Brief report

SAP gene transfer restores cellular and humoral immune function in a murine model of X-linked lymphoproliferative disease

Christine Rivat1*, Claire Booth1*, Maria Alonso-Ferrero1, Michael Blundell1, Neil J Sebire2, Adrian J Thrasher1 and H Bobby Gaspar1

1Centre for Immunodeficiency
Molecular Immunology Unit
UCL Institute of Child Health
30, Guilford Street
London WC1N 1EH

2Department of Histopathology
Great Ormond Street Hospital NHS Foundation Trust
Great Ormond Street
London WC1N 3JH

* CR and CB contributed equally to this study

Correspondence to:
Prof H Bobby Gaspar
Molecular Immunology Unit
UCL Institute of Child Health
30, Guilford Street
London WC1N 1EH
Tel: +44 2079052319
Fax: +44 2079052810
e-mail: h.gaspar@ucl.ac.uk

Running title: Gene therapy for XLP1
Abstract
X-linked lymphoproliferative disease (XLP1) arises from mutations in the gene encoding SAP and
leads to abnormalities of NKT cell development, NK cell cytotoxicity and T dependent humoral
function. Curative treatment is limited to allogeneic haematopoietic stem cell transplantation
(HSCT). We tested whether HSC gene therapy could correct the multi-lineage defects seen in SAP−/−
mice. SAP−/− murine HSCs were transduced with lentiviral vectors containing either SAP or reporter
gene before transplantation into irradiated recipients. NKT cell development was significantly higher
and NK cell cytotoxicity restored to wild type levels in mice receiving the SAP vector in comparison
to control mice. Baseline immunoglobulin levels were significantly increased and T dependent
humoral responses to NP-CGG, including germinal centre formation, were restored in SAP
transduced mice. We demonstrate for the first time that HSC gene transfer corrects the cellular and
humoral defects in SAP−/− mice providing proof of concept for gene therapy in XLP1.
Introduction

X-linked lymphoproliferative disease (XLP1) arises from mutations in the SH2D1A gene, which codes for an intracellular adaptor protein (termed SAP for SLAM associated protein) expressed predominantly in T and NK cells\(^1,2\). The lack of SAP results in defective T and NK cell cytotoxicity, NKT cell development and CD4 T follicular cell (T\(^{FHI}\)) help (reviewed in\(^3\)). The clinical phenotype is characterised by severe immunedysregulation including development of lymphoma, T cell activation defects and abnormalities in immunoglobulin production and T dependent humoral immunity\(^4,5\). Similar immune defects are seen in SAP deficient murine models\(^6-8\).

The only curative option for XLP1 is allogeneic haematopoietic stem cell (HSC) transplantation, which has high mortality in the mismatched donor setting\(^5,9\). Gene therapy using viral vector mediated gene transfer into autologous HSCs is curative in a number of severe monogenic immunodeficiencies\(^10-14\). To determine if gene therapy is a possible treatment option for XLP1, we investigated whether SAP gene transfer in HSCs could correct the multiple immunological abnormalities seen in SAP deficient mice.

Materials and methods

**Mice:** SAP deficient mice (SAP\(^{-/-}\)) have been previously described\(^7\). Animal experiments were performed under an Institutional UK Home Office licence.

**Lineage negative (Lin\(^-\)) cell purification and transduction:** Lin\(^-\) cells were isolated from SAP\(^{-/-}\) bone marrow cells using MACS\(^\circledast\) Lineage Cell Depletion Kits (Miltenyi Biotec, Woking, UK). Lin\(^-\) cells were infected over-night at an MOI of 100 according to previously described protocols\(^15\).

**Animal irradiation and reconstitution:** SAP\(^{-/-}\) mice were lethally irradiated (1100 Rad in a split dose over 2 days). 3x10\(^5\) Lin\(^-\) cells were injected into tail veins of the recipient mice. Data from two
separate experiments including 9 wild-type, 6 SAP$^+/-$ controls, 8 EFSeGFP and 9 EFS-SAPeGFP reconstituted animals were combined to generate the results. Immunisation with NP-CGG was carried out in one experiment.

(See also supplementary methods)

Results and Discussion

SAP gene transfer into murine progenitors does not affect lineage specific cell development

We generated lentiviral vectors encoding the shortened form of the elongation factor 1 $\alpha$ promoter (EFS) driving the transcription of either codon optimised human SAP cDNA linked to the enhanced green fluorescent protein (eGFP) gene (EFS-SAPeGFP) or a control vector encoding eGFP alone (EFSeGFP) (Fig 1A). Lin-ve cells from SAP$^+/-$ donor mice were transduced with either EFS-SAPeGFP or EFSeGFP and transplanted into lethally irradiated SAP$^+/-$ recipients. Transduction efficiencies for both vectors were similar with eGFP expression of 64%% and 66% for EFS-SAPeGFP and EFSeGFP respectively (supplementary Figure 1). Reconstituted animals were analysed at 13 weeks post transplant and compared to SAP$^+/-$ mice and C57BL/6 wild type (WT) littermates. The recovery of different immune cell lineages in the periphery was similar in all 4 study groups (Fig 1B). Similarly, following lin-ve cell transduction and plating in semi-solid media, there was no difference between EFS-SAPeGFP and EFSeGFP cells in the number and type of colony forming units seen (supplementary Figure 2). Together these data suggest that SAP gene transfer into murine progenitors does not affect lineage specific development.
Reconstitution of NKT and NK cellular defects in SAP-/mice

Following reconstitution at 13 weeks, expression of eGFP in the peripheral blood, bone marrow, spleen or thymus of either EFS-SAPeGFP or EFSeGFP reconstituted mice was approximately 40%-50% with no significant difference between the two groups (Fig 1C). The vector copy number in PBMCS was found to be at mean levels of 1.7 (range 0.2-5.4) vector copies per cell in EFS-eGFP mice and 2.8 (range 0.6-4.6) vector copies per cell in EFS-SAPeGFP mice (supplementary Figure 3A). SAP protein expression was clearly visible in all tissues from mice reconstituted with EFS-SAPeGFP transduced cells (supplementary Figure 3B). To compare the level of SAP expression in EFS-SAPeGFP transduced cells, we also measured intracellular expression of SAP using the same antibody in normal human PBMCs (supplementary Figure 3C). The level of SAP expression in corrected PBMCs from EFS-SAPeGFP mice is comparable to that seen in normal human PBMCs.

Analysis of thymic NKT cell development in the thymus by flow cytometry using NK1.1 and TCRβ antibodies showed a significant increase in NKT cell numbers in EFS-SAPeGFP mice in comparison to EFSeGFP or SAP-/- controls although total numbers were decreased in comparison to WT mice (Fig 1D and representative flow cytometry in supplementary Figure 4A). Analysis of the NKT cell population showed that in comparison to the overall population of PBMCs the majority (>70%) of these cells were eGFP +ve (supplementary Fig 4B left hand panel). In contrast, in other cell lineages in the peripheral blood, eGFP expression following EFS-SAPeGFP transfer was less than 50% and no different to mice transduced with EFSeGFP only vector, suggesting that only NK-T cell development was particularly dependent on successful SAP gene correction (supplementary Fig 4B right hand panel). Intrathymic T cell development in terms of proportions of double negative, double positive and single positive CD4 and CD8 thymocytes was similar in WT and in gene corrected mice (supplementary Figure 4C). Analysis of splenic NK cell cytotoxicity against RMA/S target cells in EFS-SAPeGFP mice showed similar levels of cytotoxicity to WT NK cells and significantly increased activity
compared to either EFSeGFP reconstituted mice or SAP\textsuperscript{+/−} controls (Fig 1E). These data demonstrate that SAP gene transfer allows correction of both developmental and functional cellular defects seen in SAP\textsuperscript{+/−} mice.

Reconstitution of humoral defects in SAP−/− mice

Over 50% of XLP1 patients have abnormalities of immunoglobulin production and this is the most common phenotype\textsuperscript{5}. In SAP\textsuperscript{+/−} mice, baseline immunoglobulin production is abnormal with decreased production of total IgG, IgM and IgG subclasses\textsuperscript{7}. At 13 weeks post transplant, EFS-SApEGFP reconstituted mice demonstrated significantly increased mean levels of basal IgG, IgM, IgG1, IgG2a and IgG3 in comparison to EFSeGFP reconstituted mice and SAP\textsuperscript{+/−} controls (Fig 2A). Levels of IgG1, IgG3 and IgM in EFS-SApEGFP reconstituted mice were comparable to that seen in WT mice. Following challenge with the T dependent antigen NP-CGG, EFS-SApEGFP reconstituted mice demonstrated significantly increased levels of IgG1 and IgG3 NP specific antibody in comparison to EFSeGFP or SAP\textsuperscript{+/−} controls (Fig 2B). In spleens of challenged mice, germinal center formation as evidenced by GI7 expression on splenic CD19+ B cells was significantly increased in comparison to EFSeGFP reconstituted mice and SAP\textsuperscript{+/−} controls (Fig 2C). In support of this, immunohistochemical staining of spleens with the germinal center B cell marker PNA (peanut agglutinin) showed positive staining in WT and EFS-SApEGFP reconstituted mice whereas little or no staining was visible in EFSeGFP reconstituted mice and SAP\textsuperscript{+/−} controls (Fig 2D). Specific EFS-SApEGFP reconstituted follicles showed germinal center PNA staining equivalent to WT mice (Fig 2D x100 magnifications).

We also performed transplant experiments using bone marrow from WT SAP\textsuperscript{+/−} mice transplanted into lethally irradiated SAP\textsuperscript{+/−} mice. These studies showed very similar results to the gene transfer experiments with full recovery of NK cell cytotoxicity and partial recovery of NKT cell development and immunoglobulin production (supplementary Fig 5).
Together these data demonstrate that SAP gene transfer into SAP<sup>−/−</sup> HSCs is able to reconstitute major defects in cellular and humoral immunity seen in SAP<sup>−/−</sup> mice. The mechanisms of reconstitution following gene transfer are due to the expression of SAP in a number of specific cell lineages, which now allows SAP interaction with specific SLAM family receptors, especially 2B4 and Ly108. In thymic NKT cell development, SAP gene expression is required in thymocyte-thymocyte interactions and acts to propagate positive signals and also to inhibit negative signalling through Ly108<sup>16,17</sup>. The observed generation of T dependent immune responses, is most likely related to the development of SAP expressing CD4 germinal centre T<sup>FH</sup> cells<sup>18</sup> which now allows formation of stable conjugates with B cells to allow germinal centre formation and specific antibody production<sup>17,19</sup>. Full restoration of cytotoxic function in NK cells is again dependent on SAP expression and its role in controlling positive and negative signalling through 2B4<sup>20</sup>. Thus haematopoietic stem cell gene transfer provides multi-lineage SAP expression to correct the different immunological defects.

The reason for incomplete immune recovery seen in NKT cell development and immunoglobulin production is not clear, but seems unlikely to be related to efficiency of gene transfer since copy numbers in PBMCs were not low (mean level of 2.8 vector copies per cell), and the level of SAP expression in the transduced murine PBMCs was comparable to that seen in normal human PBMCs. However it is possible that human SAP does not function optimally in a murine cell background. In addition, WT transplant experiments into SAP-deficient mice result in similar patterns of immune recovery suggesting that there may be pre-existing microenvironmental deficits that are not easily corrected by reconstitution of adult mice, or that extended time periods are necessary for full correction to be achieved.
The EFS-SAPeGFP vector used in this study constitutively expresses SAP, which may not be desirable given that SAP is not physiologically expressed in specific cell types including HSCs and B cells. However, we did not see any detrimental effect on HSC function and lineage specific development. The generation of vectors with physiologically regulated SAP gene expression may allow a fuller correction of the phenotype and may offset safety concerns and are under current development. Nevertheless, this study demonstrates for the first time a proof of concept for a HSC gene strategy approach for correction of the different immunological abnormalities seen in XLPI. These data are important in showing that HSC gene transfer can rescue T cell help and may be important in development of gene therapy for other immunodeficiencies such as CD40 ligand deficiency or ICOS deficiency.
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**Authorship contributions:** CR and CB designed and performed the experiments, gathered the data and produced the figures. MA-F generated and tested the codon optimised vector. MB was responsible for the murine injections. NJS performed the immunohistochemical analysis. HBG and AJT conceived the study, designed experiments and wrote the manuscript. All authors have reviewed the manuscript.

**Disclosure of conflict of interest:** The authors have no conflict of interest.
References


15. Montiel-Equihua CA, Zhang L, Knight S, et al. The beta-Globin Locus Control Region in Combination With the EF1alpha Short Promoter Allows Enhanced Lentiviral Vector-mediated


Figure legends

Figure 1: Immune reconstitution of SAP-deficient mice following gene transfer into hematopoietic stem cells. (A) Schematic representation of the SAP expressing lentiviral construct and the corresponding eGFP control used for the reconstitution of SAP-deficient animals. (B) Flow cytometry analysis of hematopoietic lineages in control and experimental animals 12 weeks after reconstitution. (C) Level of eGFP expression in the blood, bone marrow, spleen and thymus of all animals at the time of sacrifice. (D) Detection of NKT cells in the thymus of control and reconstituted animals by staining for the TCRVβ receptor and NK1.1 surface marker. Values for individual mice are shown as dots, and the mean of all values is represented by a horizontal line. (E) NK cells cytotoxic activity measured in a ⁵¹Chromium release assay against the radiolabelled murine T lymphoma target cells (RMA/S). Assays were done in triplicate and data shown are mean ± SEM of all values.

Figure 2: Reconstitution of the humoral defects in SAP-deficient mice after immune challenge (A) Quantification of baseline serum immunoglobulin levels by ELISA 12 weeks after reconstitution. Values for individual mice are shown as dots, and the mean of all values is represented by a horizontal line. (B) Quantification of NP-specific antibody production by ELISA at various time-points after immunisation with NP-CGG in control and reconstituted animals. (C) Detection of germinal centre B cells by flow cytometry in splenic lymphocytes stained with anti-CD19 and anti-G17 antibodies. Values for individual mice are shown as dots, and the mean of all values is represented by a horizontal line. (D) Germinal center staining in splenic follicles of control and reconstituted mice 10 days after immunisation using the germinal center B cell marker peanut agglutinin (PNA). In addition to original magnification X40 for all groups, original magnification X100 of single follicle to show degree of germinal center recovery is shown for WT and EFS-SAPEGFP mice.
Figure 1

A) Diagram showing the constructs of EFS and eGFP.

B) Bar graph showing the total PBMCs percentage for wild-type, KO, EFS-eGFP, and EFS-SAPeGFP.

C) Graph showing the percentage of GFP+ cells in blood, bone marrow, spleen, and thymus for wild-type, KO, EFS-eGFP, and EFS-SAPeGFP.

D) Box plot showing Thymic NKT cells percentage for wild-type, KO, EFS-eGFP, and EFS-SAPeGFP.

E) Graph showing the %Cr release at different effector:target ratios for wild-type, KO, EFS-eGFP, and EFS-SAPeGFP.
Figure 2

A

Total IgG

IgG1

IgG2a

IgG2b

IgG3

IgM

wt    KO    EFSe    EFSSAPeGFP

wt    KO    EFSe    EFSSAPeGFP

wt    KO    EFSe    EFSSAPeGFP

wt    KO    EFSe    EFSSAPeGFP

C

Splenic CD19+ B cells (%)

wt    KO    EFSe    EFSSAPeGFP

B

wt    KO    EFSeGFP    EFSSAPeGFP

IgG1

IgG2a

IgG2b

IgG3

IgM

IgE

d0    d5    d10

d0    d5    d10

d0    d5    d10

d0    d5    d10

D

WT x40    EFSeGFP X40    WT X100

KO X40    EFS-SAPeGFP X40    EFS-SAPeGFP X100
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