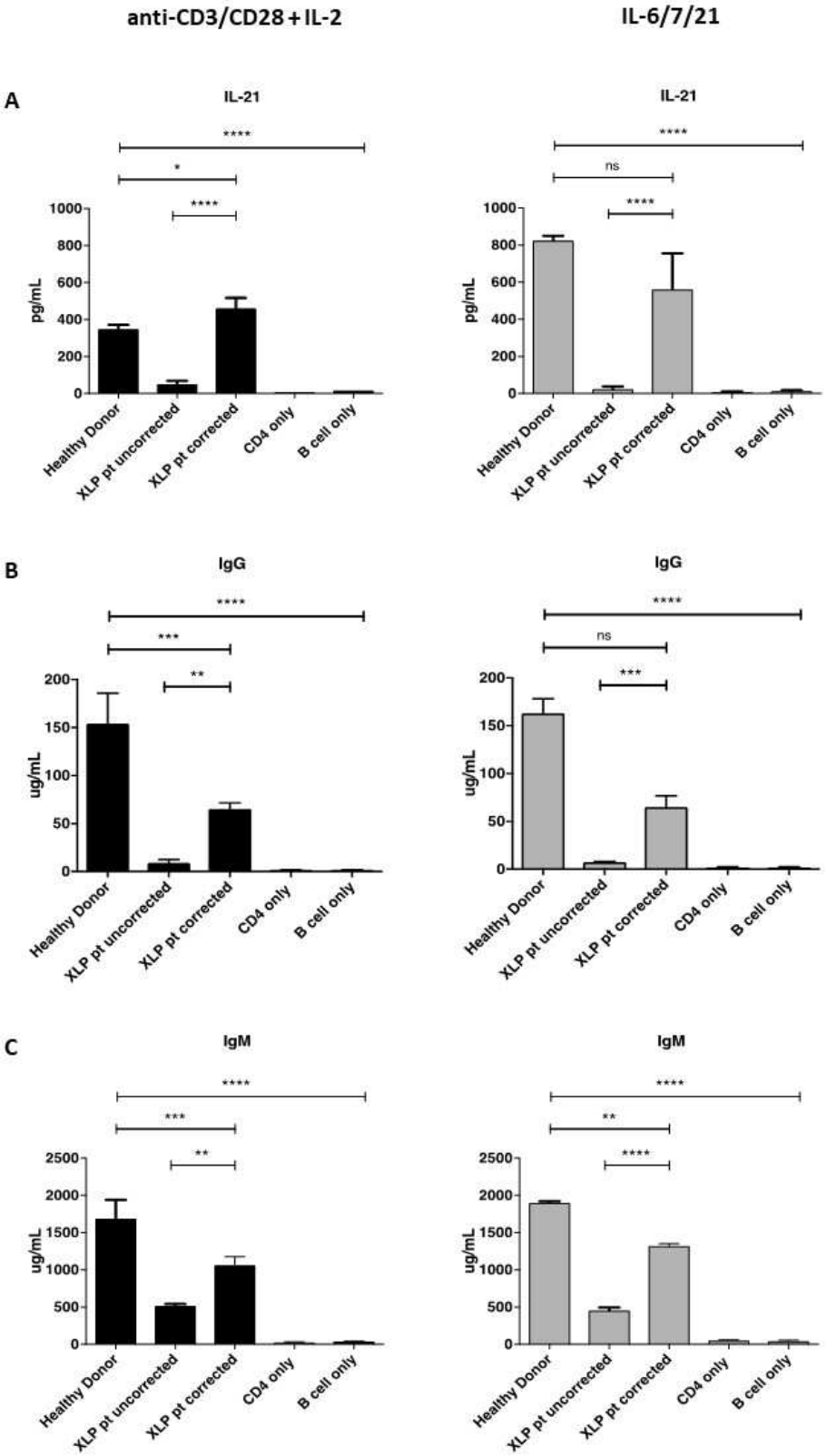
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IL-6/7/21
A
IL-21
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pg/mL
B
IgG
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IgM
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Healthy Donor
XLP pt uncorrected
XLP pt corrected
CD4 only
B cell only
ns

Figure 5

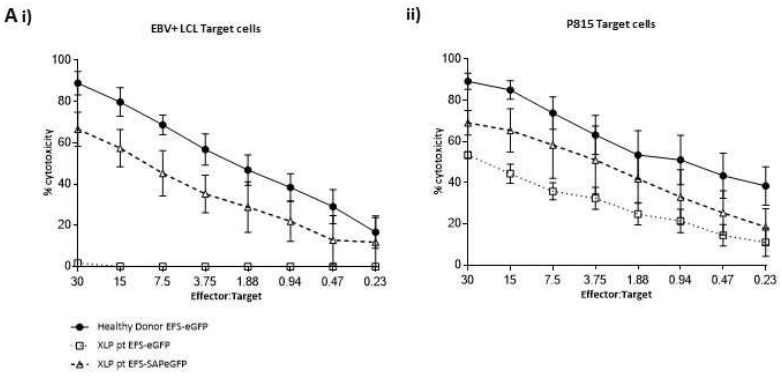

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Figure 6

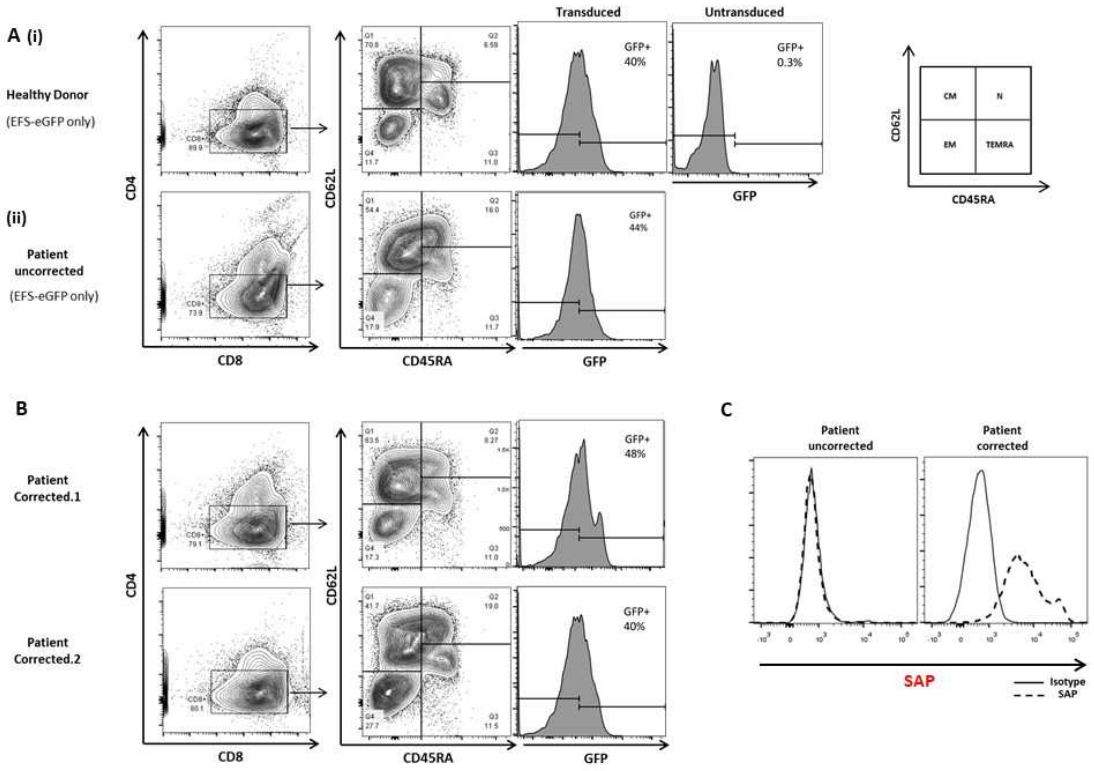

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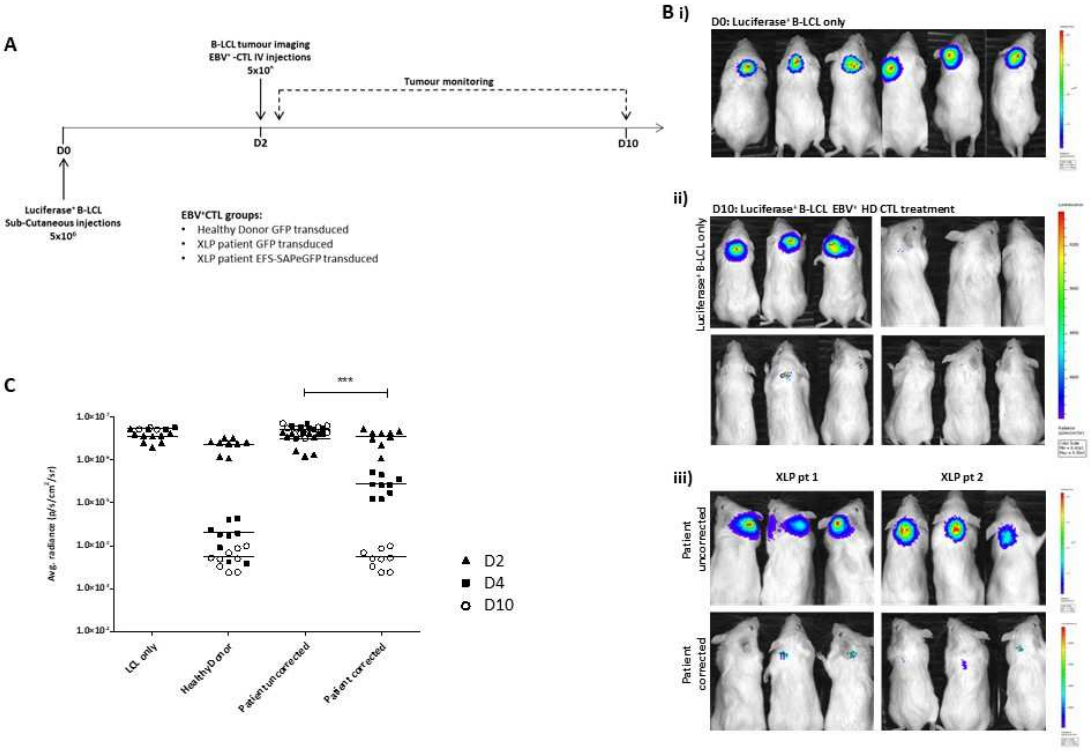
Figure 7



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SUPPLEMENTARY DATA

Supplementary Figure 1: Transduction efficiency and phenotype of murine splenic CD3$^+$ donor T cells. (A) Flow cytometry staining of murine T cells showing gating strategy for CD4+ and CD8+ lymphocyte populations. (B) Transduction efficiencies is determined by GFP expression 72h post transduction with GRV-SAP-eGFP or GRV-eGFP alone. An average transduction of 50-60% is observed across T lymphocyte compartments.

Supplementary Figure 2: Analysis of adoptively transferred T cells. (A) SAP protein expression was analysed in gene corrected murine splenic CD3$^+$ T cells post reconstitution. (B) Donor T cell engraftment was assessed in whole splenocytes using XY PCR techniques 10 weeks post reconstitution showing average engraftment levels of 40%. (C) Analysis of GFP levels in whole splenocytes and splenocyte derived T cell lineages in immunized animals.

Supplementary Figure 3: Comparison of function between HVS transformed cells and untransformed cells. One experiment was performed comparing phenotype (A), cytotoxicity (B) and T_{FH} function (C) in HVS transformed healthy donor cells, HVS transformed patient cells and non-transformed healthy donor cells which demonstrated comparability between the all samples. No significant differences were observed in both cytotoxicity and T_{FH} assay.

Supplementary Figure 4: Functional correction of XLP patient T_{FH} cells *in vitro* – IgG subclass production. Quantification of IgG subclass levels in supernatant 10 days after co-culture of naïve CD4+ T cells and allogeneic B cells by ELISA. (**$p<0.05$).

Supplementary Figure 5: *In vitro* cytotoxicity of HVS transformed CTLs using an allogeneic LCL target. PBMCs from healthy donors and EBV+ XLP patients were transformed with Herpes virus saimiri (HVS). T cell memory phenotyping was performed in both CD4 and CD8 populations to determine naive, TEMRA, effector memory and central memory populations (Ai), Bi)) . Patient transformed samples contained more terminally differentiated cells compared to healthy donor. (Aii) ^{51}Chromium release assay demonstrated functional activity in HVS transformed cells from healthy donors using an allogeneic B-LCL target with no significant difference observed in cytotoxic activity between non-transformed and HVS transformed cells. (Bii) Patient HVS transformed cells were transduced with the EFS-SAPeGFP or EFS-eGFP mock vectors and subjected to a ^{51}Chromium release assay . Uncorrected patient samples showed no cytotoxic activity against allogeneic B-LCLs compared to patient corrected CTLs which were able to kill the LCL target within the range of 40-50%. No significant differences were observed between non-transformed healthy donor CTLs and patient corrected HVS cells in cytotoxic activity when using an allogeneic B-LCL target.

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Supplementary Methods

Vector copy number and Y chromosome engraftment analysis:

Genomic DNA was extracted from either human cultured CD4/CD8 cells or murine splenic $CD3^+$ cells using Qiagen DNEasy™ blood and tissue extraction kit following manufacture's protocol. Multiplex quantitative PCR (qPCR) using gBLOCK (standards) synthesised and purchased from Integrated DNA Technologies (IDT) was carried out to determine vector copy number (VCN) and murine donor T cell engraftment.

Generation of HVS transformed human primary T cells and cytotoxicity

PMBCs from healthy donors or XLP1 patients were stimulated *in vitro* with PHA 20µg/mL then cultured in X-VIVO media with 5% human serum for a period of 72h. After 72 hours, IL-2 (20ng/mL) was added to supplement the media and cells were incubated for a further 48h. HVS virus supernatant generated using Owl Monkey Kidney cells (as previously published) was added to the cultured cells seeded at a density of $2.0x10^6$ cells per well of a 48 well plate. Phenotype of transformed cells was assessed at 2 week intervals to determine longevity and viability of cells.

Flow cytometry, immunophenotype and SAP staining:

Single-cell suspensions were prepared from human PBMCs or murine splenic tissue. Samples were incubated for 60 minutes at room temperature with various antibodies; BV421 Rat anti-Human CD185 (CXCR5) BV510 Mouse anti-Human CD279 (PD-1) or Alexa 647 Rat anti-Mouse GL7 PE Rat anti-Mouse CD19; PE Rat anti-Mouse CXCR5; BV421 Hamster anti-Mouse CD279 (PD-1) and analysed by flow cytometry for GFP and cell surface marker expression. For SAP intracellular staining, following fixation and permeabilisation (IntraPrep, Beckman Coulter), cells were incubated either with a mouse anti-human SAP (SH2D1A) antibody (Abnova clone 1C9) or with the corresponding isotype control Rat IgG_1, κ (BD Pharmingen™clone R3-34 RUO).

Immunisation and antibody response:

For immunisation animals were injected via intraperitoneal injection with NP(65)-CGG (150ug/ml per mouse) (Biosearch Technologies) mixed with alum (Serva) in a 1:2 ratio. Serum was collected via tail vein bleed at various time points after immunisation and NP-specific immunoglobulins were detected and quantitated by enzyme-linked immunosorbent assays (ELISAs) using NP(23)-BSA (Biosearch Technologies) as a capture antigen. The mouse immunoglobulin standard panel was obtained from Southern Biotechnology Associates.

To analyse germinal centre formation, immunised animals were sacrificed 10 days after NP-CGG injection and germinal centre B cells were detected by flow cytometry in single-cell suspensions of splenocytes after erythrocyte lysis using anti-CD19 and anti-GL-7 antibodies (BD Biosciences). Spleens were then fixed in formalin, sectioned and stained with haematoxylin and eosin to allow visualisation of tissue structure. Germinal centre B cells were stained by immunohistochemistry using unconjugated Peanut agglutinin (Vector Laboratories). Images were captured by Olympus

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BX51 with Olympus Paln Apo objectives, and Nikon DigitalSight DS-L1 digital microscope camera with automatic exposure and on camera white balancing. Magnifications are as stated in the figures and figure legends.

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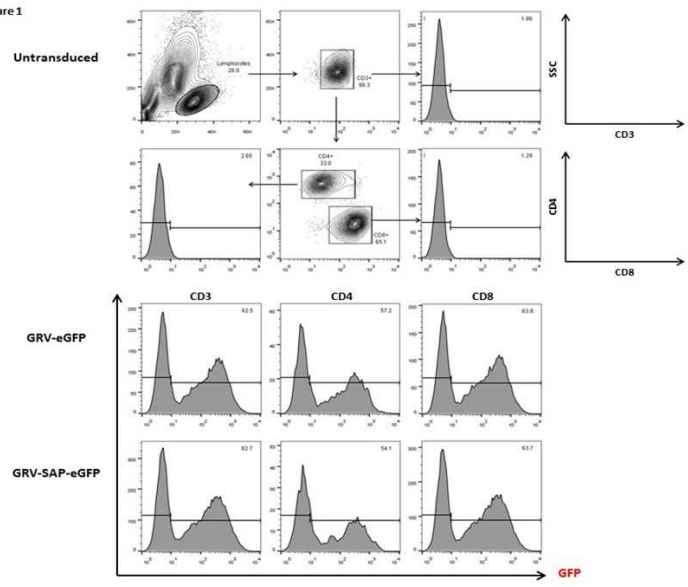
Supplementary: Figure 1



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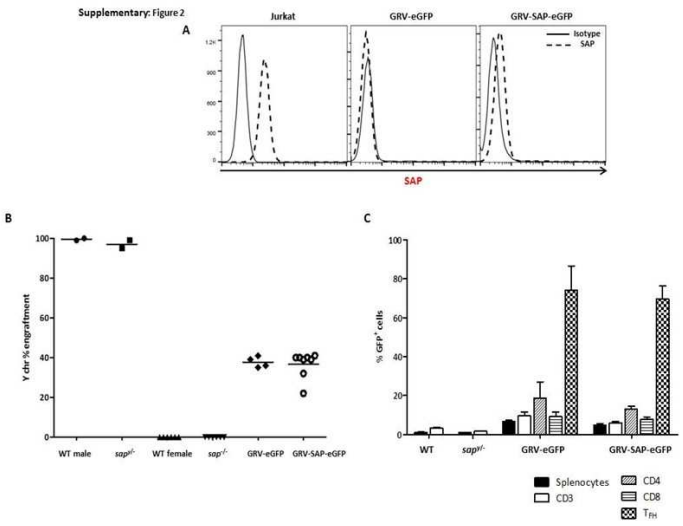

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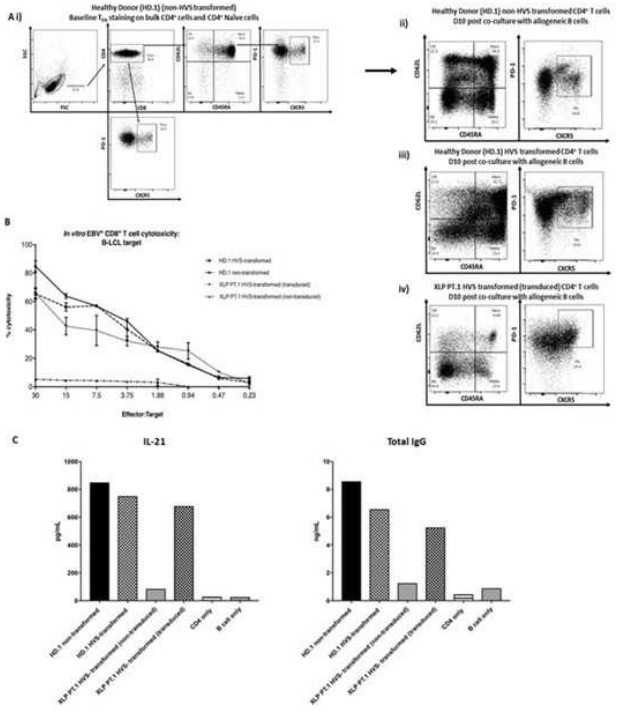

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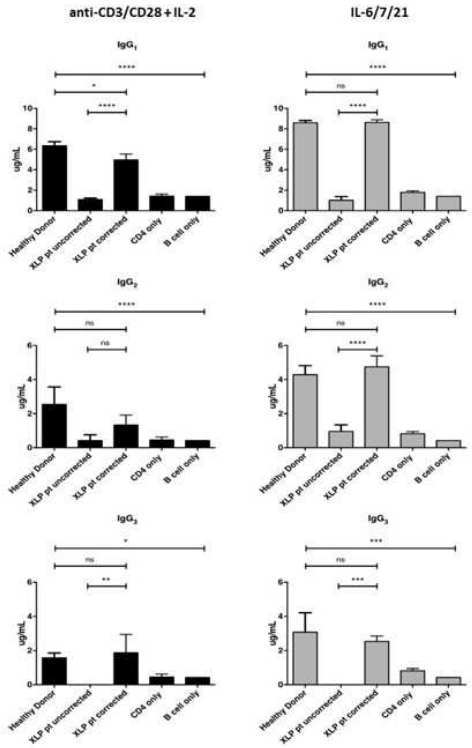

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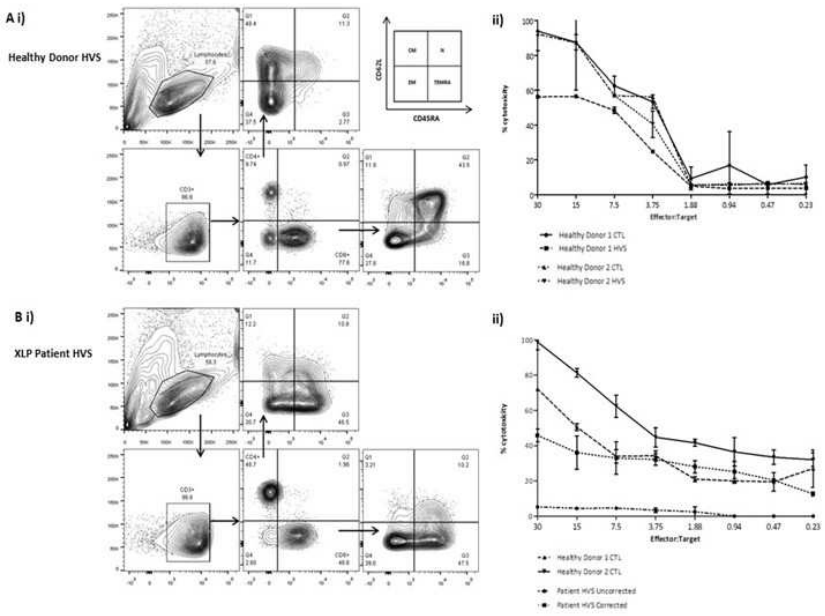
Supplementary: Figure 5



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